

Investigation of Comparative Regulation on Antioxidant Enzyme System under Copper Treatment and Drought Stress in Maize (*Zea mays* L.)

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Abstract

The present study was conducted to present the responses of drought-sensitive 'Shemal' and drought-tolerant '71MAY69' maize cultivars under drought condition (20% Polyethylene glycol, -0.40 MPa) and three different copper concentrations (0.5 mM, 1 mM, 1.5 mM CuSO₄·5H₂O) for 5 days to determine the enzymatic responses of copper treatment in maize leaves. Copper treatments alone did not change stomatal conductance, relative water content, malondialdehyde, proline, hydrogen peroxide content and abscisic acid level according to control groups. Combined treatment (drought and copper) alleviated the damage of PEG- induced drought stress in maize leaves. Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) activity increased and glutathione -S transferase (GST) activity decreased, while ascorbate peroxidase (APX) activity did not change under drought stress in the tolerant cultivar. SOD, CAT and APX were decreased and GST activities were increased while GR did not change in 'Shemal'. Also SOD, APX and CAT activity increased by copper treatment alone in both cultivars. Otherwise combined treatment increased SOD, APX and CAT activity at all concentrations, but GR and GST activity increased only by (PEG+1.5 mM) treatment when compared with PEG treatment alone in sensitive ones. As a result, exogenous copper alleviated drought stress, while it induced an oxidative damage by increasing antioxidant enzyme activities differently from drought tolerance. Copper tolerance in maize is not a common response of its defense mechanism because of different response to copper and drought in the same cultivar.

Keywords: antioxidant enzyme, copper, maize, drought stress

Abbreviations: SOD: Superoxide dismutase; CAT: Catalase; GST: glutathione -S transferase; GR: Glutathione reductase; APX: Ascorbate peroxidase; MDA: Malondialdehyde; ABA: Abscisic acid; ROS: reactive oxygen species; H₂O₂: Hydrogen peroxide; RWC: Relative water content; PEG: Polyethylene glycol; EC: Enzyme Commission

Introduction

Copper (Cu) is an essential micronutrient for the growth and development of plants. Cu plays an important role in plant metabolism like respiration, photosynthesis, protein and carbohydrate metabolism (Droppa and Horvath, 1990), but becomes toxic at high concentrations (Gaetke and Chow, 2003).

Excess copper can interfere with protein function, enzyme activity, and membrane integrity (Alaoui-Sosse *et al.*, 2004). It can induce secondary oxidative stress by catalyzing the formation of reactive oxygen species such as hydroxyl radical, superoxide anion radical and singlet oxygen (Hall, 2002). These reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide and hydroxyl radicals, are highly reactive and can cause serious damage to membrane lipids, proteins and nucleic acids (Wang *et al.*, 2004) as well as signalling dysfunctions and other metabolic disturbances (Yruela, 2005).

Plants have enzymatic defense system against oxidative stress. SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), GR (EC 1.6.4.2) and GST (EC 2.5.1.18) play role of scavenging these ROS. It is well known that the negative effects of oxidative damage could be alleviated by these enzymes activities. There are many studies related to stress tolerance and antioxidant enzyme in plant species (Gill and Tuteja, 2010). With regard to copper stress, controversial studies have been reported stating that this metal induced antioxidant enzymes in different plant species, concentrations and durations of metal exposure (Rama and Prasad, 1998). Early studies showed that excessive Cu reduced cell increment of many plant species and root biomass of maize (Ouzounidou *et al.*, 1995).

The mechanisms through which copper induces antioxidative response in plants are not clear. Sgherri *et al.* (2001) reported that it is a question whether the difference in Cu sensitivity observed between two cultivars is related to their co-tolerance towards drought stress. To evaluate this,

this study was aimed to point out the comparative regulation of antioxidant enzymes under copper treatment with stress (drought) as a control in drought-sensitive and tolerant maize leaves. SOD, APX, CAT, GR and GST enzymes activities and hydrogen peroxide, MDA, ABA, proline content, RWC, stomatal conductance and endogenous copper content were determined comparatively.

Materials and methods

Plant growth and experimental design

Seeds belonging to maize drought-sensitive 'Shemal' cultivar and drought-tolerant '71MAY69' cultivar were used as the biological material of this present study. The seeds were sown in plastic pots, each of which (width 20 cm, length 12 cm, height 18 cm) contains 30-40 seeds and the mixture of soil: clay: clay-loam in the ratio of 2:1:1, pH: 6.5). Seeds were placed in the dark for 5 days for germination. The germinated seeds were then subjected to 16 h light / 8 h dark periods. They were watered with full strength Hoagland solution (Hoagland and Arnon, 1950) and grown at 25 ± 4 °C until the seedling stage. 8 day old seedlings were divided into control (1), 20% PEG (2), Cu 0.5 mM (3), Cu 1 mM (4), Cu 1.5 mM (5), 20% PEG+Cu 0.5 mM (6), 20% PEG+Cu 1 mM (7), 20% PEG+Cu 1.5 mM (8) groups and watered with Hoagland solution for 5 days. Then, the seedlings were harvested on the 5th day and the samples were preserved at -20 °C. The osmotic potential with PEG was -0.40 MPa.

Analyses

Measurement of hydrogen peroxide and malondialdehyde content

The H₂O₂ content was determined according to Velikova *et al.* (2000). Fresh leaves (0.1 g) were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 min. The supernatant (0.5 ml) was then mixed with 0.5 ml of buffer (10 mM potassium phosphate, pH 7) and 1 ml of 1 M KI. The absorbance reading was taken at 390 nm. The level of lipid peroxidation in leaf samples was determined in terms of the malondialdehyde (MDA) content according to the method specified by Madhava and Sresty (2000). The MDA content, an end-product of lipid peroxidation, was determined by using the thiobarbituric acid reaction. The MDA concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. An extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to determine the MDA concentration.

Relative water content and stomatal conductance assay

The relative water content (RWC) was calculated in accordance with Smart and Bingham (1974). Harvested leaves were weighed to determine their fresh weights (FW). The seedlings were floated on de-ionized water for 5 h under low irradiance and then the turgid tissue was quickly blotted to remove excess water and their turgid weights (TW) were determined. Dry weights (DW) were determined after the leaves were dried in an oven at 70 °C for 72 h. Stomatal conductance was measured on the 0th and 5th day of stress treatment using a portable steady-state porometer (SC-1). The data were collected from six sample leaves per replicate.

Measurement of Cu²⁺ ion content

The Cu²⁺ content was obtained by wet oxidation of dried leaf tissue with nitric and perchloric acids in accordance with the method adapted by Hanlon (1998) (AAS), Kacar and Inal (2008) (AAS, ICP-OES). The digest was diluted with 0.1 N perchloric acid, and Cu concentrations were determined by atomic absorption spectrophotometry.

Measurement of ABA level

ABA content levels were determined in accordance with Flores *et al.* (2011) with an UHPLCMS/MS. Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg/L) were prepared by exact weighing of the powder and they were dissolved in methanol (HPLC-grade).

Measurement of proline content

The proline contents of leaves were determined according to Claussen (2005). For each treatment, 0.5 g leaf sample was ground in a mortar after addition of a small amount of glasspowder and 5 mL of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was filtered through two layers of glass-fibers. To the filtrate (1 mL), glacial acetic acid and ninhydrin reagent (1 ml each) were added. The closed test tubes containing the reaction mixture were kept in a boiling water bath for 1 h before the reaction was terminated at room temperature (22 °C) for 5 min. The absorbance of the reaction mixture was determined at 546 nm. The proline concentration was determined from a standard curve and calculated on freshweight basis ($\mu\text{g proline g}^{-1} \text{ FW}$).

Antioxidant enzyme and isoenzyme activity

The superoxide dismutase (EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm (Beauchamp and Fridovich, 1971). Equal amounts of protein were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), except that sodium dodecyl sulfate was omitted. For the separation of SOD isoenzymes, 4.5% stacking and 12.5% separating gels under constant current (60 mA) at 4 °C were used. SOD activity was detected by photochemical staining with riboflavin and NBT as described by Beauchamp and Fridovich (1971). Gels stained for SOD activities were photographed with an Image Acquisition and Analysis Software (4.6.00.0; UVP Bioluminescence systems, UK). In densitometric analyses of SOD activities of control plants were taken as 100% and % of control values for each treatment are shown. The values are the average of data from 3 independent gels. The ascorbate peroxidase (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The assay depended on the decrease in absorbance at 290 nm as the ascorbate was oxidized. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA Na₂, 1.2 mM H₂O₂, and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of APX was defined as 1 mmol ml⁻¹ ascorbate oxidized min⁻¹. The catalase (EC 1.11.1.6) activity was assayed in a reaction mixture (2 ml) containing 50 mM K-phosphate buffer (pH 7.0). Then, 12.2 mM H₂O₂ was added, and the reaction was

started by adding 100 μ l supernatant. The activity was determined by monitoring the degradation of H_2O_2 at 240 nm over 2 min against a supernatant-free blank. Enzyme-specific activities were expressed as μ mol of H_2O_2 oxidized $min^{-1}mg^{-1}$ protein (Bergmeyer, 1970). GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). The assay medium contained 25 mM Na-phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM NADPH. Na_4 , and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient of NADPH ($6.2\text{ mM}^{-1}\text{ cm}^{-1}$). One unit of GR was defined as 1 $mmol\text{ ml}^{-1}$ GSSG reduced min^{-1} . The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein. GST (EC 2.5.1.18) activity was determined by the method of Habig *et al.* (1974) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH).

Statistical analysis

Statistical variance analysis of the data was performed using ANOVA and differences among treatments were compared using Tukey's post-hoc analysis with least significant differences at the 5% level.

Results and discussion

Under drought stress, a reduction in RWC was a common response in plants (Pérez-Pérez *et al.*, 2007). In the results, PEG treatment reduced RWC in both cultivars when compared with the control samples (Tab. 1) Reduction in RWC results in loss of turgidity, which leads to stomatal closure and reduced photosynthetic rates (Kramer and Boyer 1995). Parallel to this, in our findings, tolerant cv. '71MAY69' showed higher reduction (23.7%) in stomatal conductance than in the sensitive one (9%) to prevent water loss (Fig. 3). However, copper treatment alone did not change RWC content, when compared with the control group. Parallel to this result, Chen *et al.* (2001) observed no change in RWC under Cu treatment in rice leaves. In contrast, excess copper treatment reduced RWC in sunflower leaves as reported by Kastori *et al.* (2008). In this work, stomatal conductance was also not affected under copper treatment alone, in parallel to the results of RWC. Otherwise, combined treatment (PEG + 0.5 mM, 1 mM, 1.5 mM) increased RWC according to PEG treatment alone, but the highest alleviation was under PEG+ 1 mM Cu, PEG+ 1.5 mM Cu in cv. 'Shemal'. Accordingly, stomatal conductance was nearly the same as control levels under combined treatment (Fig. 3). It could be explained that copper protects leaves from dehydration by increasing compatible solutes such as proline (Ku *et al.*, 2012). Interestingly, copper treatment alone did not change proline level, while combined treatment increased proline content in both cultivars (Fig. 5). These results showed us that proline accumulation could be related only with drought tolerant in maize leaves.

ABA is produced under dehydration conditions and plays pivotal roles in response to drought stress (Shinozaki and Yamaguchi-Shinozaki, 2000). Direct correlation between ABA content and stomatal conductance has been

demonstrated (Socias *et al.*, 1997). In the present study, ABA level was increased (51.69% and 45.4%) by drought stress in both cultivars and this could lead to stomatal closure (Fig. 4). These results are in agreement with the reports of Garcia-Mata and Lamattina (2001) and Bright *et al.* (2006). Otherwise, copper treatment alone did not change the endogenous ABA level. In contrast, Zengin and Kirbag (2007) reported that excess copper treatment increased ABA content in sunflower. As mentioned before, stomatal conductance did not change under combined treatment in both cultivars according to PEG treatment alone. But combined treatment increased ABA level according to PEG treatment alone in 'Shemal', while it decreased in '71MAY69'. It could be explained that the increase in ABA accumulation was not efficient to close stomata in such cells and this also determines that there was no stress under the combined treatment. PEG treatment induces ABA level under combined treatment independent of the copper effect. As in earlier works (Chen *et al.*, 2001), we demonstrated that copper treatment increased proline content independent of ABA accumulation. In this study, alone copper treatment did not cause any water stress in maize leaves.

MDA is a product of lipid peroxidation; elevation in MDA content determines cell wall damage. In this study, the results showed that PEG increased oxidative damage in both cultivars (18.95% and 43.8%) (Fig. 2). Many reports indicate that drought stress induced MDA content in leaves and roots (Reddy *et al.*, 2004). Otherwise, in results, copper treatment alone in all concentrations did not lead to change in MDA content. This result could be explained by the fact that copper concentrations used in the experiment did not induce oxidative damage by inducing antioxidant enzymes in maize leaves. These results are also in agreement with Posmyk *et al.* (2009) who determined that higher copper concentration (2.5 mM) increased oxidative damage in red cabbage seedlings. Similarly, Fidalgo *et al.* (2013) observed that enhanced lipid peroxidation in *Solanum nigrum* L. roots and leaves depend on Cu concentrations. Apart from this, we determined that MDA level reduced under combined treatment by 52% in 'Shemal' (PEG+1 mM Cu) and 42% in '71MAY69' (PEG+1.5 mM Cu) (Fig. 2). These findings imply that copper concentrations used in this work (1 mM and 1.5 mM) were effective to protect maize leaves from drought damage. Regarding H_2O_2 , PEG treatment increased hydrogen peroxide levels in both cultivars. Similar to our results, there was increased in hydrogen peroxide level observed in maize (Chung *et al.*, 2011) and barley (Hossain, 2013) under drought stress.

In this work, copper treatment did not affect the H_2O_2 content in both cultivars, but combined treatment (1.5 mM) reduced (34.1% and 67.3%) H_2O_2 content, when compared with PEG treatment alone (Fig. 1). In contrast to our results, 50 and 100 μ M copper treatment increased hydrogen peroxide content in rice leaves grown in hydroponic culture (Thounaojan, 2012). It could be suggested that copper concentrations showed the same reaction within H_2O_2 parallel to MDA results.

SOD (EC1.15.1.1) catalyzes the dismutation of two molecules of superoxide into oxygen and hydrogen peroxide (Fridovich, 1975), the first step in ROS scavenging systems.

In this study, SOD enzyme activity increased in '71MAY69' by PEG treatment, but decreased in cv. 'Shemal', when compared with the control samples (Fig. 6). Our results are consistent with other studies reporting increased SOD activity in response to drought stress in sunflower (Gunes *et al.*, 2008). In our results, increased copper concentration induced it in '71MAY69', but the highest induction (2.5 fold) was at 1.5 mM in 'Shemal' according to the control group. Otherwise, the induction of SOD activity was not changed between 0.5 and 1 mM treatments in 'Shemal'. These results are parallel to the report of Tanyolac *et al.* (2007) who determined that 1.5 mM copper treatment

Tab. 1. Effects of copper treatment under drought stress on relative water content (RWC) (%) in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars

Treatments*	Cultivars	
	'71 May 69'	'Shemal'
Control	92.48±3.12 ^b	94.09±1.95 ^b
PEG	87.84±4.74 ^a	90.68±5.07 ^a
0.5 mM Cu	92.79±1.73 ^b	92.44±4.17 ^b
1 mM Cu	91.85±3.85 ^b	94.60±2.37 ^b
1.5 mM Cu	91.80±3.07 ^b	94.07±1.39 ^b
PEG+0.5 mM Cu	90.54±1.01 ^b	92.94±2.52 ^b
PEG+1 mM Cu	90.7±4.13 ^b	93.30±2.54 ^b
PEG+1.5 mM Cu	91.12±1.97 ^b	93.89±3.76 ^b

*Values with different letters are significantly different (p < 0.05) (n=6)

Tab. 2. Effects of copper treatment under drought stress on Cu²⁺ (ppm) in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars

Treatments*	Cultivars	
	'71 May 69'	'Shemal'
Control	7.3±0.02 ^a	13.8±0.05 ^b
PEG	7.7±0.04 ^a	7.7±0.005 ^a
0.5 mM Cu	13.4±0.05 ^b	15.9±0.02 ^b
1 mM Cu	11.7±0.02 ^b	15.9±0.03 ^b
1.5 mM Cu	23±0.05 ^c	39.7±0.04 ^c
PEG+0.5mMCu	20.5±0.05 ^c	29.6±0.03 ^c
PEG+1 mMCu	19.8±0.01 ^c	23.7±0.04 ^c
PEG+1.5mMCu	21±0.01 ^c	20.7±0.09 ^c

*Values with different letters are significantly different (p < 0.05) (n=6)

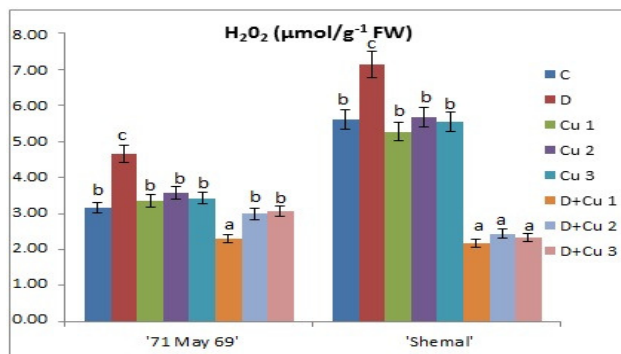


Fig. 1. Effects of copper treatment under drought stress on hydrogen peroxide (H₂O₂) content in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different (P < 0.05) values

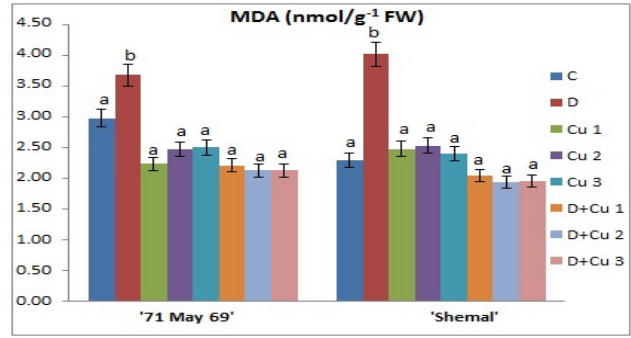


Fig. 2. Effects of copper treatment under drought stress on malondialdehyde (MDA) content in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different (P < 0.05) values

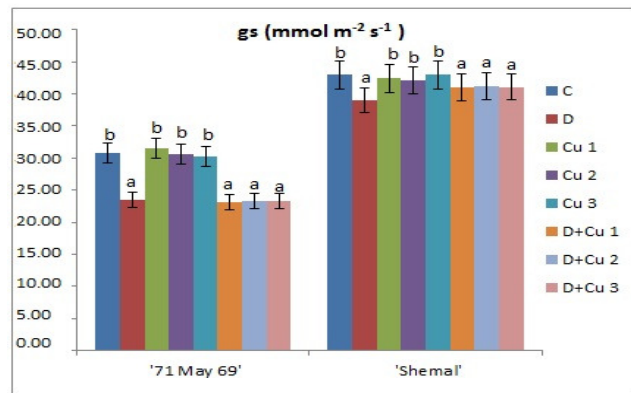


Fig. 3. Effects of copper treatment under drought stress on stomatal conductance (gs) in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Different letters represent significantly different (P < 0.05) values

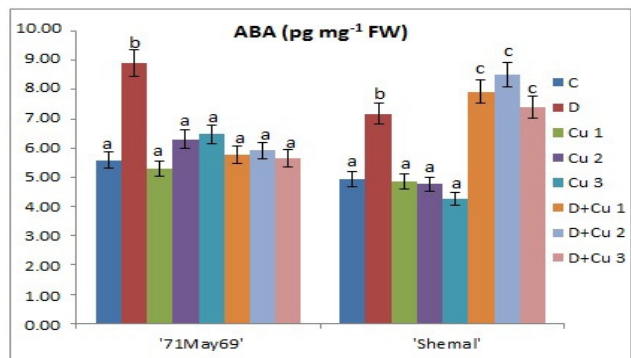


Fig. 4. Effects of copper treatment under drought stress on abscisic acid (ABA) content in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different (P < 0.05) values

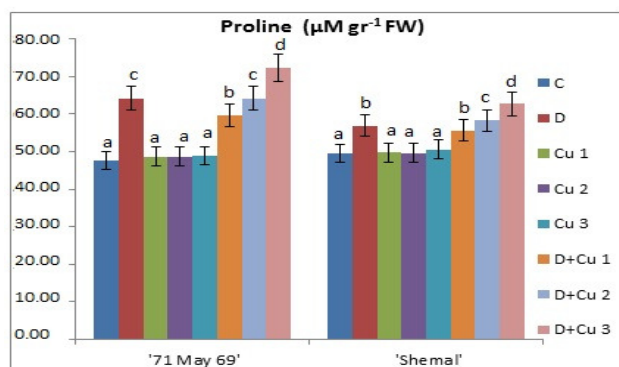


Fig. 5. Effects of copper treatment under drought stress on proline content tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

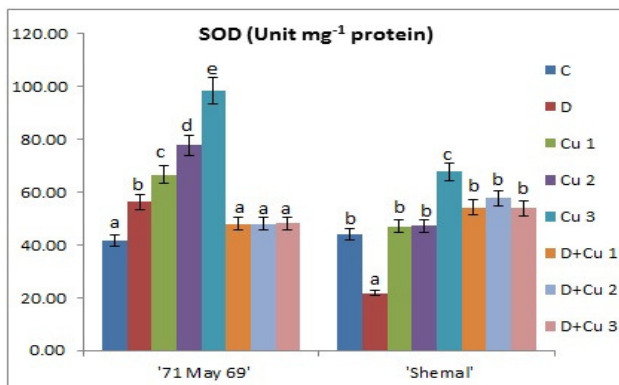


Fig. 6. Effects of copper treatment under drought stress on superoxide dismutase (SOD) enzyme activity in tolerant '71May 69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

induced SOD activity more in tolerant maize cultivar. Also, Fidalgo *et al.* (2013) determined unchanged SOD activity under lower copper treatment in *Solanum nigrum*. In Sgherri *et al.* (2001) report, SOD did not change in wheat leaves under sub-symptomatic copper doses. It was also previously observed in Cu-treated seedlings (Palma *et al.*, 1984). However, combined treatment increased SOD enzyme and isoenzyme activity in Shemal under all concentrations while it was decreased in '71MAY69' (Fig. 6a, 6b). Thounaojam *et al.* (2012) reported that SOD induced under copper treatment in rice shoots, but it could be changed by difference in concentrations. These differences could be originated in different sensitivities in response to copper in maize leaves. According to the results, it could be suggested that copper treatment protected the sensitive leaves from drought-induced damage by increasing SOD activity according to PEG treatment alone, but 1.5 mM Cu was not subsymptomatic for 'Shemal' (Fig. 6).

CAT activity increased in tolerant cultivar (41.5%), but decreased (33.8%) in sensitive one under PEG treatment alone according to the control groups. In accordance with this result, Chung *et al.* (2011) determined that CAT activity increased in tolerant maize and decreased in sensitive one under stress. This result is also in agreement with the result of hydrogen peroxide, which had been increased under PEG treatment in both cultivars. CAT is an important enzyme, which could scavenge hydrogen peroxide in plant cell (Li *et al.*, 2009). The decrease in SOD and CAT activity leads to increase in H_2O_2 content, especially in sensitive leaves. With regard to copper treatment, there was increase in CAT activity of both cultivars according to the control group (Fig. 7). Similarly, Posmyk *et al.* (2009) and Brahim and Mohamed (2001) observed that copper treatment induced CAT activity in red cabbage and *Atriplex halimus*. In contrast, some papers indicated that this metal inhibited CAT activity in *Brassica juncea* (Wang *et al.*, 2004) and sunflower (Jouli and El Feriani, 2003). However, in our results, combined treatment induced CAT activity according to PEG treatment alone in both cultivars. It could be clearly observed that copper could induce CAT activity more under combined treatment than PEG induced alone. This result showed that copper treatment plays a role for inducing antioxidant enzymes, which is independent of its tolerant to drought stress. PEG treatment alone did not change APX activity in '71MAY69', but decreased in 'Shemal' according to the control groups (Fig. 8). In the previous studies, APX activity increased in drought-tolerant bentgrass (DaCosta and Huang, 2007). Similarly, an increase in APX activity was observed in drought-stressed rice seedlings. In this study, the decrease in APX activity could lead to higher hydrogen peroxide in sensitive maize leaves, while it was less in tolerant ones. Otherwise, copper treatment alone induced APX activity in both cultivars (45.6% and 41.9%) when compared with the control group, but did not change into each other (Fig. 8). Similarly, Tewari *et al.* (2006) and Andrade *et al.* (2010) reported the enhancement of APX activity under copper stress. This could be related with unchanged hydrogen peroxide and MDA content under copper treatment. Like CAT activity results, combined treatment increased APX activity according to PEG treatment alone in both cultivars, but this increase was higher in sensitive cultivar (Fig. 8). As a result, APX activity was more effective under copper treatment according to PEG treatment. As in SOD, CAT activity, APX was also effective for detoxifying the hydrogen peroxide content. Otherwise, only SOD activity was effective depending on Cu concentrations.

Glutathione reductase activity is one of the important antioxidant enzymes for detoxifying ROS (Yousuf *et al.* 2012). As a result, PEG induced GR activity according to the control group '71MAY69', while it was not changed in 'Shemal' (Fig. 9). Parallel to our results, GR activity increased under drought stress in sensitive *Hibiscus esculentus* L. cultivars (Kusvuran, 2012). With regard to copper treatments, the highest increase was under 1.5 mM concentrations. Similarly, Tanyolaç *et al.* (2007) reported that 1.5 mM concentration induced GR activity in maize plants. Otherwise, combined treatment induced GR activity

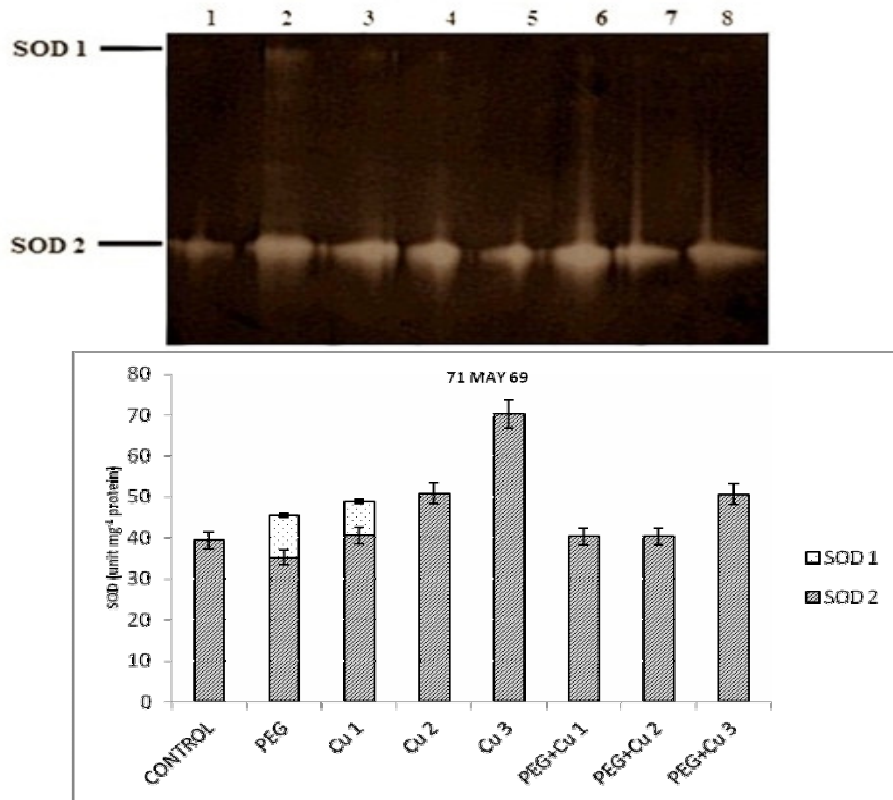


Fig. 6a. Activity staining and % induction of SOD isoenzymes in tolerant '71May69' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3)

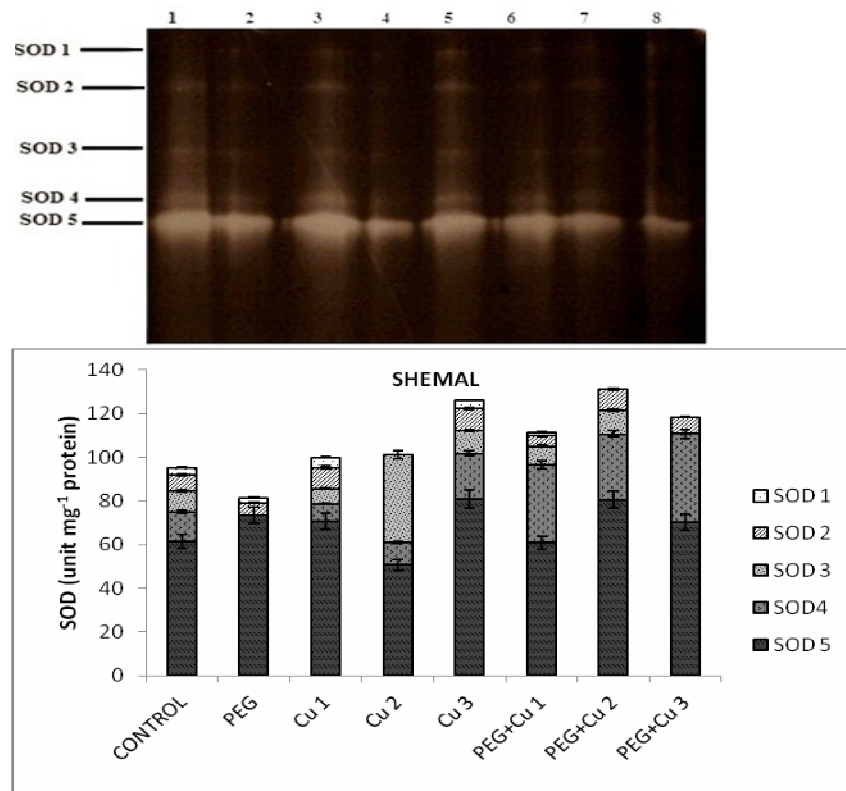


Fig. 6b. Activity staining and % induction of SOD isoenzymes in sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3)

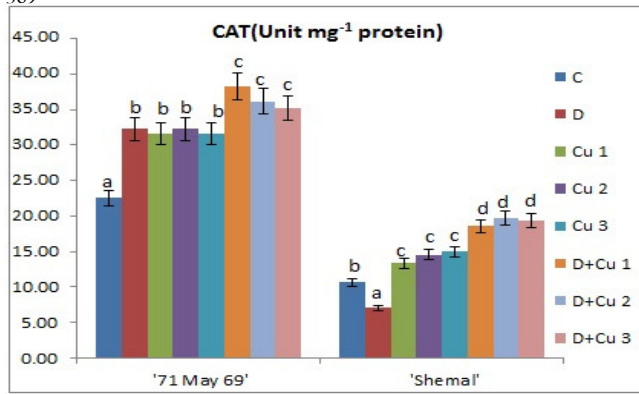


Fig. 7. Effects of copper treatment under drought stress on catalase (CAT) enzyme activity in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

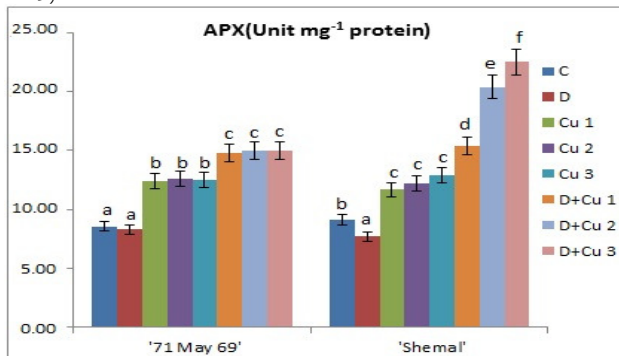


Fig. 8. Effects of copper treatment under drought stress on ascorbate peroxidase (APX) enzyme activity in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

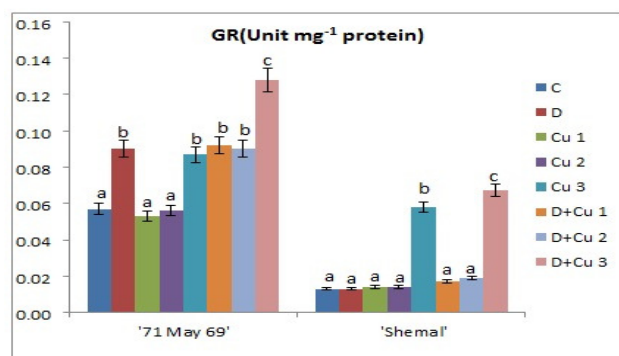


Fig. 9. Effects of copper treatment under drought stress on glutathione reductase (GR) enzyme activity in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

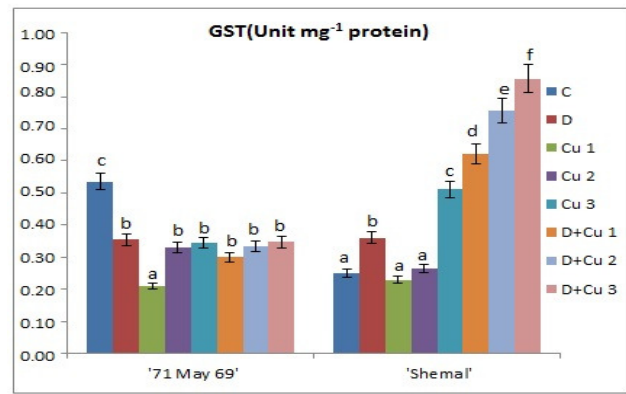


Fig. 10. Effects of copper treatment under drought stress on glutathione *S*-transferase (GST) enzyme activity in tolerant '71May69' and sensitive 'Shemal' maize (*Zea mays*) cultivars. Control group (C), 20% PEG (D), Cu 0.5 mM (Cu1), Cu 1 mM (Cu2), Cu 1.5 mM (Cu3), 20% PEG+Cu 0.5 mM (D+Cu1), 20% PEG+Cu 1 mM (D+Cu2), 20% PEG+Cu 1.5 mM (D+Cu3). Columns with different letters are significantly different ($P < 0.05$) values

according to PEG treatment alone in both cultivars (42.2% and 5.1 fold) (Fig. 9). Glutathione *S*-transferases (GST, EC 2.5.1.18), a group of dimeric, multifunctional enzymes, catalyze conjugation of glutathione (GSH) with xenobiotic compounds for detoxification (Tausz *et al.*, 2004). In this work, GST enzyme activity increased in 'Shemal' by PEG treatment but decreased in cv. '71MAY69', when compared with the control samples (Fig. 10). Csizár *et al.* (2007) also reported that GST activity increased with decreasing water content in *Allium sp.* Copper treatment alone reduced the GST activity in '71MAY69', but it increased under highest copper concentration (1.5 mM) in cv. 'Shemal' according to the control samples. These findings showed that only the highest concentration could be achieved to induce GST activity in maize leaves. Similar to our results, there was no change in GST activity observed in wheat seedlings (Gajewska and Sklodowska, 2005). Combined treatment increased GST activity (PEG+ 1.5 mM copper treatment, 2.4 fold) when compared with PEG treatment alone in 'Shemal', while it did not change in '71MAY69'. From this result, it could be suggested that GST enzyme activity did not change in '71MAY69' for its tolerant capacity against drought stress.

Conclusions

Collectively, our results showed that copper concentration did not cause oxidative damage in maize leaves, but it could alleviate the drought-induced oxidative damage, especially in sensitive cultivar. Otherwise, SOD, APX, CAT, and GR enzyme activities were induced by copper treatment alone, while they reduced under PEG treatment alone. The increase in enzyme activity under PEG treatment could be explained by endogenous ABA accumulation, but it is not the same with copper treatment because there was no change in ABA, hydrogen peroxide, proline content and MDA level. It could be suggested that there could be another signal mechanism to induce enzyme activities with copper. These findings determined that

copper response of plants is not a common signal but specific in maize leaves. Exogenous copper alleviated drought stress induced oxidative damage by increasing antioxidant enzyme activities by different signals from drought tolerance.

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