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Regulation of glutathione *S*-transferase enzyme activity with salt pre-treatment under heat stress in maize leaves

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The present study was conducted to present the responses of heat-sensitive (Shemal) and heat-tolerant (71May69) maize cultivars under heat stress (at 40 °C for 2 hours in 2 days) with pre-salinity treatment (50 mM NaCl) to determine the relation of salicylic acid (SA) and glutathione *S*-transferase (GST) enzyme activity in those responses. Relative water content (RWC), chlorophyll content (CHL), stomatal conductance (g_s), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) content, GST enzyme and isoenzyme activity and internal SA levels were investigated. While heat treatments resulted in low RWC, CHL g_s and high MDA and H_2O_2 levels in sensitive cultivar, all these values were lower in tolerant cultivar. Compared to single heat stress treatments, heat stress with pre-salinity treatment increased GST enzyme and isozyme activity of both cultivars. Results also indicated that pre-salinity treatment triggered internal SA levels and hydrogen peroxide contents in sensitive leaves according to control groups. Such hydrogen peroxide levels also decreased by increasing total GST and isozyme activities under heat alone, pre-salinity treatment alone. As a result, it could be said that SA is a good signal for the changes of GST enzyme activity under heat stress treatment.

Keywords: glutathione-*s*-transferase, heat stress, salicylic acid, salt stress, maize

Glutathione *S*- transferase (GST; EC 2.5.1.18) is a diverse enzyme group best known with their ability to catalyze the conjugation of tripeptide glutathione (GSH) to unfamiliar electrophilic and hydrophobic substrates to form less toxic or non-toxic peptide derivatives. Previous studies indicated the role of this enzyme in elimination of oxidative damages induced by environmental stress conditions (Dixit, 2001; Lannelli *et al*., 2002; Haluskova *et al*., 2009). Plant GST enzyme was reported to play a significant role in plant response to biotic and abiotic stresses, hormones and other developmental changes (Dixon *et al*., 2002).

A literature review on GST enzyme and hormones revealed that there were limited number of studies including auxin, ethylene, salicylic acid, abscisic acid and nitric oxide (Moons, 2005). It was reported in literature that ethylene hormone played a role in arrangement of GST gene expressions (Zhou *et al.,* 1993) and a strong relationship was observed between *GST* gene expression and development of ethylene-induced root hairs in *Arabidopsis* plant (Mang *et al*., 2004). In a study carried out with rice roots, it was observed under

salt stress that ABA resulted in accumulation of *OsGSTU3* gene transcript (Moons, 2003). Similarly in a study carried out with wheat seedlings under salt stress, increased GST enzyme activity was observed with pre-treatments of NO-releasing substance (SNP) (Hasanuzzaman *et al*., 2011). External salicylic acid treatments under pathogen stress also resulted in expression of nine different GST genes of *Arabidopsis* plant (Wagner *et al*., 2002).

High heat-stress may damage physiological and biochemical process and decrease plant growth and product quality of plants (Burke, 1990). Such abiotic stress conditions, especially with 1.5-6 °C increase in optimum growth temperatures (Houghton *et al,*. 2001), may inhibit photosynthesis (Abdul Karim, 1999), damage cell membranes (Marcum, 1998), limit plant growth and development and ultimately result in die-off. Therefore, adaptation is used to improve the adaptation capacities of the plants to various stress conditions. It is a significant process since it targets to reduce yield losses. There are previous studies in literature carried out with low salt concentrations, nitric oxide, salicylic acid or hydrogen peroxide to improve stress tolerance of the plants (Amzallag *et al*., 1990; Bethke and Drew, 1992). Beside them, there are also other studies reporting cross-protections with pre-stress treatments (Silva *et al*., 2013; Hossain *et al*., 2013). Adaptation of plant to a stress factor may result in improved response of the plant against the other stress factors. Increasing antioxidant enzyme activities under heat stress are closely related to plant tolerance against stress factors.

Glutathione *S* - transferase (GST; EC 2.5.1.18) is an important antioxidant enzyme and it was investigated in various enzyme-related studies (Haluskova *et al*., 2009). Although several studies have been performed on GST enzymes and biotic stress conditions, there are limited studies in literature about abiotic stress conditions. Therefore, it was considered significant to investigate GST enzyme activities under heat stress with salt pre-treatments. Thus in the present study, physiological and biochemical changes in two different maize cultivars (Shemal, 71MAY69) under heat stress with salt pre- treatments and the relation of GST enzyme in those changes were investigated firstly.

Material and methods

Plant Culture and Experimental Design

Heat-sensitive (Shemal) and heat-tolerant (71May69) maize seeds, supplied from MAY-Agro seed company, were used as the plant material of the present study. Seeds were placed in dark for 5 days for germination. Germinated seed were than subjected to 16 hours light / 8 hours dark periods. They were watered with Hoagland solution for a week and grown at 27 °C until the seedling stage. Seedlings were divided into control (1), salt pre- treatment (2), heat (3), heat + salt pre- treatment (4) groups. Salinity (2) and heat + salinity (4) groups were subjected to 50 mM NaCl Hoagland solution for 5 days. Heat (3) and heat + salinity (4) groups were subjected to heat stress through 40 °C temperature for 2 hours in 2 days. Then, the seedlings were harvested at 0h (0) and 4h (4) hours and samples were preserved at -20 °C.

Relative water content, stomatal conductance

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 5h 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 leaves were dried in an oven at 70 ^{0}C for 72 h). Stomatal conductance was measured at 0h, 4h of heat stress treatment using a portable steady-state porometer (SC-1). The data were collected from six sample leaves per replicate.

Hydrogen peroxide content, malondialdehyde content, total GST enzyme activity, GST isoenzyme activity

Hydrogen peroxide levels were determined in accordance with Velikova *et al*. (2000). The level of lipid peroxidation in the leaf samples was determined in terms of malondialdehyde (MDA) content according to the method specified Rao *et al.* (2000). GST 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). Containing equal amount of protein were run 10 % native PAGE according to the method of Laemmli (1970) and stained for GST activity using the method of Ricci et al. (1984). Briefly after electrophoretic run, the gel was equilibrated 0.1M potassium phosphate buffer , pH. 6.5 for 10 min and transferred to reaction mixture containing 4.5 mM GSH, 1 mM CDNB and 1 mM nitrobluetetrazolium in 0.1 M potassium-phosphate buffer, p H. 6.5 at 37 ^{o}C for 10 min. Further the gel was incubated in at room temperature 0.1 M Tris/HCl, pH 9.6, containing 3 mM phenazine methosulphate. Gels stained for GST activities were photographed with a Image Acquisition and Analysis Software, 4.6.00.0 version. In densitometric analyses of GST activities, 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.

SA levels

SA content levels were determined in accordance with Flores *et al.* (2011) with an UHPLC-MS/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) from Sigma.

Statistics were performed with SPSS.22 package programme. Kolmogorov-Smirnov test is used for variables normality. Comparions between variables are tested with independent samples t-tests if they were distributed normal.

Table 1: Effects of pre-salt treatment under heat stress on RWC (%) and chlorophyll (mg g^{-1} FW) content in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars.

		71MAY69		SHEMAL	
		0h	**4h**	**0h**	**4h**
	C	63.40±3.8^{a}	64.1±1.8.7^{a}	41.47±8.9^{b}	41.47±8.9^{b}
CHL	**H**	64.42±1.6^{a}	64.2±4.7^{a}	38.75±4.4^{a}	33.78±4.2^{a}
(mg g ^{1} FW)	**S**	63.87±1.4^{a}	64.2±2.5^{a}	38.79±7.4^{a}	42.25±2.5^{b}
	H +S	64.19±3.7^{a}	64.1±4.8^{a}	38.15±1.1^{a}	43.15±1.6^{b}
RWC (%)	**C**	92.3±0.15^{a}	93±0.15^{a}	93±0.17^{a}	93±0.17^{a}
	H	91.2±0.16^{a}	90±0.12^{c}	92.8±0.14^{a}	89.3±0.12^{b}
	S	88±0.10^{b}	89±0.12^{b}	89±0.12^{b}	89±0.18^{b}
	H +S	91.5±0.15^{a}	93±0.16^{a}	92.4±0.11^{a}	92.3±0.10^{a}

C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. The different letters are significantly different ($p < 0.05$) values

Results and Discussion

Relative water content decreased ratios under salt pre-treatments (50 mM NaCl) in (Shemal) (3.72 %) and (71MAY69) (3.68 %) compared to the control treatment (Table 1). Parallel to these findings, Kaya *et al.* (2013) observed distinctive decreases in relative water contents of maize plants under salt stress (100 mM NaCl) as compared to the control treatment. Such findings indicated that salt pre-treatments did not result in high level damages on maize plant. Nevertheless, heat treatments yielded lower relative water contents than the control treatment and such decreases were more distinctive in Shemal. Complying with the

current findings, Carmo-silva *et al.* (2012) reported decreased relative water content values for cotton plants under heat stress. Compared to single heat treatments, heat stress with salt pre- treatments yielded increases in relative water contents of maize cultivars. Similarly, heat stress with pre-salinity treatments resulted in closer relative water content values to the control treatments for *Jatropha curcas* L. plant. Such findings indicate that pre-salinity treatments might increase heat tolerance of maize plants (Silva *et al*., 2013).

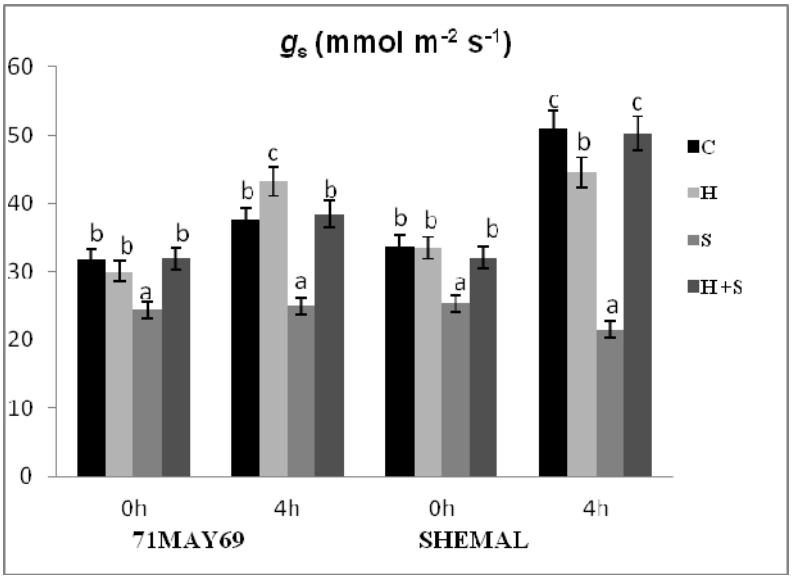

Fig 1: Effects of salt pre-treatment under heat stress on stomatal conductance (gs) in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. Different letters represent significantly different ($P < 0.05$) values.

Salt pre-treatment did not change in CHL content in both cultivar while it reduced only in sensitive maize under heat stress (Table 1). Similarly, Hernandez *et al.* (1995) reported that salt stress (70 mM) reduced CHL in pea. Liu and Huang (1999) reported that heat stress reduced CHL content in bentgrass leaves. Otherwise, salt pre-treatment alleviated the decrease in CHL content in sensitive ones. This was in accordance with the results of RWC and stomatal conductance. In case of a heat stress, plants open their stomas and transpire at higher rates to remove the heat rise within the plant. Therefore, a heat stress-induced decrease is observed in leaf water content (Suzuki *et al*., 2005; Mittler, 2006). The changes in stomatal conductance and leaf water content vary based on the intensity of the stress exerted on the plant (Reddy *et al*., 2004). In the present study, while there was 8.36% decrease in stomatal conductance of Shemal at 0h with pre-salinity treatments, the ratio was 7.4% in 71MAY69 compared to the control treatment, such ratios at 4h were respectively observed as 29.5 and 12.5% (Fig. 1). Parallel to these findings, Carcamo *et al*. (2012) also observed decreasing stomatal conductance values of maize plants under salt stress (100 mM NaCl). Contrarily, Rahnama *et al.* (2010) reported increased stomatal conductance values for different durum wheat genotypes under NaCl (50 mM) treatments. Such different responses against the similar salt treatments were mainly because of the type of plant experimented in those researches.

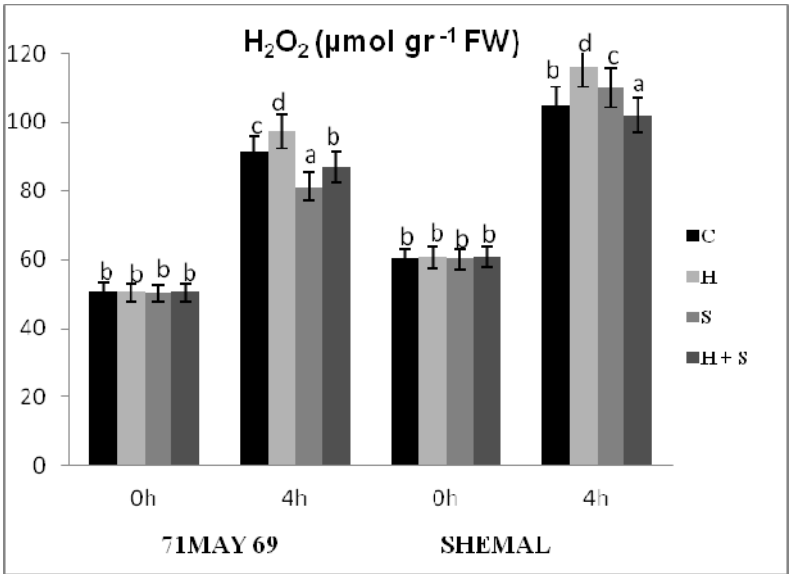

Fig. 2: Effects of salt pre-treatment under heat stress on hydrogen peroxide (H_2O_2) content in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. Different letters represent significantly different ($P < 0.05$) values

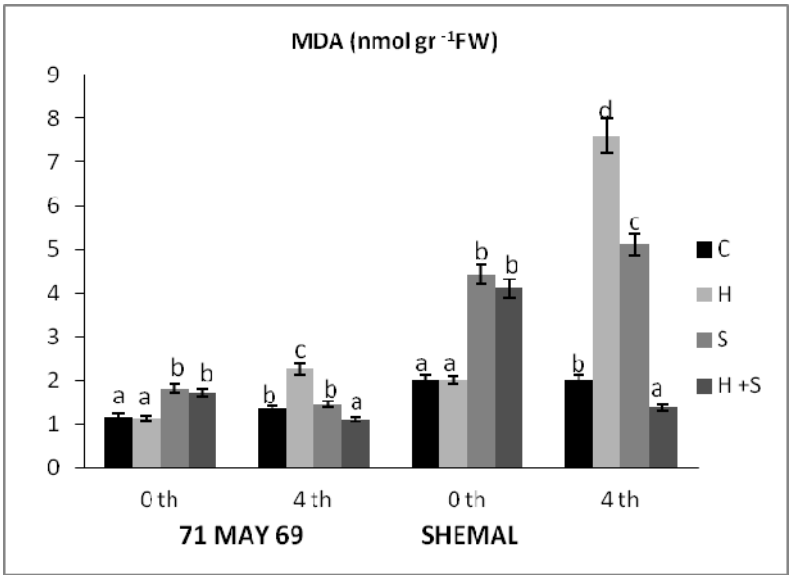

Fig. 3: Effects of salt pre-treatment under heat stress on malondialdehyde (MDA) content in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. Different letters represent significantly different ($P < 0.05$) values

When the stomatal conductance values of the control and heat treatments of the present study were compared, it was observed that while there was 16.2% increase in 71MAY69, 12.7% decrease was observed in Shemal. Similarly, Li *et al*. (2011) also observed increases in stomatal conductance values of *Suaeda salsa* plants. Higher increase ratios in stomatal conductance of tolerant maize cultivar than the sensitive one may be explained by plant tendency to open stomas and try to cool the leaves through respiration under heat stress (Ferreira-Silva, 2011). In the present study,

stomatal conductance values of both cultivars under heat stress with pre-salinity treatments were not different from the values of control treatment. Such unchanged values in both tolerant and sensitive cultivars indicated that pre-salinity treatments increased the resistance to heat stress. In addition, compared to single heat treatment, heat stress with pre-salinity treatment resulted in 34.8% increase in stomatal conductance of the tolerant cultivar and 12.7 % increase in the sensitive cultivar. Such findings indicated that pre-salinity treatment improved the decrease in stomatal conductance of sensitive cultivar. Present findings comply with the results of previous studies conducted on *Jatropa cucas* and *Pisum sativum* L. plants (Silva *et al*., 2013; Pandolfi *et al*., 2012).

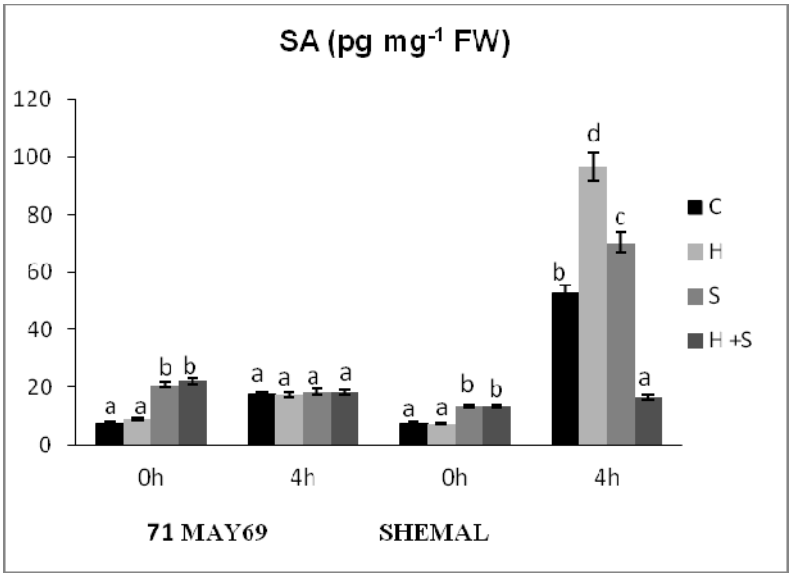

Fig. 4: Effects of salt pre-treatment under heat stress on salicylic acid (SA) content in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. Different letters represent significantly different ($P < 0.05$) values

Plants produce excessive reactive oxygen species under stress conditions. Plants also convert free-oxygen radicals into less harmful forms through antioxidant systems to prevent oxidative damages (Sharma *et al*., 2012). They convert them into H_2O_2, less harmful than $^{\cdot}O_2$, with the help of superoxide dismutase (SOD) and catalase (CAT) enzymes. They are detoxified through ascorbate-glutathione cycle with the impacts of ascorbate peroxidase (APX) and glutathione reductase (GR) and glutathione s-transferase (GST) enzymes with oxidation-reduction potentials (Shim *et al*., 2003). H_2O_2 levels did not change under salt pre-treatments compared to the control groups in both cultivar (Fig. 2). Parallel to current findings, salt stress-induced increases were also observed in H_2O_2 levels of rice and maize plants (Uchida *et al*., 2002; Gunes *et al*., 2007). Heat-induced increase in H_2O_2 levels was higher in the sensitive cultivar than the tolerant one. Similar increases in H_2O_2 levels of maize plants because of heat stress were also reported by Gong *et al.* (2001) and Mahmood *et al*. (2012). Compared to single heat treatment, while heat stress with pre-salinity treatment resulted in increase in H_2O_2 content of both cultivar (Fig. 2). These findings comply with the results of a previous study carried out with *Jatropa cucas* plants under salinity and heat stress (Silva *et al*., 2013).

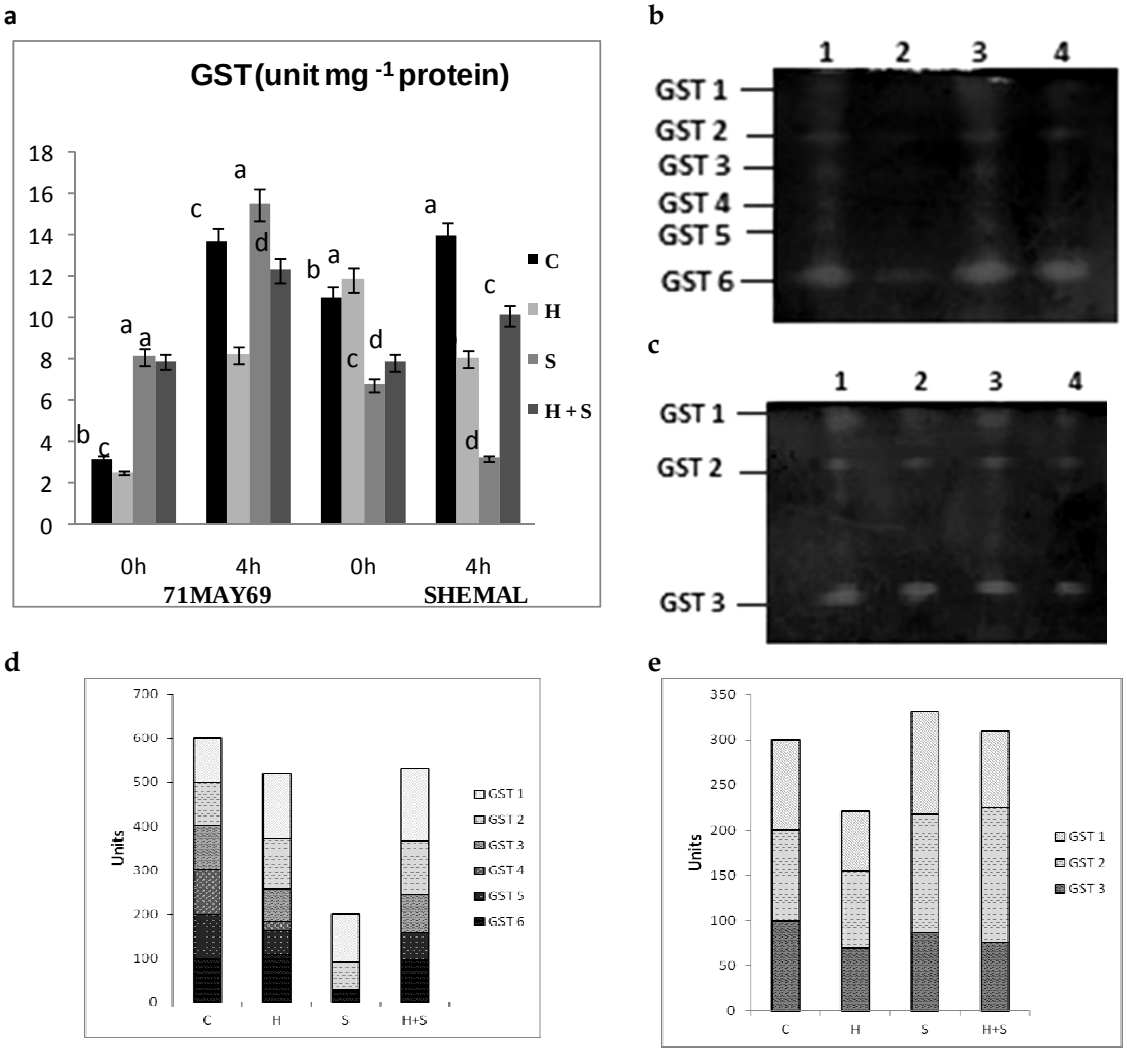

Figure 5a: Effects of salt pre-treatment under heat stress on total GST enzyme activity in tolerant (71May 69) and sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. Different letters represent significantly different ($P < 0.05$) values. **Fig. 5b:** Effects of salt pre-treatment under heat stress on GST isoenyzmes in sensitive (Shemal) maize (*Zea mays* L.) cultivars. **1:** 4 th Control **2 :** 4 th NaCl **3:** 4 th Heat **4:** 4 th NaCl + Heat. In densitometric analyses of GST activites, 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. F**ig. 5c:** Relative proportion of GST isoenzymes detected in sensitive (Shemal) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt. **Fig. 5d:** Effects of salt pre-treatment under heat stress on GST isoenzymes in tolerant (71May69) maize (*Zea mays* L.) cultivars. **1:** 4 th Control **2 :** 4 th NaCl **3:** 4 th Heat **4:** 4 th NaCl + Heat. In densitometric analyses of GST activites, 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. **Fig. 5e:** Relative proportion of GST isoenzymes detected in tolerant (71May69) maize (*Zea mays* L.) cultivars. C: Control, H:Heat, S: NaCl, H+S: Heat + Salt.

Malondialdehyde (MDA) is a lipid peroxidation product and oxidative damage on maize leaves of the present study were determined based on (MDA) contents while salt pre- treatments did not result in significant changes in MDA content of 71MAY69 at 0h and 4h hour compared to the control treatment, while 48 % and 62.9 increased in MDA content in Shemal (Fig. 3). Increases in MDA content of maize leaves were also reported in previous studies (Azevedo Neto *et al*., 2006). Similarly, current findings indicated that salt pre- treatments (50 mM) slightly increased MDA content of the sensitive maize cultivar and did not yielded any significant changes in MDA content of the tolerant cultivar. Maintenance of resistance also under salt stress of heat tolerant cultivar may be resulted from combined response to stress factors. Heat treatment increased MDA content in 71MAY69 by 69.2 %, while 3.7 fold in Shemal.MDA content decreased 51.3 % in 71MAY69 under pre-salinity with heat treatment at 4h compared to single heat treatment, while this decrease ratio in Shemal was 82.8 %. Such findings indicated that pre-salinity treatments improved salt-stress damage. Similar to current findings, hydrogen peroxide, salicylic acid and NaCl like substances were used to improve stress tolerance of plants (Pandolfi *et al*., 2012; Azevedo Neto, 2005).

The highest increase in internal SA levels was observed in sensitive cultivar at 4h (Fig. 4). Salicylic acid plays a role in plant defense system against pathogen stress. However, various other abiotic stress conditions also play a role in defense system (Shakirova *et al*., 2003). Borsani *et al*. (2001) reported that external salicylic acid treatments provided a protection to *Arabidopsis* leaves against salt stress. Distinctive increases in internal SA levels of the sensitive cultivar with pre-salinity treatments may indicate that this hormone was a stress indicator for maize leaves under salt stress. These findings also comply with malondialdehyde, a lipid peroxidation product indicating oxidative damage, findings of the study (Fig. 3). Salicylic acid may stimulate hydrogen peroxide and allows the plant to detect the stress factors (Rao *et al*., 1997). It was also observed in the present study that increased SA values triggered hydrogen peroxide contents exactly in Shemal (Fig. 4). Compared to the control treatment, single heat treatments resulted in increase in SA values of the sensitive cultivar at 4h, but there were not any significant changes in the tolerant cultivar. Similarly, Dat *et al*. (1998) reported that salicylic acid increased the plant resistance against heat stress-induced damage. Nevertheless, compared to single heat treatment, heat stress with salt pre- treatment decreased internal SA values of the sensitive cultivar and did not change the values of the tolerant cultivar. These findings indicated that heat stress increased internal salicylic acid content of maize leaves, but salt pre-treatments degraded such impacts.

Glutathione-*S* transferase (GST; EC 2.5.1.18) is an enzyme group effective against adverse environmental conditions (Dixit, 2001; Lannelli *et al*., 2002; Haluskova *et al*., 2009). It is commonly evaluated against biotic stress conditions, but recently has been evaluated against abiotic stress factors, too. In the present study, while pre-salinity treatments decreased GST enzyme activity of the sensitive cultivar, an increase was observed in GST enzyme activity of the tolerant cultivar. Such a difference in tolerant one can be explained by antioxidant defense system against oxidative damages. Parallel to these findings, Gapinska *et al*. (2008) also reported increasing GST enzyme activities of tomato plants under salt stress. Compared to single heat treatment, heat stress with pre-salinity treatment increased GST enzyme activities of both cultivars at 4h (Fig. 5a, 5c). These results indicated that the damage of heat stress on maize leaves was somehow eliminated by pre-salinity treatments through improving GST enzyme activity. Similarly, increasing GST enzyme

activities were also reported for wheat seedlings under heat stress (Haluskova *et al*., 2009). The increase in GST enzyme activities of the present study may be explained by low MDA contents under heat stress with pre-salinity treatments. In our results, the number isoenzymes were higher in Shemal than 71MAY69 (5a, 5c). Parallel to current findings, Polidoros and Scandolios (1999) also reported that higher hydrogen peroxide levels induces *GST1* gene expression. Also GST isoenzymes were agreement with total GST activity results. Salt pre-treatment increased GST1 and GST2 activities in 71MAY69 but it decreased GST2 and 6 in Shemal. Moreover, heat stress reduced GST isoenzymes activities in both of cultivar, while it was higher in Shemal. At 4h, heat stress with pre-salinity treatment, compared to single heat treatment increased GST1 and GST2 of both cultivars at 4h (Fig. 5b, 5d).

Conclusion

The present findings put forth, the promoting effect of salt pre- treatments on increasing heat stress tolerance of maize plants. Otherwise, for the first time, it was concluded that GST enzyme may be triggered by low-concentration NaCl treatments under heat stress and internal SA levels may increase hydrogen peroxide contents in sensitive leaves. Otherwise increased total GST enzyme and isoenzyme activity in combined treatments according to treatment alone (salt, heat) lead to decrease the hydrogen peroxide levels. Collectively, it could be said that SA plays role a signal molecule for GST activity changes by decreasing it with salt, heat treatment alone but increasing in the heat and salt treatment.

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