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Temperature induced changes in anti-oxidative stress metabolism in maize

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ABSTRACT

Anti-oxidative system in plants comprising of enzymatic and non-enzymatic antioxidants imparts stress tolerance by scavenging/detoxification of excess reactive oxygen species (ROS) produced under high temperature stress. Present investigation deals with the estimation of metabolites and anti-oxidative enzyme activities in four inbred maize lines; NSJ221, NSJ189, PSRJ13099 and RJR270 in response to high temperature stress imposed at reproductive stage by staggered sowing. An increase in H_2O_2 and malondialdehyde (MDA) was observed in all the genotypes, however the increase was higher in PSRJ13099 and RJR270. The activities of studied enzymes increased in NSJ189 and NSJ221 while a decrease was observed in PSRJ13099 and RJR270. Under heat stress isoforms of SOD increased in NSJ189 and NSJ221 while a concomitant decrease was observed in PSRJ13099 and RJR270. Two new SOD-isoforms were also observed in NSJ221. GPX showed more number of high mobility isoforms with low activity in NSJ221 and less mobile isoforms with higher activity in both NSJ189 and NSJ221. Whereas, PSRJ13099 and RJR270 showed decrease band intensity of less mobile GPX-isoforms under heat stress. Activity of CAT-isoforms increased to a similar extent across the genotypes under heat stress. In case of non-enzymatic antioxidants, non-protein thiols increased in all the genotypes while the level of carotenoids depleted in all the genotypes except NSJ221. Ascorbate (AsA) levels depleted in PSRJ13099 and RJR270 and increased in NSJ189 and NSJ221 under heat stress. Understanding the intricate regulatory pathways in crop plants under heat stress will help in developing genotypes with enhanced stress tolerance.

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Anti-oxidative metabolism; heat stress tolerance; maize; climate change

Abbreviations

SOD – super oxide dismutase; APX – ascorbate peroxidase; POD – peroxidase; GPX – guaiacol peroxidase; CAT – catalase; AsA – ascorbate; NT – normal temperature; HT – high temperature

Introduction

One of the major aspects of global climatic variability that directly affects crop growth and production is rising atmospheric temperature. Atmospheric temperature is expected to rise by about 1 to 3.7°C by the year 2114 as per the report of the Intergovernmental Panel on Climatic Change (IPCC 2013). Predicted global warming has become a serious threat for sustainable agriculture worldwide and imposed an increasing challenge to improve plant tolerance to temperature elevation. Significant coherent increasing trends in mean temperature were observed in Southern states of India (Rathore et al. 2013). Substantial variation has been observed in maize germplasm in response to high temperature stress (Yadav et al. 2016). Plant adaptation to high temperature stress involves mechanisms at various levels, including changes in molecular, cellular, biochemical, physiological and growth responses (Wahid et al. 2007). Under heat stress production and consumption of energy is often unbalanced due to more severe delay in the dark reactions of photosynthesis than the absorption of light and electron transport (Dash and Mohanty 2002). The electron flow driven by PS I was stimulated in contrast to sharp inhibition of that mediated by PS II (Foyer and Noctor 2005). Unbalanced flow of electrons causes the generation of reactive oxygen species (ROS). ROS are produced in response to heat-induced oxidative stress, including superoxide radicals (O_2-), hydrogen peroxide

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(H_2O_2), hydroxyl free radical ($OH^•$), and singlet oxygen ($^1O_2$). Additional to abiotic/biotic stress induction, plants produce ROS as primary metabolic by-products of chloroplastic and mitochondrial electron transport system. Simultaneously, plants have excellent ROS detoxification system, collectively called antioxidative defense system, to maintain cellular redox homeostasis (Noctor 2006). Aberrations in environmental patterns disturb the equilibrium state of redox balance by causing a quick increase in ROS production. The cellular redox equilibrium state has been anticipated to be an environmental sensor and signal among a variety of aspects of plant metabolism (Yadav et al. 2016). No sooner a plant senses a redox imbalance, an acclimation response is induced and irreversible damage is avoided.

The prevailing view is that, high temperature causes an up-regulation of antioxidative metabolism in plants (Sedlak and Lindsay 1968; Yadav et al. 2015). Nevertheless, the direct evidences for this up-regulation are variable, dependent on the temperature intensity, duration of high temperature, rate of raise in temperature and the plant species and components of antioxidative metabolism investigated. Similarly, reports investigating the effects of elevated temperature on crop production show contrasting responses of individual components of the antioxidant system (Lowry et al. 1951). To have a better universal understandings of the effects of heat stress on the antioxidant system and to find out biochemical indices and mechanisms of heat tolerance, which can further be used to develop maize varieties which could be grown under relatively warmer environments. The objective of the present investigation was to assess the effect of high temperature stress on anti-oxidative stress metabolism during reproductive stage in maize and also to determine the variability in response in the four genotypes.

Materials and methods

Plant growth conditions and temperature treatment

Four maize (*Zea mays* L.) genotypes; PSRJ 13099, RJR 270, NSJ 189 and NSJ 221) with variable sensitivity to high temperature stress were selected for the present study. Genotypes PSRJ 13099 and RJR 270 were observed to be relatively sensitive to high temperature while NSJ 189 and NSJ 221were found to be tolerant, especially during fertilization and grain filling stage. Maize genotypes used in the present study were received from ICAR-NBPGR, New Delhi. The experiment was carried out at ICAR-Central Research Institute for Dryland Agriculture, Hyderabad, situated on the latitude of 17° 35` North and longitude of 78° 50` East. Maize plants were raised in field on two different dates of sowing, one in the end of January (normal sown) and other in the end of February (late sown) during 2015 with 30 days interval in such a manner that the February sown crop was exposed to high temperature stress during reproductive stage (Fig. 1A). A template was used for sowing to maintain the uniformity in distance and depth of seeds. Two seeds per hole were sown to ensure uniform population. After germination, one healthy seedling was retained at each plant position subsequent to thinning/gap filling. For gap filling, seeds imbibed with water were used.

Recommended dose of NPK (300–150–125 kg ha^{-1}) was applied. Full dose of P, K and 1/8 of total N was applied at sowing, while remaining 1/5 N at four-leaf stage, 1/3 N at grand growth stage and 1/3 N was applied just before flowering (Rehman et al. 2011). All the recommended cultural practices were kept constant for all the genotypes for uniform growth.

The plants were irrigated regularly to avoid any visible water deficit symptoms. These experiments were laid out in adjacent field to minimize drift in soil conditions. The data were recorded on 3 randomly selected plants of each genotype for both sowing dates.

Sampling

Leaf samples (0.2 g to 0.5 g) were collected for various biochemical parameters at grain filling stage. Fresh leaf tissues from third/fourth fully expanded leaf were collected from selected plants of each genotype at sampling dates to measure enzymatic and non-enzymatic antioxidants, soluble proteins and dry weight. Samples for enzyme analysis were dipped in liquid nitrogen to freeze the biological activities instantly and stored at –30°C until use.

H_2O_2 and MDA estimation

For the estimation of hydrogen peroxide and MDA content the extraction was made by homogenizing fresh leaves in 0.1% trichloroacetic acid (TCA) under cooling conditions. H_2O_2 was estimated according to Velikova et al. (2000). Briefly supernatant was

Figure 1. Agrometeorology Data; A. Temperature, B. Relative humidity and C. Sun shine hours for the months during cropping season for the year 2015. Data presented as average of each 10 days during the cropping season. MAXT = maximum temperature; MINT = minimum temperature; RH1 and RH2 = relative humidity; SS = sun shine hours; Av T = average temperature for the complete month.

incubated with the reaction mixture containing 0.1% TCA, 100 mM K-phosphate buffer and 1 M potassium iodide (KI) for 1 hr in dark. The blank probe consisted of 0.1% TCA in the absence of leaf extract. After incubation, absorbance was recorded at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentration of H_2O_2. Lipid peroxidation level in maize leaves was estimated in terms of MDA content as described by De Vos et al. (1991). The final concentration of MDA was calculated using an extinction coefficient € = 155/mM/cm and expressed as µmole/g dry wt content.

Determination of carotenoids and ascorbate

Total carotenoids were quantified as per method described by Kirk and Allen (1965). The amount of carotenoids was calculated by using formula

$$\text{Carotenoids (µg/g fr wt)} = A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})$$

where, A = Absorbance at respective wave length

Ascorbate content was quantified as per method described by Jagota and Dani (1982). For ascorbate estimation, 0.2 g fresh leaves were homogenized in extraction solution containing 3% TCA amended with 1mM EDTA and centrifuged at 10,000 g for 15 min. Absorbance was recorded at 720 nm and ascorbate concentration was calculated by using standard curve for L-Ascorbate.

Non-protein thiol quantification

Non-protein (acid soluble) thiols were measured according to Sedlak and Lindsay 1968 by using Elman`s reagent (5–5, dithiobistrinitrobenzoic acid, DTNB). Leaf samples (0.2 g) were homogenized in 0.2% trichloroacetic acid (TCA) containing 20 mM EDTA, centrifuged at 12000 g for 15 min. One ml supernatant was added in 9 ml of reaction mixture (containing 5 mM EDTA and 0.6 mM DTNB in 120 mM sodium phosphate buffer, pH

7.5), mixed thoroughly and incubated for 15 min and absorbance was recorded at 420 nm. Concentration of non-protein thiol was calculated by using reduced GSH as standard and expressed as mg/g dry wt.

Antioxidative enzyme assays

The activities of four anti-oxidative enzymes, Catalase, Peroxidase, Guaiacol Peroxidase and Superoxide Dismutase were measured in crude extracts following standard assay procedure described below. Extracts and temperature-sensitive reagents were maintained at 4°C until individual assays were performed. In order to express enzyme activity on a protein basis, the protein content of the extracts was estimated following Lowry et al. (1951).

Superoxide dismutase (SOD)

Plant leaves were homogenized in potassium-phosphate buffer (50 mM, pH 7.8) containing EDTA (1 mM) and a pinch of polyvinyl poly pyrrolidone (PVPP). Homogenate was centrifuged at 15,000 g for 15 min. The SOD activity was determined by measuring its ability to inhibit the photo-reduction of nitro blue tetrazolium chloride (NBT), as described by Beyer and Fridovich (1987). Three cm^3 reaction mixture contained 13 mM methionine, 25 µM nitro blue tertazolium, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium bicarbonate and 100 µl crude enzyme. The reaction was initiated by the addition of 2 µM riboflavin into reaction mixture and incubated at 30°C for 15 min under two 15 W florescent lamps. Two enzyme blanks were run parallel to the reaction mixture. One blank was irradiated under light while another was kept in dark. Irradiated enzyme blank developed maximum color. Reaction was stopped by switching off the light. Color intensity of the chromogen in the reaction mixture was measured at 560 nm against non-irradiated blank. One unit of SOD activity (U) was defined as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with irradiated blank and the results were expressed as µmol/min/mg protein or U/mg protein.

Guaiacol peroxidase (GPX)

GPX activity was determined by the method of Tatiana et al. (1999). The reaction mixture contained 1% guaiacol, 0.3% H_2O_2, appropriate concentration of buffer and enzyme extract. The reaction was started by the addition of H_2O_2. Increase in absorbance (A_{470}) because of the oxidation of guaiacol was recorded at every 30 sec interval for 3 min. Concentration of oxidized guaiacol was calculated by using its molar extinction coefficient € = 26.6/mM/cm. One GPX unit was defined as the amount of enzyme that produced 1µmol/min oxidized guaiacol and expressed as µmol/min/mg protein or U/mg protein.

Catalase (CAT)

CAT activity was measured by determining H_2O_2 consumption following Summermatter et al. (1995). Leaf sample (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing EDTA (20 mM) with pinch of polyvinyl poly pyrrolidone (PVP). Homogenate was centrifuged at 15,000 g for 20 min at 4°C. The reaction mixture contained potassium-phosphate buffer (50 mM, pH 7.0), EDTA (0.1 µM) H_2O_2 (60 mM) and enzyme extract. Linear change in $A_{240}$was monitored at 15 sec interval for 3 min. The decrease in absorbance A_{240} due to breakdown of H_2O_2 was calculated using molar extinction coefficient € 36/mM/cm. One CAT unit was defined as the amount of enzyme that hydrolyzed 1 µmole H_2O_2/min and expressed as µmol/min/mg protein or U/mg protein.

Peroxidase (POD) assay

Crude enzymes extraction was done by the homogenizing leaf samples in 50 mM Tris-buffer (pH 7.5) amended with 1% polyvinyl pyrrolidine (PVP). The homogenate was centrifuged at 10,000 g for 10 min at 4°C and the clear supernatant was taken for peroxidase (POD) assay. Peroxidase activity was determined according to Chance and Maehly1995. The reaction mixture contained 50 mM sodium-acetate buffer (pH 5.4), O-dianisidine solution (0.5%) and H_2O_2 (0.042%). The increase in absorbance at 460 nm was recorded for 3 min at 15 sec interval. One POD unit was expressed as change in absorbance/min/mg protein and quantified by its molar extinction coefficient € = 11.3/mM/cm.

Native PAGE and in gel activity assay

Crude extracts for the respective enzymes were used for activity gel analysis. Samples were applied with Laemmli (1970) buffer without SDS. Electrophoretic separation of SOD and GPX was performed at a constant voltage of 200 V while for APX at 100 V at 4°C. For SOD, 4% stacking and 10% resolving non-denaturing,

non-reducing polyacrylamide gels were used whereas for GPX and CAT, 3% and 5% stacking and 8% resolving polyacrylamide gels, respectively, were used. After electrophoresis gels were stained according to respective protocols for the enzyme.

SOD activity was detected according to Rao et al. (1996) with slight modifications. After completion of electrophoresis the gels were incubated in a solution containing 2.45 mM NBT for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM riboflavin and 28 mM TEMED under dark. Expression of SOD isoforms (achromatic bands against dark blue background) was achieved by light exposure for 10–20 min at room temperature.

GPX was assayed according to Ros Barcelo et al. (1987) with slight modifications. Gels were incubated in guaiacol-benzidine solution for 5 min and subsequently H_2O_2 was added to the same solution. GPX isoforms appeared as brown-orange bands against a white background.

CAT activity was stained by the method described by Clare et al. (1984). Followed by electrophoresis, gels were rinsed in distilled water. Washed gels were incubated in 50 mM phosphate buffer, pH 7 amended with HRP for 45 min in dark. Subsequently, 5 mM H_2O_2 was added in the above solution and incubation was resumed for 10 min. Again gels were washed twice with distilled water and incubated in phosphate buffer containing DAB up to the appearance achromatic bands against brown-orange background.

Statistical analysis

The experimental data were statistically analyzed using two-way analysis of variance (ANOVA).

Results

The prevailing environmental conditions during the crop growth period are shown in Fig. 1A–C. ANOVA results of the studied parameters showed significant differences among genotypes, treatments and their interactions (Table 1). H_2O_2 is a signal molecule during abiotic stress and lipid peroxidation is widely used stress indicator of plant membranes got accumulated significantly under high temperature stress (second sowing) in all the genotypes. Mean values confirmed that high temperature stress led to increased H_2O_2 content in all the genotypes and it ranged from 0.24 to 0.73 mg/g dry wt. It was higher in the sensitive genotypes as compared to tolerant ones and highest H_2O_2 content was observed in RJR 270 under second sowing as compared to first sowing [0.26 mg/g dry wt] (Table 2). Among sensitive genotypes the increase observed in H_2O_2 content was much higher (up to 183% in RJR 270) as compared to the tolerant ones. Similar trends were also observed for MDA content with a substantial accumulation in sensitive genotypes. The results were highly significant for the two sowing dates. Highest increase in MDA content was observed in PSRJ 13099 followed by RJR 270 with highest percent increase in PSRJ 13099 (172%) followed by RJR 270(157%) (Table 2).

Non-enzymatic antioxidants

The non-protein thiol content in the leaves of tolerant genotypes was observed to be highest under second date of sowing. Factorial analysis results indicated that the maximum and minimum content of thiol was observed in NSJ 221 [1.41 and 0.52 mg/g dry wt] in second and first sowing, respectively. This highest increase of 169% under stress condition when compared to control is a clear indication of the involvement of the parameter in imparting tolerance. The same trend was followed by NSJ 189 with an increase of 107%. Heat sensitive genotypes (PSRJ 13099 and RJR 270) showed relatively lower accumulation of non-protein thiol content, 47% and 86%, respectively (Table 3).

Table 1. Analysis of variance for enzymatic and non-enzymatic antioxidants in maize genotypes exposed to high temperature stress by sowing on two different dates (DOS).

Source	df	SOD	GPX	CAT	POD	MDA	H_2O_2	Non-protein thiols	Ascorbate	Carotenoids
Replications	2	0.0012	0.18	0.47	5486.21	7.70	0.000002	0.12	0.24	0.02
Genotypes	3	4.01**	2.31**	2810.35**	337109.09**	512.88**	0.11**	0.24**	63.02**	0.40**
DOS	1	0.034*	8.45**	12.14^{NS}	190939.74**	28831.57**	0.05**	4.57**	907.37**	4.07**
Genotypes X DOS	3	1.91**	13.80**	500.25**	1835744.38**	497.31**	0.025**	0.08*	221.15**	2.04**
Error	14	0.01	0.04	5.47	19842.89	13.49	0.0002	0.02	0.22	0.01
Total	23	0.78	2.51	435.71	304272.74	1394.19	0.02	0.26	76.67	0.50

NS non-significant; ** Significant at 1 % level; * significant at 5 % level

Table 2. Mean, standard Error and percent change for the characters evaluated in four maize genotypes sown on two different dates. '–' indicates % increase.

Character	First date of sowing (NT)				Second date of sowing (HT)				% Change			
	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	PSRJ 13099	RJR 270	NSJ 189	NSJ 221
SOD U/mg protein	5.6 ±0.05	5.0 ±0.02	5.1 ±0.06	6.2 ±0.09	4.9 ±0.02	4.1 ±0.03	6.6 ±0.03	7.7 ±0.08	12	20	–29	–23
GPX U/mg protein	6.0 ±0.13	5.5 ±0.09	4.4 ±0.14	3.7 ±0.08	2.7 ±0.09	1.6 ±0.33	6.2 ±0.13	6.4 ±0.20	55	72	–41	–72
CAT U/mg protein	89.6 ±1.83	59.8 ±1.53	84.9 ±1.03	40.7 ±1.11	65.1 ±0.88	70.8 ±1.65	101.7 ±2.02	43.2 ±1.95	27	–18	–20	–6
POD U/mg protein	3814.2 ±51.5	3727.3 ±130.5	3128.0 ±88.1	3290.9 ±104.6	3021.3 ±65.0	2961.4 ±112.1	4295.3 ±129.2	4395.9 ±17.7	21	21	–37	–34
MDA µmole/g dry wt	48.7 ±2.53	55.0 ±1.03	57.1 ±1.34	48.7 ±1.91	132.4 ±2.52	141.2 ±3.21	107.9 ±3.70	105.4 ±2.79	–172	–157	–89	–117
H_2O_2 mg/g dry wt	0.24 ±0.01	0.26 ±0.00	0.25 ±0.01	0.29 ±0.01	0.55 ±0.01	0.73 ±0.00	0.38 ±0.01	0.35 ±0.01	–129	–183	–51	–21
Non-protein thiols mg/g dry wt	0.56 ±0.03	0.65 ±0.02	0.60 ±0.03	0.52 ±0.01	0.83 ±0.06	1.21 ±0.14	1.25 ±0.31	1.41 ±0.11	–47	–86	–107	–169
Ascorbate mg/g dry wt	20.3 ±0.68	17.1 ±0.05	14.7 ±0.09	19.3 ±0.12	7.7 ±0.00	7.2 ±0.16	17.5 ±0.05	17.5 ±0.38	62	58	–19	9
Carotenoids mg/g dry wt	3.8 ±0.07	3.0 ±0.05	3.0 ±0.15	2.1 ±0.05	1.5 ±0.01	1.8 ±0.00	2.7 ±0.08	2.5 ±0.06	60	40	9	–22

The results were statistically significant for AsA content. The mean values of data show randomness in AsA content with respect to genotypes and treatments. The interaction studies concluded that the AsA content increased only in NSJ 189 under high temperature stress (19%), while it decreased in the other three genotypes, NSJ 221, RJR 270 and PSRJ 13099 by 9%, 58% and 62%, respectively.

The data on the effect of heat stress on carotenoids content was observed to be highly significant. It got reduced significantly in sensitive genotypes under high temperature stress. However, tolerant genotypes retained higher carotenoids level even under high temperature stress. High temperature reduced carotenoids content in genotypes PSRJ 13099 and RJR 270 by 60 and 40% respectively, while NSJ 221 reported an increased by 22%.

Antioxidant enzymes

The results presented in Table 1 revealed a variable degree of stimulation in the activities of SOD, GPX, CAT and POD in leaves of maize under high temperature stress. Enzyme induction was significantly correlated with the high temperature stress. Results for NSJ 189 and NSJ 221 indicated stimulation in the activity of all the ROS scavenging enzymes under high temperature stress conditions. Data presented in Table 2 shows trends of the activity of superoxide dismutase in the non-stressed and heat stressed conditions. For superoxide dismutase the mean values varied from 4.1 to 7.7. SOD activity increased under high temperature stress, indicating high temperature stress had a stimulatory effect on the enzymes except in sensitive genotype RJR 270 followed by PSRJ 13099. The tolerant genotypes NSJ 189 and NSJ 221 recorded an increase in the activity of SOD by 29% and 23%, respectively.

High temperature stress stimulated the POD activity by 34% and 37% in NSJ 221 and NSJ 189 with the mean values of 4396 and 4295 as compared to first sowing values 3291 and 3128, respectively. Whereas a significant decrease of around 21% was observed in PSRJ 13099 and RJR 270 under second sowing compared to first sowing. Similarly an increase in GPX activity was observed in the leaf tissue of tolerant genotypes under high temperature stress. Genotype NSJ 221 showed greater up-regulation in GPX activity (72%) than NSJ 189 (41%). Among sensitive genotypes, RJR 270 was more severely affected and high temperature stress led to significant inhibition of GPX activity (Table 2). PSRJ

13099 was comparatively less affected and showed a decline of 55% in leaf GPX activity.

High temperature stress did not cause any substantial change in CAT activity in sensitive and tolerant genotypes. However, all the genotypes except PSRJ 13099 showed an increase in CAT activity under high temperature stress.

Electrophoretic analysis of enzyme activities

When leaf extracts were subjected to native PAGE and examined for SOD activity, variable number of SOD isoforms was observed across the genotypes in first sowing as well as second sowing samples indicating the influence of temperature (Fig. 2). Variation in intensity and number of SOD isoforms was noticed among genotypes under high temperature conditions. Under the influence of high temperature, the intensities of Mn-SOD, Fe-SOD and Cu/Zn-SOD increased in all the genotypes except PSRJ 13099. However, under the influence of high temperature, Mn-SOD (SOD-2), Fe-SOD-2 and Cu/Zn-SOD-1,2 disappeared in PSRJ 13099 but the intensities of other Cu/Zn-SOD-3,4 isoforms were selectively enhanced (Fig. 2). Significant enhancement in SOD isoforms intensity was observed in NSJ 189 and NSJ 221 under high temperature however, genotypes PSRJ 13099 showed decreased SOD activity pattern when compared with respective control. The intensity of SOD isoforms in different samples was well correlated with the levels of previously assayed enzyme activities.

Besides quantitative changes in the enzyme levels, variations were also observed in the intensities and number of isozyme bands during stress. In gel activity assays indicated variation in intensities and number of isozyme bands during high temperature stress. The GPX isozyme bands got intensified under stress (Fig. 3). Isozyme patterns of GPX showed marked variation in both number of bands and band intensities relative to their respective controls.

The native PAGE and in gel activity staining for CAT revealed the presence of two isoforms in all the samples. Exposure to high temperature enhanced the intensity of both the isoforms to a similar extent (Fig. 4). However, no new CAT isoform was observed under heat stress.

Discussion

Plants show a variety of responses to high temperature stress which are evident by unique symptomatic

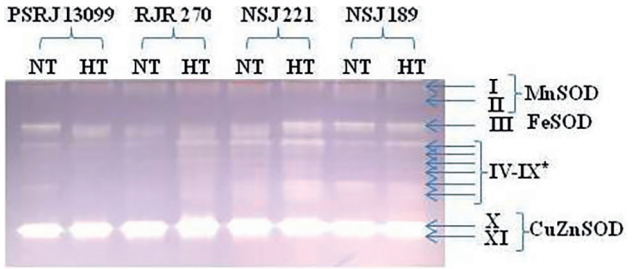

Figure 2. Electrophoretic mobility profiling of superoxide dismutase isoforms in maize genotypes at anthesis-silking stage of growth under normal (NT) and high temperature stress (HT) conditions, Clear zones depict the activity of SOD isoenzymes on the gel. Asterisks mark the location of some minor activities that were seen only occasionally.

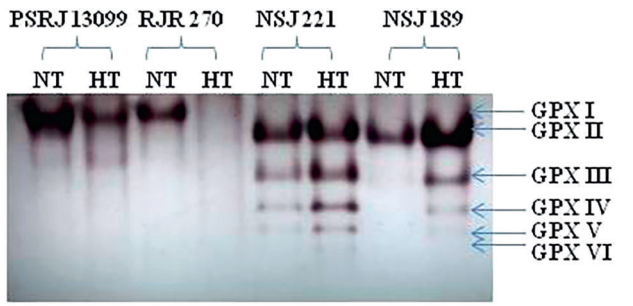

Figure 3. Isoenzymic pattern profiling of GPX in maize genotypes at anthesis-silking stage under NT and HT conditions, brown bands on gel depict the activity of GPX isoenzymes as shown by arrows.

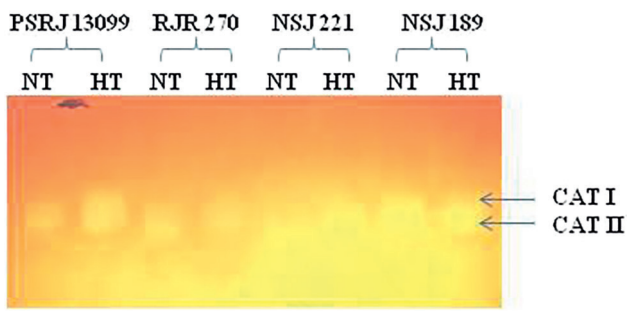

Figure 4. In-gel assay of CAT isoenzymes during Normal (NT) and High Temperature stress (HT) conditions in maize leaves at reproductive stage, Clear zones depict the activity of CAT isoenzymes on the gel.

and qualitative changes in growth and morphology at each phenophase of a crop species. In the present investigation, high temperature stress led to a significant increase in MDA content in all the genotypes (Table 2). However, the tolerant genotypes showed less increment as compared to the sensitive genotypes. Enhanced lipid peroxidation after high temperature stress suggests that high temperature caused oxidative damage; it showed a state of oxidative stress most likely due to generation of ROS. Under high

temperature stress, ROS generation is higher, inducing oxidative damage at cellular level. In plants, ROS are formed during electron transport in mitochondria, chloroplasts, peroxisomes and glyoxisomes (Jiang and Zhang 2002). These ROS are highly reactive and swiftly disrupt the normal cellular homeostasis. Cell membrane disruption is generally ascribed to lipid peroxidation, due to increased production of ROS. Membrane lipid peroxidation level is considered the best criterion to estimate the damage caused by rising ROS generation. Lipid peroxidation occurs when $OH^{\cdot}$ radicals, generated close to cellular membranes attack the polyunsaturated fatty acids (PUFA) side chains in membrane lipids. Resultant of the reaction is formation of lipid hydroperoxides (Bestwick et al. 2011). Increase in the level of lipid hydroperoxides in membranes disrupts their normal functions and can cause their collapse, leading to electrolyte leakage and the defeat of selective permeability (Saelim and Zwiazek 2000).

H_2O_2 is a toxic compound which is injurious to the cell, resulting in lipid peroxidation and membrane injury (Saelim and Zwiazek 2000). In the present study, H_2O_2 levels significantly increased under high temperature. Changes in H_2O_2 levels due to high temperature stress suggested that it influenced the antioxidant response mechanisms in maize. The genotypes; PSRJ 13099 and RJR 270, having higher H_2O_2 concentration showed higher lipid peroxidation in terms of MDA content. Similar trends have been observed earlier under sudden and extended heat stress in maize seedlings (Yadav et al. 2016). The lower values of MDA and H_2O_2 in genotypes NSJ 221 and NSJ 189 (Table 2) indicate that at cellular level these genotypes are better equipped with efficient ROS scavenging system that efficiently combated the excess of oxidants.

Plants have a well evolved antioxidant defense system to combat the threats posed by the stress induced oxidative damage. Since oxidative stress is triggered by several biotic/abiotic stresses, the biological activities of antioxidant defense system play a very vital role in neutralizing the negative impact of stress induced oxidative events. Antioxidant defense system includes both enzymatic and non-enzymatic components. Enzymatic antioxidant defense system comprised mainly of SOD, POD, GPX, CAT and APX (Meloni et al. 2003). Alterations in the plant antioxidant defense system have been observed to improve plant tolerance to oxidative stress (Yadav et al. 2016). Alterations in enzymatic antioxidants may be due to the increased synthesis of new isozymes or improvement of the pre-existing enzymes activity for the detoxification of ROS (Kang et al. 1999). In present study, swing in the levels of activities of SOD, POD, GPX and CAT were recorded in maize leaves (Table 1) indicated that these enzymes play a vital role in antioxidant defense.

To detoxify the reactive oxygen species, SOD, CAT and APX contribute together and interact in a very complex and coordinated manner. These interactions also engage other peroxidases like GPX and POD (Noctor and Foyer 1998). The extent of oxidative stress causing cellular damage might possibly differ depending on the stress imposed and the plants ability to combat. Plants may respond in different ways to different environmental stresses and also to the same stress and response would depend on intensity and duration of stress and phenophase of the crop. SOD serves as the first line of defense against ROS-mediated toxicity. SOD catalyzes the dismutation of superoxide into O_2 and H_2O_2. Under high temperature stress, increased in SOD activity suggested preparedness of plants to detoxify H_2O_2 as and when generated. Further H_2O_2 is hydrolysed by peroxidases and catalase depending on their location in cell. Catalase hydrolyses H_2O_2 into H_2O. Peroxidases detoxify H_2O_2 by the oxidation of co-substrates such as phenolic compounds or by using the antioxidants (Blokhina et al. 2003). Antioxidants like ascorbate, non-protein thiols and carotenoids are involved in scavenging H_2O_2 mainly via the Halliwell-Asada pathway (Horemans et al. 2000). It has been suggested that hydrogen peroxide must be effectively scavenged in order to minimize cytotoxicity by ROS in the presence of increased SOD activity (Scot et al. 1987). Coordinated increase of ROS detoxifying enzymes in NSJ 221 and NSJ 189 was effective in protecting the plant from the accumulation of ROS under high temperatures, thus averting membrane and cellular damage under adverse conditions. However, a rise in accumulation of reactive oxygen species in PSRJ 13099 and RJR 270 was not accompanied by a corresponding increase in anti-oxidant defense system indicating vulnerability of these genotypes to high temperature stress. As a result, the level of MDA under high temperature stress was higher in genotypes PSRJ 13099 and RJR 270 than the genotypes NSJ 221 and NSJ 189. However, the lesser degree of lipid peroxidation reflected by low MDA content and the coordinated increase in the ROS detoxifying enzymes was observed under

high temperature stress. This indicated that the genotypes NSJ 221 and NSJ 189 have a higher capacity for scavenging ROS at high temperature than the genotypes PSRJ 13099 and RJR 270. The diverse response of enzymatic antioxidants due to high temperature stress suggested their possible role in imparting stress tolerance in four maize genotypes. It appears that the difference in SOD, CAT, POD and GPX activity has a direct relation to the tolerance of the two NSJ 221 and NSJ 189.

Peroxidase activity estimation is very crucial under abiotic stress conditions because it is an essential enzyme for a variety of cellular functions such as cell wall biosynthesis, plasticity and lignifications which may be altered upon exposure to relatively high temperature. Rice cultivars exposed to chilling stress showed decreased CAT and POD activity in sensitive cultivars whereas increased activities of these enzymes have been found in tolerant cultivars (Saruyama and Tanida 1995). Similarly in maize at low temperature, the activity of SOD and APX of the tolerant variety were higher than in controls. Differential responses of the activities of SOD, APX, CAT and POX to drought stress were observed in drought sensitive and tolerant maize seedlings (Chugh et al. 2011). Effect of thermal stress on the antioxidant system in *Eupatorium* species also showed different responses of antioxidative enzymes in tolerant and sensitive species. Several environmental stress studies in plants are known to induce the expression and increasing levels of antioxidative enzymes and their mRNAs (Jithesh et al. 2006).

Findings from the present study suggested that there was a coordinated and differential increase in the activity of ROS detoxifying enzymes in the four genotypes of maize which might be necessary to protect plants from the accumulation of ROS at high temperatures. Differences in activities of the enzymatic antioxidants, content of non-enzymatic antioxidants, H_2O_2 and MDA content in the genotypes studied may be ascribed to differences in mechanisms underlying oxidative stress injury and subsequent tolerance to high temperature stress.

Although, induction and increased expression of antioxidative enzymes have been reported earlier, the mechanism by which the antioxidant system get activated under high temperature stress is not yet well understood. Further studies on the regulation of gene expression of antioxidative enzymes, identification of the genes that regulate antioxidative enzymes under high temperature are needed for making a better understanding of the mechanisms of high temperature stress tolerances. Future goal involves studying the long-term responses of the antioxidant system, to further elucidate the role of enzymatic and non-enzymatic components in imparting high temperature stress tolerance in maize.

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