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Exogenous Application of Bio-Regulators for Alleviation of Heat Stress in Seedlings of Maize

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Research Article

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Abstract

High temperature is an important environmental stress that influences the growth and development of crop plants thus adversely affecting their production and productivity. An experiment was carried out to investigate the protective role of bioregulators viz., Putrescine (Put), Thiourea (TU) and Hydrogen Peroxide (H_2O_2) against high temperature stress in maize at early vegetative stage. Maize genotypes earlier identified as heat tolerant (NSJ 221 and NSJ 189) and heat sensitive (RJR 270 and PSRJ 13099) were chosen for the present study. Independent foliar spray of Put (4 mM), TU (20mM) and H_2O_2 (1.2 mM) was observed to have significant difference ($P>0.01$) between genotypes, treatments and their interaction when applied 72 hrs prior to heat exposure. High temperature stress led to disruption of cellular membrane by increasing cell membrane injury, lipid peroxidation and H_2O_2 contents. It led to decrease in total chlorophyll content, soluble proteins, quantum yield and POD activity. Increased SOD activity in heat stressed seedlings was recorded with genotypes NSJ 189 and NSJ 221. Foliar application of Put, TU and H_2O_2 ameliorated heat-induced damages by stimulating the antioxidant enzyme system through decrease in lipid peroxidation, membrane injury and H_2O_2 contents in all the genotypes when compared with untreated heat stressed seedlings. Spray with these chemicals resulted in an increase in chlorophyll content, quantum yield and activities of anti-oxidative stress enzymes. Genotypes RJR 270 and PSRJ 13099 recorded improved heat tolerance with spray either of these chemicals by enhancing their biochemical potential. Spray of Put or TU was observed to be more effective of improving heat stress tolerance of maize seedlings.

Keywords: Anti-oxidative enzymes; Foliar spray; Maize; High temperature

Introduction

With increasing frequency of climate change and variability, plants are frequently subjected to various environmental stresses such as water deficit, freezing, heat and salt stress [1-7]. Temperature is an important environmental stress with both low and high temperatures affecting plant growth and development at whole plant level, tissue and cell level and even at sub-cellular level. High temperature induced a marked

decrease, although variable, in growth of maize genotypes [8]. Elevated temperature caused substantial decrease in shoot dry mass, relative growth rate (RGR) and net assimilation rate (NAR) of maize [9]. Direct injuries due to high temperatures in plants include protein denaturation and their aggregation and increased fluidity of membrane lipids. Indirect injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity. High temperature induces numerous biochemical responses including increased production of reactive oxygen species (ROS) that disrupts normal metabolism of plants causing lipid peroxidation, protein denaturation and DNA damage [10,11]. ROS are harmful to all cellular compounds and negatively influence cellular metabolic processes. Nature has provided all the organisms with self repair mechanisms varying in extent to alleviate the damage by high temperature stress. The detoxification of these ROS is very important and plants have evolved complex strategies to deal with them. The plant cells typically respond to increases in ROS levels by increasing the expression and activity of ROS-scavenging enzymes and increasing the production of antioxidants in order to maintain redox homeostasis.

Plant stress tolerance can be improved with the exogenous use of stress alleviating chemicals. Some of the important plant bioregulators tested act in low concentrations to inhibit, promote or modify the morphological, physiological and biochemical processes of the plants. These substances can be applied directly to plant leaves, fruit and seed provoking alterations of vital and structural processes [12]. Previous research evidences suggested that exogenous application of Polyamines (di- and tri- and tetra-amines) regulate the enzyme activities and also be exploited for increasing tolerance to salinity, cold, drought, heavy metal, osmotic stress, high-temperature, water logging flooding tolerance and involved in nearly all developmental process in various crop plants [13]. These regulations may be attributed to the potential effect of putrescine (Put) which acts as free radical scavenger and plant cell membrane stabilizer [14]. Thiourea (TU) is a non-physiological thiol and has also been employed by various researchers to impart stress tolerance and improve yield of crops like wheat, mungbean, potato and maize [15-18].

Many stress alleviating agents including thiols are crucial for enhancing the crop productivity as they improve the metabolic imbalances produced in a cell during stress. Thiols are well-known to maintain the redox state (-SH/-S-S- ratio) of the cell and its proper functioning under stress conditions [19]. Improvement in plant growth and development under different stresses due to application of thiourea has been observed in crops like maize, wheat, pearl millet and cluster bean [15,20-22]. Hydrogen peroxide (H_2O_2) plays a key role in cellular signaling because of which it is also termed as a second messenger [23]. However, at higher levels, it causes oxidative stress thereby causing lipid peroxidation of biological membranes and leakage of ions. The production of H_2O_2 has been reported to rise in response to different biotic and abiotic stresses [5,24].

Exogenous application of diverse chemicals on different crop plants offers an effective strategy to mitigate various abiotic stresses in crop plants. In the present study, the selected bio-regulators have different characteristics which make them suitable choice for mitigating the damage induced by high temperature stress. Putrescine is also known to be a free radical scavenger and plant cell membrane stabilizer. Thiourea is a non-physiological thiol having a role in alleviating abiotic stress. H_2O_2 is a cellular signaling molecule and is also termed as secondary messenger. Manuscript describes the changes in various physiological and biochemical functions after spray of Put, TU and H_2O_2 on seedlings of tolerant and susceptible maize genotypes exposed to high temperature stress.

Materials and Methods

Maize genotypes identified earlier [25] as heat tolerant (NSJ 221 and NSJ 189) and heat susceptible (PSRJ 13099, RJR 270) were used in the present study. The seeds of these four maize genotypes were surface sterilized with 0.1% $HgCl_2$ for 1 min, rinsed thoroughly with distilled water and sown in pots (37 cm in diameter and 40 cm in height) filled with soil mixture containing red soil, sand and farm yard manure in 1:1:1 ratio. Forty eight pots were arranged in completely randomized block design (CRBD). Plants were observed daily and water was applied manually with the help of sprinkler whenever needed. However, precaution was taken to avoid excessive irrigation. A recommended dose of NPK was applied to all the treatments. Special attention was given to spacing between plants in pots in order to reduce plant competition and shade avoidance. After complete emergence, thinning was carried out leaving four seedlings per pot. Then, pots were divided into three sets; first set represented plants grown under normal conditions. Second set represented plants exposed to high

temperature stress (48°C for 3 hrs) daily and third set had seedlings sprayed with Put (4 mM), TU (20mM) and H_2O_2 (1.2 mM) and exposed to high temperature stress (48°C for 3 hrs) after 72 hours of spray. The experiment was conducted with three replications. To understand the possible effect of sprayed chemicals on various physiological and biochemical parameters, leaf samples were analyzed for membrane integrity, chlorophyll content, Fv/Fm and oxidative stress indicators (MDA, H_2O_2), total protein content and assay of activities of SOD and POD following standard procedures.

Cell Membrane Injury (CMI)

Membrane permeability was estimated by electrolyte leakage (EL) as described by Yadav et al., 2016 [24]. Leaves samples (0.1 g) were excised, washed with deionized water, and placed in test tubes containing 10 ml distilled ionized water and incubated at room temperature for 1 hr and subsequently at 4°C for next 24 hrs. The electrical conductivity of bathing solution (L1) was determined. The samples were then autoclaved at 120°C for 60 min to release all electrolytes, cooled to 25°C and the final electrical conductivity (L2) was determined. The EL was expressed following the formula EL= (L1/L2) × 100.

Malondialdehyde Content (MDA) or Lipid Peroxidation

The level of lipid peroxidation was measured by determining the levels of malondialdehyde (MDA) content using the method of Hodges, et al. 1999 [26]. Leaf sample (200 mg) was homogenized, in 10 ml of 5 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15000 x g for 10 min. To a aliquot of 2.0 ml supernatant, 4.0 ml of 0.5 % thiobarbaturic acid (TBA) in 20 % TCA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath and centrifuged at 10000 x g for 10 min. The absorbance of supernatant was recorded at 532 nm by spectrophotometer (Genesis 6). The value for non specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as µmol (MDA) g^{-1} dry weight.

Hydrogen Peroxide (H_2O_2)

The H_2O_2 concentration was determined as described by Velikova et al. (2000) [27]. Freshly cut leaf samples (0.1 g) were homogenized in an ice bath with 1 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000x g for 15 min. Supernatant (0.5 ml) was added to 10 mM potassium phosphate buffer (pH 7.0) (0.5 ml) and 1 M potassium iodide (1 ml) and vortexed to mix the contents well. The absorbance was read at 390 nm using water as blank. The H_2O_2 concentration was determined from standard curve prepared by using 30% H_2O_2.

Determination of Chlorophyll Content

Total chlorophyll content was extracted and estimated according to the method of Lichtenthaler (1987) [28]. About 0.2 g leaves was cut into tiny segments and kept in 10ml of chilled 80% acetone in a capped glass tube. After 48h extraction in dark at 4°C, the leaf segments were well-extracted for residual pigments. The total chlorophyll content was measured at 645 and 663 and pigment contents were calculated and expressed in mg g^{-1} FW.

Fv/Fm (Quantum Yield): Maximum Quantum yield, PSII photochemistry (F_v/F_m) was measured by using Fluorpen (*FluorPen FP 100*) during noon hours.

Antioxidant Activity: Fresh leaf material (0.5 g) was ground in 10 mL of chilled phosphate buffer (pH 7.8) in an ice bath. The extract was filtered and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was used for the determination of activity of antioxidant enzyme SOD and total soluble proteins.

Superoxide Dismutase (SOD): The activity of SOD was determined following Giannopolitis and Reis (1997) [29] on the basis of inhibition of photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture (3 ml) contained 50 µl of enzyme extract with 50 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 75 mM EDTA and 50 mM phosphate buffer (pH 7.8). The cuvettes containing samples were illuminated under 15 W fluorescence lamp light for 15 min. The absorbance of the irradiated solution was recorded at 570 nm using a UV-visible spectrophotometer. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photo reduction and expressed as U mg^{-1} protein.

Peroxidase (POD): Enzymes extraction was carried out by the homogenization of leaf samples (0.5 g) in 50mM Tris-buffer (pH, 7.5) containing 1% polyvinyl pyrrolidone. The extract was centrifuged at 10,000g for 10 min at 4ºC and the clear supernatant was used for peroxidase (POD) assay. Peroxidase activity was determined through Chance and Maehly (1955) [30] method by adding enzymes extract into reaction mixture, containing

sodium-acetate buffer (pH 5.4, 50mM), freshly prepared O-dianisidine solution (0.5 %) and H_2O_2. The increase in absorbance at every 15 sec interval for 3min was recorded using spectrophotometer (Genesys 6, Thermo Spectronic, USA) at 460nm. One POD unit was expressed as change in absorbance U mg^{-1} protein and quantified.

Protein Content

The Bradford 1976 [31] method was employed to determine the soluble protein content in fresh leaf samples using Bovine Serum Albumin Fraction V as a standard.

Statistical Analysis

Experiment was laid out in complete randomized block design with three replications. Data was analyzed for analysis of variance by factorial complete randomized design and least significant differences (LSD) were calculated at 0.05 and 0.01 levels.

Result and Discussion

The results indicated that exogenous application of Put, TU and H_2O_2 resulted in an overall increase in improvement in heat stress tolerance in maize. Spray of these chemicals significantly increased antioxidant enzyme activity (SOD and POD), protein content and maintained the Fv/Fm and chlorophyll content as compared to control. The factorial ANOVA analysis was highly significant (P> 0.01) for all studied parameters. However, the response of tested genotypes was differential under heat stress conditions; NSJ 221 and NSJ 189 performed better under the high temperature stress than the genotypes PSRJ 13099 and RJR 270. Wide range

of studies on crop plants has shown an ample range of physiological and biochemical responses to heat stress. Only a few have explored how these processes are linked to heat resistance of the whole plant under field conditions. In the present study, both lipid peroxidation (MDA) and H_2O_2 contents increased upon exposure to heat stress (Figures 2 & 3) in all the genotypes.

Genotypes NSJ 221 and NSJ 189 showed minimum cell membrane injury (%) and minimum changes in MDA contents indicating that they have a better protection mechanism to withstand oxidative stress caused by heat stress.

Cell Membrane Stability

Genetic variation among genotypes for membrane stability can be utilized to identify the tolerant and susceptible genotypes under heat-stressed environments. It is well evident that high temperature reduces cell membrane stability (CMS), genotypes showing high CMS are considered as tolerant. Higher CMS values indicated lower cell membrane injury (CMI) and hence higher ability to prevent the membrane protein denaturation, inhibition of electrolyte leakage and tolerance to prevailing heat stress [24]. High temperature stress led to increased cell membrane injury (CMI) in all the genotypes studied. Genotype PSRJ13099 sprayed with H_2O_2 showed least membrane injury followed by RJR 270 and NSJ 221 when sprayed with Put. CMS was higher in NSJ 189 and NSJ 221 under heat stress compared to RJR 270 and PSRJ 13099 (Table 1) (Figure 1).

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	100.00 ±0.00 a	100.00 ±0.00 a	100.00 ±0.00 a	100.00 ±0.00 a	100
Heat stress	45.04 ±1.09 j	40.74 ±1.14 i	63.07 ±0.93 k	59.66 ±0.52 i	52.13
H_2O_2	76.54 ±1.13 e	84.69 ±0.94 gh	69.64 ±1.03 c	66.48 ±2.29 g	74.34
Putrescine	72.79 ±0.68 ef	88.02 ±0.59 d	80.57 ±0.66 b	87.06 ±4.64 cd	82.11
Thiourea	69.95 ±0.48 fg	82.52 ±0.51 h	64.78 ±0.26 c	83.10 ±0.82 cd	75.09
Mean	72.87	79.2	75.61	79.27	
SED CD (0.05) CD (0.01)					
Genotypes 0.6934** 1.40144** 1.87527**					
Treatments 0.77524** 1.56686** 2.09662**					
Genotype x Treatments 1.55048** 3.13371** 4.19324**					

Table 1: Effect of plant growth regulating chemicals on CMS (%) of maize genotypes under high temperature stress.

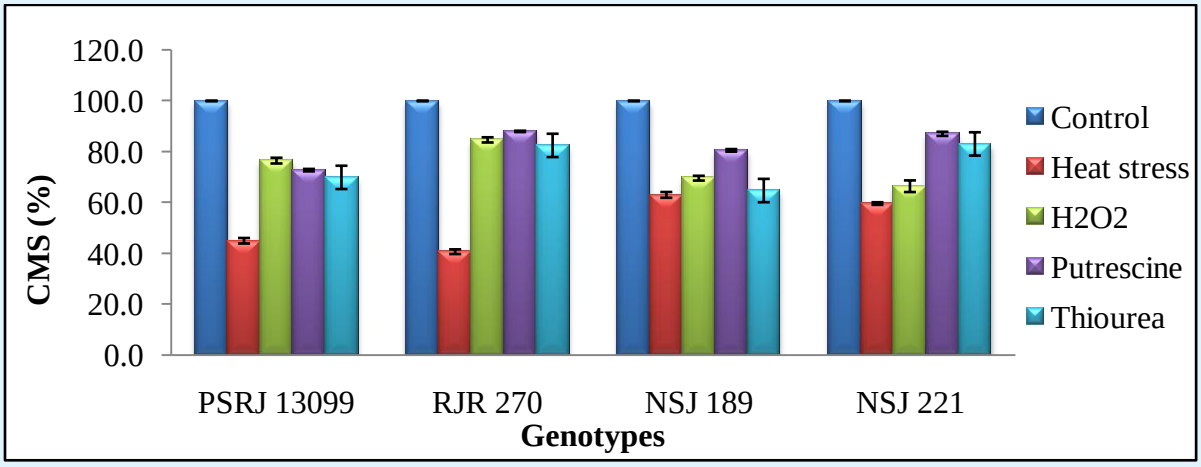

Figure 1: Effect of foliar application of H_2O_2, Putrescine and Thiourea on cell membrane stability (CMS %) of maize seedlings exposed to high temperature stress

Sprays with Put, TU and H_2O_2 improved CMS in all the genotypes studied to varying extent. The improvement was more evident in RJR 270 and PSRJ 13099. Results indicated that H_2O_2 and putrescine acclimated seedlings had lower CMI for both susceptible and tolerant group of genotypes. These results were in agreement with Ranjeet, et al. 2014 [32]. Jiang & Huang, 2000 [33] found that CMI led to more permeability to ions by increased solubilization and peroxidation of membrane lipids under heat stress. Cell membrane stability is now a well established index for screening crop plants against heat and drought tolerance. In the present study, high temperature stress induced damage in maize was observed to be associated with reduced membrane integrity and increased electrolyte leakage, indicating concurrence with previous reports of Shi et al., 2006; Bala et al., 2010 [34,3].

Lipid Peroxidation

The level of lipid peroxidation, expressed as MDA content, has been described as an indicator of free radical damage of cell membranes. A sharp increase in MDA content was observed in the high temperature stressed seedlings of all the genotypes (Figure 2).

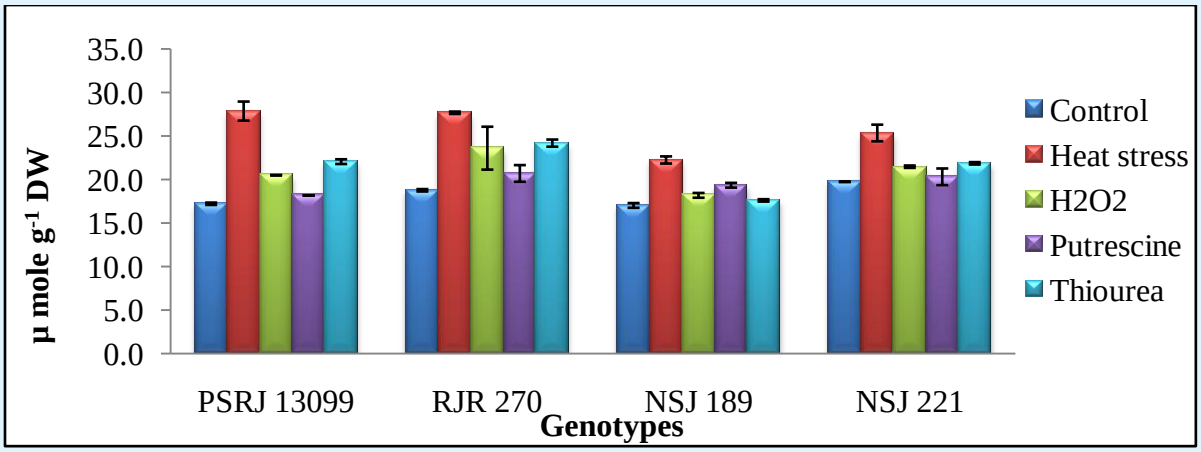

Figure 2: Effect of foliar application of H_2O_2, Putrescine and Thiourea lipid on peroxidation (MDA content) in maize seedlings exposed to high temperature stress.

The contents of MDA decreased significantly in maize seedlings under all the foliar spray treatments when compared unsprayed controls. The decline of MDA content was more with foliar spray of Put followed by H_2O_2 and TU. Ranjeet et al., 2014 [32] reported that the combined treatment of heat stress with 2.5mM Put reduced MDA content and the results were in agreement with previous reports of Nayyar and Chander (2004) [35]. Polyamines act as antioxidants by inhibiting lipid peroxidation of plants [36]. At the cellular level, polyamine could mitigate the lipid peroxidation by down regulating the NAD (P) H-oxidase/NAD (P) H peroxidase activity. Also, lipid peroxidation decreased as a result of external supply of different concentrations of PAs on wheat [37]. Combined ANOVA showed highly significant (P>0.01) (Table 2) reduction in MDA content.

Treatments/ Genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	17.23 ±0.14 kl	18.77 ±0.14 l	17.03 ±0.27 ijk	19.74 ±0.00 hij	18.19
Heat stress	27.87 ±1.10 a	27.6 ±0.14 de	22.26 ±0.41 a	25.35 ±0.96 b	25.77
H_2O_2	20.52 ±0.00 fgh	23.61 ±2.46 jkl	18.19 ±0.27 cd	21.48 ±0.14 efg	20.95
Putrescine	18.19 ±0.00 jkl	20.71 ±0.96 hij	19.35 ±0.27 efgh	20.32 ±0.96 ghi	19.64
Thiourea	22.06 ±0.27 defg	24.19 ±0.41 kl	17.61 ±0.14 bc	21.87 ±0.14 efg	21.43
Mean	21.17	22.99	18.89	21.75	
SED CD (0.05) CD (0.01)					
Genotypes 0.37895** 0.76590** 1.02485**					
Treatments 0.42368** 0.85630** 1.14582**					
Genotype x Treatments 0.84735** 1.71260** 2.29163**					

Table 2: Effect of foliar application of plant growth regulating chemicals on MDA content (µ mole g^{-1} DW) of maize genotypes under high temperature stress.

Significantly lower oxidative stress was observed in plants sprayed with Put, TU and H_2O_2 and the results are in agreement with their possible role in quenching ROS and protecting the cells from lipid peroxidation [38]. Khalil et al., 2009 [39] reported that the high temperature stress decreased antioxidant enzymes activity leading to accumulation of H_2O_2 and consequently increased lipid peroxidation.

Induction of H_2O_2 as a Signaling Molecule

All the genotypes indicated an induction in H_2O_2 under high temperature stress conditions. The analysis of variance of genotypes, treatments and their interaction was highly significant at (P>0.01) level (Table 3).

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	53.12±7.68 i	41.76±3.67 j	77.17±5.01e	83.85±5.01 cd	63.98
Heat stress	78.51±4.34 e	85.86±2.34 c	103.90±5.68 b	119.93±3.01 a	97.05
H_2O_2	72.83±10.02 f	86.86±0.67 c	80.85±4.68 de	72.49±1.67 f	78.26
Putrescine	36.75±2.00 k	67.82±1.67 g	83.18±13.03 cd	80.51±3.67 de	67.07
Thiourea	61.47±1.34 h	60.47±3.01 h	57.80±1.67 h	86.53±1.67 c	66.57
Mean	60.53	68.55	80.58	88.66	
SED CD (0.05) CD (0.01)					
Genotypes 0.881** 1.780** 2.383**					
Treatments 0.985** 1.991** 2.664**					
Genotype x Treatments 1.970** 3.982** 5.328**					

Table 3: Effect of foliar application of plant growth regulating chemicals on H_2O_2 content (µ mole g^{-1} DW) of maize genotypes under high temperature stress.

Highest induction of H_2O_2 was observed in NSJ 221 followed by NSJ 189 and within the treatments, the control seedlings recorded lowest values for H_2O_2 in RJR 270 and PSRJ 13099. Application of H_2O_2, TU and Put led to alleviation of induction of H_2O_2 in all the genotypes (Figure 3).

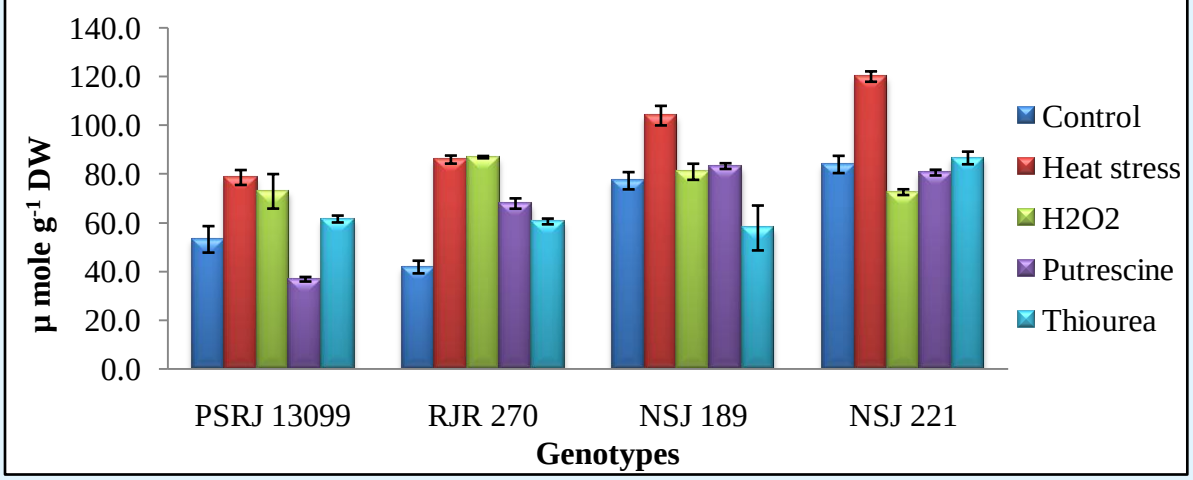

Figure 3: Effect of foliar application of H_2O_2, Putrescine and Thiourea foliar application on H_2O_2 accumulation in maize seedlings under high temperature stress.

H_2O_2 is the most important ROS generated in plants. Higher levels of H_2O_2 being toxicant, it has been regarded as an important regulator of the expression of some genes/proteins in cells [40]. According to Hernandez et al., 2000 [41], H_2O_2 control of expression of different genes which includes, antioxidant, cell defense, signaling, stress protein and transcription factors. H_2O_2 played a signaling role in triggering cross adaptation of maize seedling to various stresses [42]. Exogenous application of Polyamines has been shown to reduce the H_2O_2 levels and raise the levels of antioxidants in chickpea [35]. Ranjeet et al., 2014 [32] observed a decrease in H_2O_2 accumulation under heat stress combined with 2.5mM Put treatment.

Chlorophyll Content

The chlorophyll content in the seedlings of maize significantly decreased under heat stress when compared with control (Figure 4).

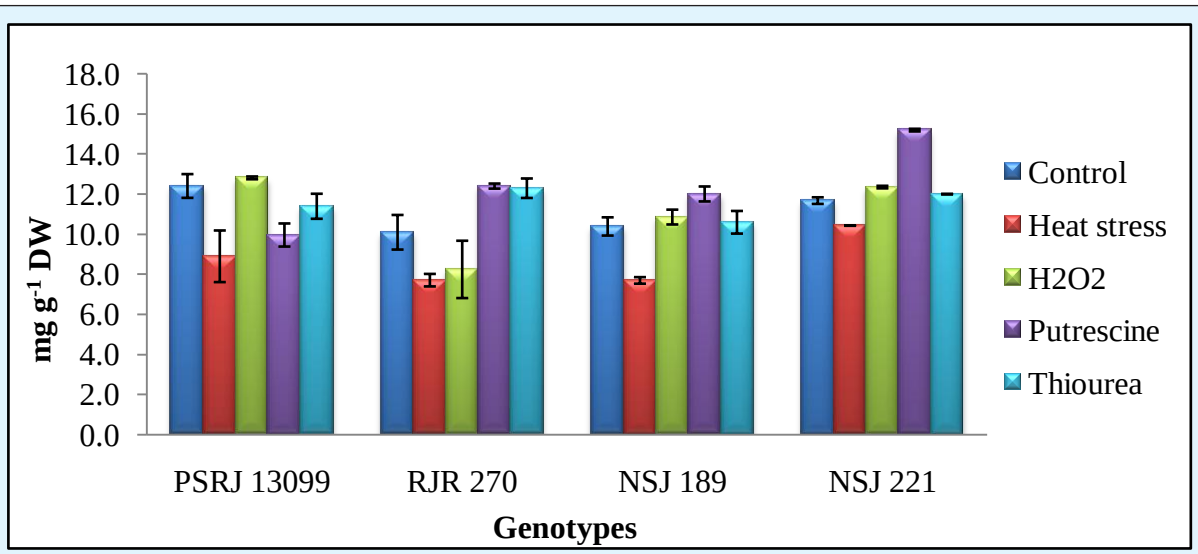

Figure 4: Effect of foliar application of H_2O_2, Putrescine and Thiourea on total chlorophyll content in maize seedlings exposed to high temperature stress.

Exogenous application of Put, TU and H_2O_2 showed great protection effect on the chlorophyll content in the seedlings. Irrespective of genotypes, high temperature stressed maize seedlings sprayed with Put, TU and H_2O_2 invariably led to an improvement in chlorophyll content. According to mean values of genotypes, the tolerant genotype NSJ 221 recorded the maximum chlorophyll content of 12.32 followed by 11.08 in susceptible genotype PSRJ 13099 while the overall mean for genotypes was 10.96. The mean values of treatments showed that the Put recorded the maximum chlorophyll content of 12.38 followed by 11.56 with TU. The interaction analysis of variance (Table 4) was highly significant and the chlorophyll content in NSJ 221 improved with Put treatment (15.19) followed by PSRJ 13099 sprayed with H_2O_2 (12. 80).

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	12.40±0.59 bc	10.09±0.86 fgh	10.38±0.46 efg	11.67±0.16 bcde	11.14
Heat stress	8.89±0.07 hg	7.69±0.31 g	7.68±0.16 g	10.41±0.01 efg	8.67
H_2O_2	12.80±0.07 b	8.23±1.43 g	10.84±0.37 defg	12.34±0.06 bc	11.05
Putrescine	9.95±0.58 gh	12.39±0.13 bc	12.00±0.37 bcd	15.19±0.06 ab	12.38
Thiourea	11.38±0.63 cdef	12.28±0.49 bc	10.58±0.57 efg	11.99±0.01cd	11.56
Mean	11.08	10.14	10.3	12.32	
SED CD (0.05) CD (0.01)					
Genotypes 0.300** 0.606** 0.811**					
Treatments 0.335** 0.678** 0.907**					
Genotype x Treatments 0.671** 1.356** 1.814**					

Table 4: Effect of foliar application of plant growth regulating chemicals on chlorophyll content (mg g^{-1} DW) of maize genotypes under high temperature stress.

Our results conclude that the foliar spray of these chemicals showed significant improvement in chlorophyll content. Sharma et al. (2008) [43] reported an increase in chlorophyll content along with other yield attributes in wheat with use of bio-regulators and thiourea foliar spray. Emmanuel et al. (2010) [44] reported an increase in chlorophyll content due to treatment of rare earth elements in wheat. Heat stress led to decrease in chlorophyll content and similar results have been obtained earlier by Morales et al., 2003 [45]. Garg (2003) [4] has shown that photosynthetic efficiency of thiourea applied plants was better in mung bean with higher contents of total chlorophyll as compared to the control.

Quantum Yield and ROS Generation

The maximal quantum yield of PSII photochemistry, Fv/Fm was significantly reduced under heat stress for all the genotypes, which was up to 84 % lower than the control (Table 5).

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	0.793±0.01 a	0.692±0.02 b	0.793±0.01 b	0.758±0.02 a	0.76
Heat stress	0.033±0.02 h	0.045±0.07 h	0.120±0.04 h	0.260±0.05 h	0.11
H_2O_2	0.328±0.04 ef	0.242±0.01f g	0.230±0.08 fg	0.460±0.02 g	0.32
Putrescine	0.408±0.10 cde	0.212±0.04 g	0.370±0.04 g	0.603±0.09 de	0.4
Thiourea	0.443±0.12 cd	0.243±0.01 fg	0.260±0.09 fg	0.490±0.08 c	0.36
Mean	0.401	0.286	0.355	0.514	
SED CD (0.05) CD (0.01)					
Genotypes 0.019** 0.039** 0.052**					
Treatments 0.022** 0.044** 0.058**					
Genotype x Treatments 0.043* 0.087* 0.117*					

Table 5: Effect of foliar application of plant growth regulating chemicals on Fv/Fm (quantum yield) of maize genotypes under high temperature stress.

However, the decline in Fv/Fm was significantly alleviated by foliar application of Put which maintained the Fv/Fm at the 0.40 level as compared to non-stressed control (0.76). TU treated seedlings maintained Fv/Fm at 0.36. However, the parameter Fv/Fm was not able to recover to the control level in any of the foliar sprayed seedlings. The mean values of genotypes showed that, NSJ 211 had maximum Fv/Fm of 0.51 followed by PSRJ 13099 (0.40). Two factorial ANOVA of genotypes and treatments were highly significant at P>0.01 level. The control seedlings with all the genotypes showed higher Fv/Fm values. The highest value of 0.80 was recorded by NSJ 189 and for rest of the genotypes descending order was PSRJ 13099>NSJ 221> RJR 270. The Fv/Fm improved adequately in NSJ 221 with Put (0.60), TU (0.49) and H_2O_2 (0.46). Photosystem-II (PSII) is most sensitive to high temperature [46]. Asada, (2006) [2] suggested that heat stress uncoupled enzymes and metabolic pathways leading to the generation and accumulation of unwanted and harmful ROS most commonly singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) which were responsible for oxidative stress. The reaction centers of PSI and PSII in chloroplasts were the major sites of ROS generation, though ROS were also generated in other organelles viz. peroxisomes and mitochondria [6]. A linear relationship exists between maximal efficiency of PSII and the accumulated ROS (Figure 5) (Table 6).

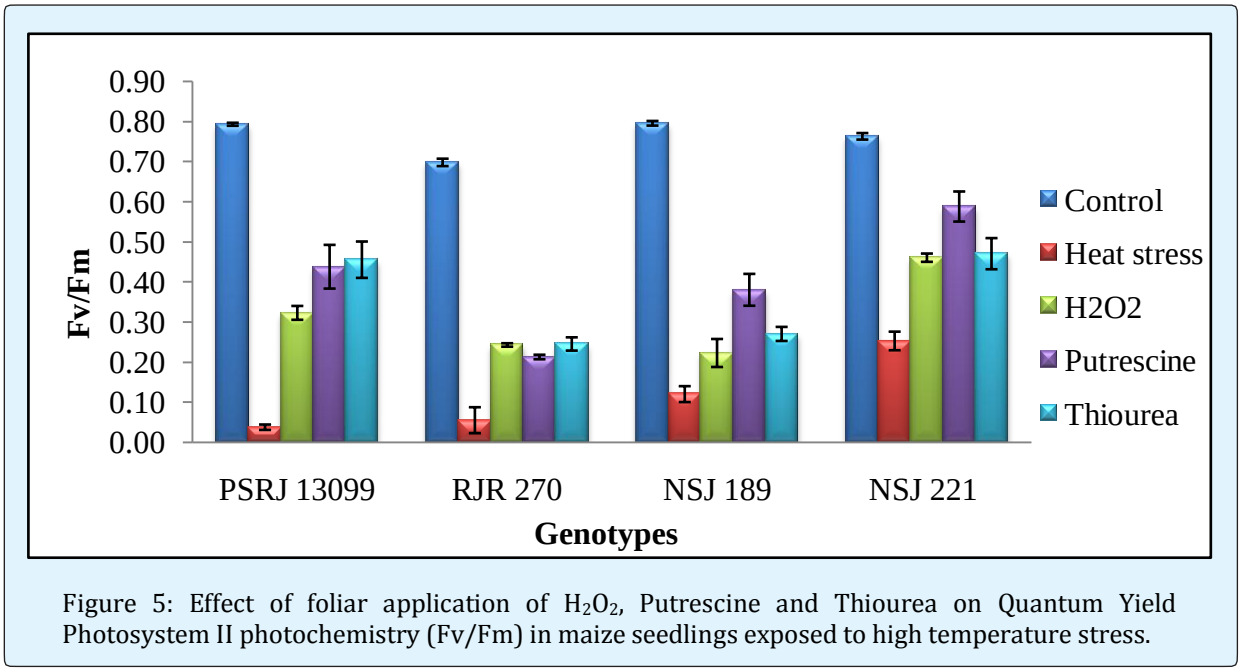

Figure 5: Effect of foliar application of H_2O_2, Putrescine and Thiourea on Quantum Yield Photosystem II photochemistry (Fv/Fm) in maize seedlings exposed to high temperature stress.

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	88.86±3.06 c	79.18±1.42 e	79.37±2.49 e	55.81±0.24 i	75.81
Heat stress	68.71±1.33 d	55.52±3.14 ei	89.45±1.48 cd	73.54±0.38 f	71.81
H_2O_2	98.27±0.83 b	78.61±6.04 e	75.04±2.91 f	61.76±2.95 h	78.41
Putrescine	90.94±2.62 c	70.04±7.08 g	97.19±1.25 b	73.48±1.88 f	82.91
Thiourea	79.66±1.08 e	80.18±1.37 e	102.06±0.00 a	72.66±3.10f g	83.64
Mean	85.29	72.7	88.62	67.45	
SED CD (0.05) CD (0.01)					
Genotypes 0.663** 1.342** 1.795**					
Treatments 0.742** 1.500** 2.007**					
Genotype x Treatments 1.485** 3.000** 4.015**					

Table 6: Effect of foliar application of plant growth regulating chemicals on the activity of SOD (U mg^{-1} Protein) of maize genotypes under high temperature stress.

Antioxidant Enzymes SOD and POD as Scavengers of ROS:

Excessive ROS production can cause oxidative stress, which damages plant metabolism at various levels. ROS-scavenging antioxidant enzymes, such as SOD and POD, play a vital role in removing these destructive oxidant species. By catalyzing the detoxification of $O_2^{\cdot-}$ to O_2 and H_2O_2, SOD inhibits cell damage caused by $O_2^{\cdot-}$. POD break down H_2O_2 to H_2O and O_2. When plants are exposed to oxidative stress, antioxidant systems become active and begin to scavenge ROS. Antioxidant defense system play vital role in helping plants tolerate stressful conditions. Highly significant changes in SOD and POD activities were observed in seedlings of all the genotypes sprayed with various bio-regulators (P>0.01) (Figure 6).

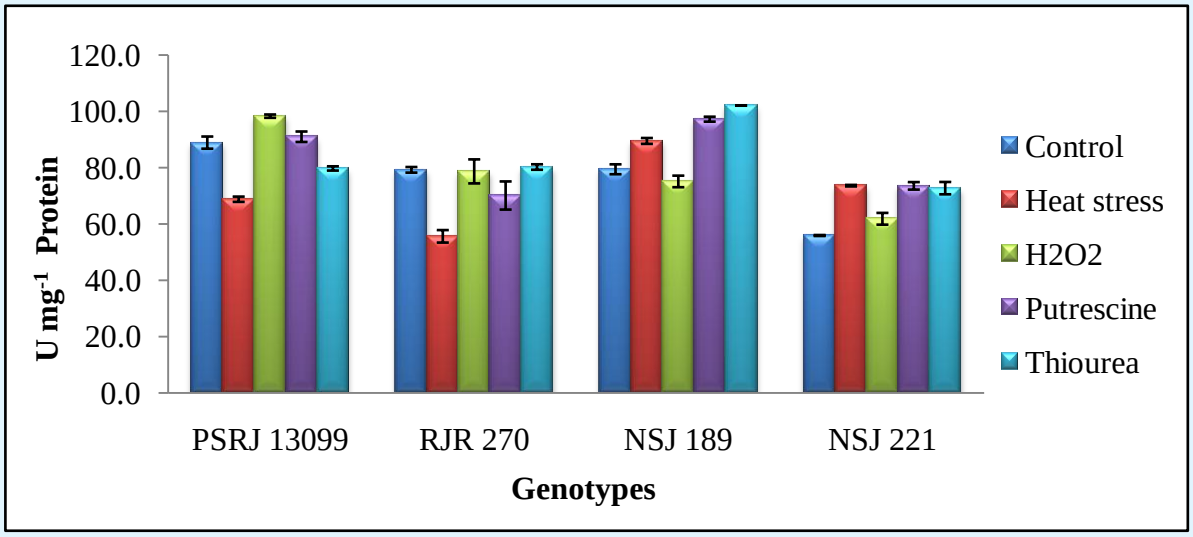

Figure 6: Quantification of SOD enzyme activity in maize seedlings upon exposure to high temperature stress. Prior to heat exposure seedlings were treated with H_2O_2, Putrescine and Thiourea foliar application.

High temperature stress led to an increase in SOD activity in stressed seedlings of tolerant genotypes NSJ 189 and NSJ 221 as compared to respective controls whereas a decrease in activity was observed in susceptible genotypes, PSRJ 13099 and RJR 270. Susceptible genotypes sprayed with various bio-regulators exhibited better SOD activity. Interaction analysis of variance showed significant increase in the activity of SOD in NSJ 189 treated with TU followed by Put. Foliar application of different chemicals improved SOD activity in all the genotypes under high temperature stress and net improvement was in the order of NSJ 189 > PSRJ 13099> RJR 270>NSJ 221 and for treatments, it was TU> Put> H_2O_2 > Control> Heat stress. Results indicated that the genotype NSJ 221 may have natural resistance towards heat stress which might be the reason for least generation of ROS under heat stress. Ranjeet et al. (2014) [32] found that SOD activity was higher in response to heat stress alone and combined treatment of heat stress and Put. The results are positively correlating with the lowest MDA content of the genotype NSJ 221. The differences between genotypes regarding the enzyme activity and other parameters studied varied significantly which might be due to genotypic specificity or variation. According to Chakraborty and Pradhan, 2011 [47], the activities of enzymes also differ depending upon tolerance or susceptibility of different crop varieties, their growth stages and growing season.

Irrespective of genotypes, foliar spray on maize seedling with Put, TU and H_2O_2 at 72 hrs before high temperature treatment (48ºC for 3hrs) resulted in the maximum increase in POD activity compared with controls and heat stressed seedlings respectively, (Figure 7) (Table 7).

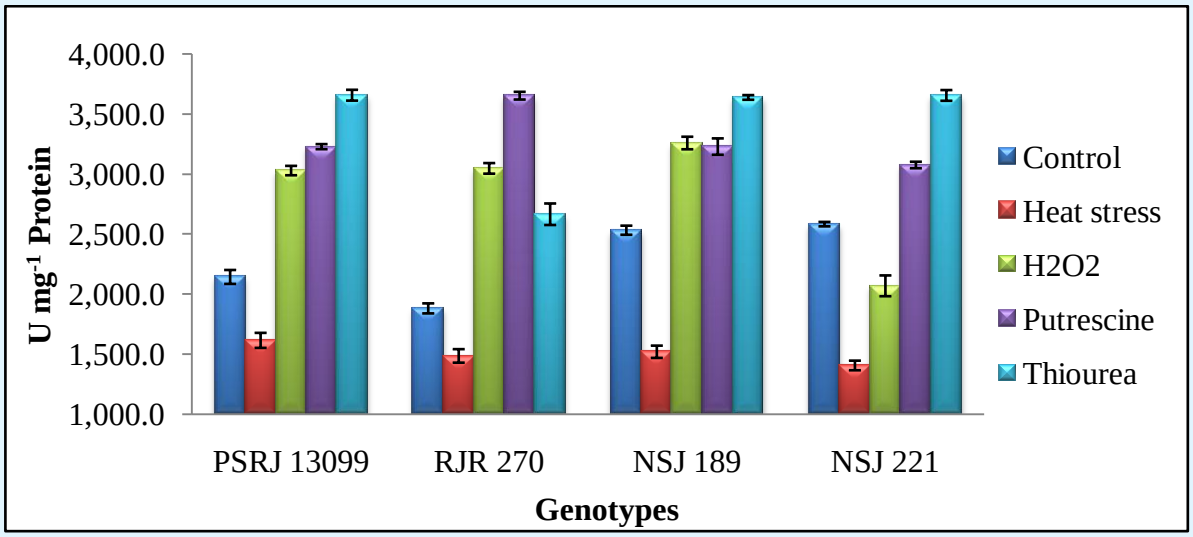

Figure 7: Quantification of peroxidase (POD) activity in maize seedlings upon exposure to high temperature stress. Prior to heat exposure seedlings were treated with H_2O_2, Putrescine and Thiourea foliar application.

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	2143.04±82.18 f	1881.25±58.96 g	2532.32±53.62 e	2582.88±26.08 de	2284.87
Heat stress	1614.68±88.53 h	1485.40±79.50 i	1519.78±72.39h i	1405.83±56.67 i	1506.42
H_2O_2	3029.03±55.19 c	3047.28±62.34 c	3258.52±73.88 b	2069.11±122.37 f	2850.98
Putrescine	3228.27±63.63 b	3652.79±126.75 a	3228.79±27.41 b	3075.44±62.99 c	3296.32
Thiourea	3656.66±29.90 a	2665.35±45.36 d	3638.12±96.53 ab	3654.85±38.25 a	3403.74
Mean	2734.34	2546.41	2835.5	2557.62	
SED CD (0.05) CD (0.01)					
Genotypes 26.12** 52.79** 70.64**					
Treatments 29.20** 59.02** 78.97**					
Genotype x Treatments 58.40** 118.04** 157.95**					

Table 7: Effect of foliar application of plant growth regulating chemicals on POD (U mg^{-1} Protein) of maize genotypes under high temperature stress.

Application of 4mM Put produced the highest increase in the activity of POD in RJR 270 followed by 20mM TU in PSRJ 13099. The genotype mean values showed a greater improvement of POD activity in NSJ 189 followed by PSRJ 13099 and NSJ 221. Tolerant genotypes (NSJ 221 and NSJ 189) showed high POD activity in control seedlings when compared with susceptible genotypes. Here it may be inferred that tolerant genotypes can endure heat stress because they possess natural tolerance. Little improvement in POD activity with spray treatments might be enough to withstand the stressed conditions. Bavita and Akash (2011) [48] observed that high temperature led to increase in POD activity in both root and shoot and it was higher in sprayed samples. It has been reported that the abiotic stresses cause increase in the production of reactive oxygen species, ionic imbalance, damage to membranes and macromolecules [49]. PAs play an important role in regulation of these processes [50]. Interaction of different stresses might increase the cellular concentration of ROS, which was finally converted to H_2O_2 [51]. The results of the present study explain how the stressed seedlings treated with TU and Put had enhanced SOD and POD activities, indicating that there was efficient ROS scavenging activity in the treated seedlings. The combined action of SOD and POD efficiently eliminated superoxide and hydrogen peroxide and

indirectly protected plants against toxic hydroxyl radicals. These results are in accordance with Almeselmani et al. (2009) [1] who observed that the activities of SOD, APX, CAT, GR, and POX increased significantly at all stages of growth in heat tolerant cultivars of wheat plants in response to heat stress treatment. TU is able to facilitate the biosynthesis of antioxidant enzymes. The alleviation of oxidative damage by using thiourea appears to be due to the presence of a thiol group [52], which is very important in the scavenging of reactive oxygen species. Looking at the structure of thiourea, both "imino" and "thiol" functional groups have great implications in abiotic stress tolerance.

Level of Soluble Proteins

The mean values of data (Table 8) showed that highest protein content was recorded in genotype PSRJ 13099 followed by NSJ 221 (Figure 8).

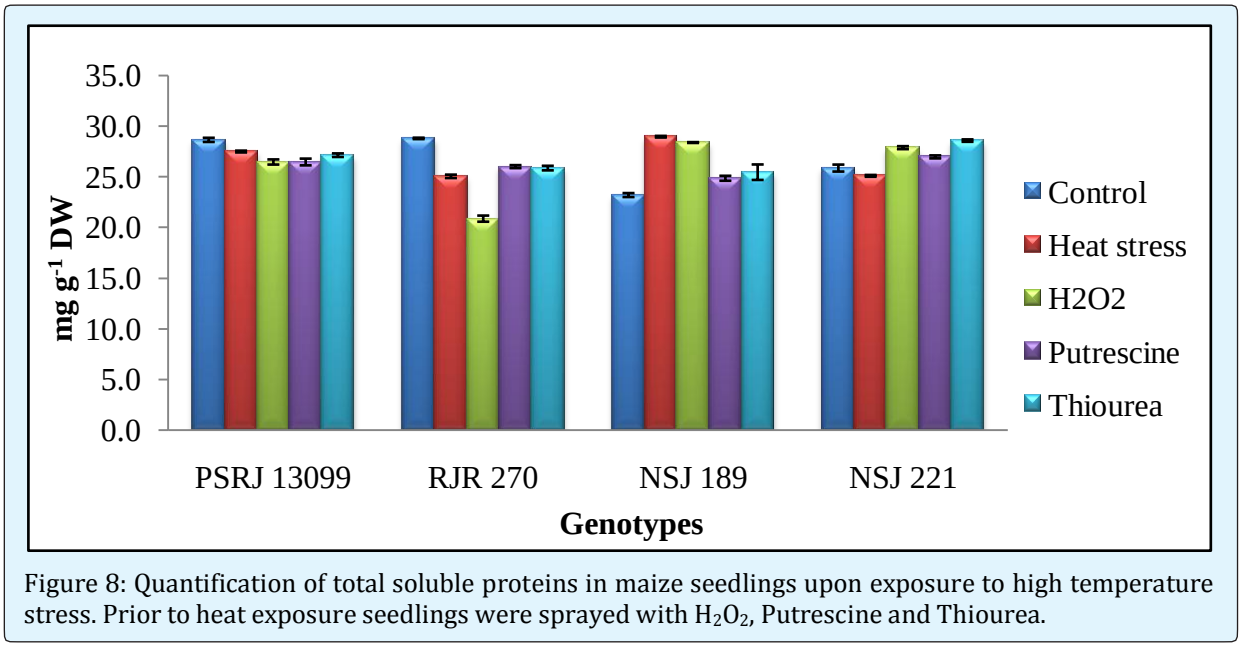

Figure 8: Quantification of total soluble proteins in maize seedlings upon exposure to high temperature stress. Prior to heat exposure seedlings were sprayed with H_2O_2, Putrescine and Thiourea.

Treatments/ genotypes	PSRJ 13099	RJR 270	NSJ 189	NSJ 221	Mean
Control	28.63±28.63ab	28.78±23.18ab	23.18±28.78j	25.85±25.85ghi	26.61
Heat stress	27.48±27.48cde	25.03±28.94hi	28.94±25.03a	25.08±25.08hi	26.63
H_2O_2	26.45±26.45efg	20.86±28.37k	28.37±20.86abc	27.86±27.86cd	25.89
Putrescine	26.45±26.45de	25.99±24.84ghi	24.84±25.99ghi	26.95±26.95ab	26.06
Thiourea	27.12±27.12efg	25.85±25.44fgh	25.44±25.85i	28.56±28.56def	26.74
Mean	27.23	25.3	26.16	26.86	
SED CD (0.05) CD (0.01)					
Genotypes 0.230** 0.465** 0.623**					
Treatments 0.257* 0.520* 0.696*					
Genotype x Treatments 0.515** 1.041** 1.393**					

Table 8: Effect of foliar application of plant growth regulating chemicals on Protein content (mg g^{-1} DW) of maize genotypes under high temperature stress.

Means ± St dev (n = 3) sharing with same letter did not differ significantly P < 0.05. Significant effects at ($P \leq 0.05$)* and ($P \leq 0.01$)** of genotypes, treatments and their interaction.

Thiourea application increased the protein content significantly. However, combined ANOVA concluded that the tolerant genotypes sprayed with Put, TU and H_2O_2 greatly increased the protein content control. It is observed to be vice-versa in susceptible genotypes. Maximum protein content was observed in heat stressed seedlings of NSJ 189 (28.94) followed by NSJ 221(28.56) with foliar application of TU. Increase in soluble protein in pollen of rice under heat stress was also observed by Tang et al. (2008) [53], which contributed to maintain cell structure and functions under stressed conditions. Mahatma et al. (2009) [38] reported increase in amino acid contents with the exogenous application of TU. Significant increase in amino acid contents with both seed treatment and foliar spray of TU has also been reported by Garg et al. (2006) [22]. Abdlkader et al., 2012 [54] reported that spraying of 2.5-5.0 mmol TU on wheat leaves at heading stage led to increase in photosynthetic pigments, antioxidant enzyme activity and metabolites including growth promoters. Mazid et al., 2011 [55] have reported regulation of H_2O_2 mediated abiotic stress tolerance in plants by nitric acid. Results in the present manuscript suggested that at vegetative stage foliar application of Put, TU and H_2O_2 improved the plant growth potential. This could be attributed to maintenance of redox homeostasis through its broad range of ROS scavenging antioxidant enzyme (SOD and POD) activities due to the sprayed chemicals.

Conclusion

Pretreatment of maize plants with different chemicals conferred protection of membrane integrity through reduced electrolyte leakage and MDA content and improved chlorophyll content, Fv/Fm and protein concentration. The induced heat stress tolerance response may be directly correlated with the regulated antioxidant enzyme activities and reduced lipid peroxidation and H_2O_2 levels. All the studied genotypes showed good improvement in various parameters over controls. Genotypes NSJ 221 and NSJ 189 possessed heat tolerant traits while genotypes PSRJ 13099 and RJR 270 susceptible to heat stress could mitigate the adverse affects of high temperature stress with the spray of Put, TU and H_2O_2. Stress alleviation was observed to be better with Put and TU. However, to validate further, the role of spray to alleviate heat stress in maize, field experiments need to be carried out to better understand the behavior of various genotypes exposed to high temperature stress.

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