*Agric Res J* **61** (4): 633-646, *August* 2024
*DOI: 10.5958/2395-146X.2024.00078.7*

# ENHANCING ANTIOXIDANT ACTIVITY AND OSMOLYTE ACCUMULATION IN HEAT-STRESSED MAIZE HYBRIDS WITH PLANT GROWTH PROMOTERS AND THERMOTOLERANCE-INDUCED SEEDS

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## ABSTRACT

**High-temperature stress significantly hampers maize production by reducing both yield and crop quality. This study investigated strategies to mitigate heat stress in maize under natural summer conditions. A split-plot design was used, with two maize hybrids (RCRMH-2 and 900MG) as the main treatments and several heat stress mitigation measures as sub-treatments, including foliar applications of salicylic acid (200 ppm), orthosilicic acid (200 ppm), calcium chloride (2000 ppm), benzyl adenine (20 ppm), and thermotolerance-induced seed treatment. The results showed that the hybrid RCRMH-2 outperformed 900MG, exhibiting higher osmolyte accumulation and enhanced antioxidant activities. RCRMH-2 accumulated 2.80% more proline than 900MG, with cytokinin treatment increasing proline by 46.6%. Calcium chloride led to a 47.16% increase in protein accumulation, while thermotolerance-induced seed treatment boosted free amino acids by 30.62%. In terms of sugar accumulation, seed treatment reduced sugar levels by 53.90%, while RCRMH-2 showed higher non-reducing sugar accumulation compared to 900MG. Salicylic acid was most effective in reducing reducing sugars, with a decrease of 17.71%. These findings suggest that specific mitigation strategies, including seed treatments and foliar applications, can significantly enhance maize heat tolerance by improving osmolyte synthesis and antioxidant defense. The study offers practical approaches to mitigate heat stress, with implications for improving maize resilience amid global climate change.**

**Keywords:** Cytokinin application, Heat stress mitigation, Maize, Osmolytes

The ongoing threat of global warming presents a serious challenge to life worldwide. A recent report from the Intergovernmental Panel on Climate Change (IPCC) highlights heat stress as a major risk to global crop production. From 2011 to 2020, the global surface temperature increased by 1.09°C (±0.1°C) compared to the baseline period of 1850 to 1900. The IPCC (2023) predicts that the global mean temperature will continue to rise by 0.3°C per decade, potentially reaching 3°C above current levels by 2100. This warming trend is primarily driven by human activities, especially the emission of greenhouse gases. Regardless of the emission scenarios considered, the IPCC projects that global surface temperatures will continue to rise at least until the mid-21$^{st}$ century. A study by the Swiss Re Institute estimates that climate change could reduce the global economy's total value by 10% by 2050 (Guo *et al.*, 2022).

Maize (*Zea mays* L.), often referred to as the "queen of cereals," is a highly adaptable crop grown in diverse climates. It ranks as the world's second most cultivated crop, with global production reaching approximately 1147.7 million metric tonnes from 193.7 million hectares, yielding an average of 5.75 t ha$^{-1}$ (FAOSTAT, 2021). The global consumption of maize is distributed as follows: 61% for feed, 17% for food, and 22% for industrial purposes. Maize plays a pivotal role in industries such as animal feed, starch production, and biofuels, accounting for 83% of its total production. Additionally, maize is a key ingredient in over 3,000 products, making it a cornerstone of the global agricultural economy (IIMR, 2022). However, NASA forecasts that by 2030, rising greenhouse gas emissions could significantly reduce maize yields, with potential declines of up to 24%. A 20% drop in global maize production could have severe consequences for food security (Gray, 2021).

Maize is especially vulnerable to the effects of global warming, with average yield reductions of 7.4

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Date of receipt: 12.06.2024, Date of acceptance: 27.08.2024

± 4.5% for every 1°C rise in global mean temperature (Zhao *et al.*, 2017). Heat stress causes irreversible damage to maize growth and development, impairing processes such as photosynthesis, leaf expansion, seedling establishment, and biomass accumulation. These stress-induced alterations disrupt the metabolic and cellular mechanisms of the plant, leading to lower test weight and overall yield (Li *et al.*, 2020; Mathur *et al.*, 2021; El-Sappah *et al.*, 2022). The reproductive phase, in particular, is highly susceptible to heat stress, which disrupts key processes such as pollen dehiscence, fertility, silk emergence, stigma receptivity, seed setting, and grain filling, ultimately leading to reduced grain yield and quality (Tiwari and Yadav, 2019).

Heat stress also increases the accumulation of reactive oxygen species (ROS), such as malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$). For instance, under heat stress conditions, MDA levels rise, indicating severe membrane damage. A study by Li *et al.* (2020) found that heat stress treatment significantly increased both MDA and $H_2O_2$ contents in maize hybrids, with the hybrid XY335 showing the most pronounced membrane damage. Shao *et al.* (2021) reported significant changes in MDA levels, as well as catalase, peroxidase, and superoxide dismutase (SOD) activities at the tasselling stage, particularly in maize ears. High temperatures of 35°C and 42°C resulted in increased $H_2O_2$ production, with plants exposed to 42°C for two hours showing 1.5 times higher $H_2O_2$ content compared to control plants grown at 25°C (Lee *et al.*, 2018). Heat stress also disrupts cellular structures, including chloroplasts and mitochondria, especially during the 12$^{th}$ leaf stage of maize. This damage leads to increased MDA and ROS content, further exacerbating stress responses (Li *et al*., 2020). As ROS accumulate in the leaves, they accelerate the breakdown of thylakoid components, which in turn hastens leaf senescence, further limiting photosynthetic capacity and overall plant health (Yang *et al*., 2018).

Heat tolerance in plants involves a complex interplay of various cellular components, with two crucial factors contributing to this tolerance: osmolytes and antioxidant enzymes. Osmolytes are small organic molecules that help maintain cellular homeostasis by stabilizing membrane proteins and cellular structures, preserving cell turgor, and supporting growth under stress. These include amino acids such as proline and betaine, small sugars, and polyols (Yancey, 2005; Hand *et al.*, 2007). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and peroxidases, play a critical role in scavenging reactive oxygen species (ROS) generated during heat stress. ROS can induce oxidative damage to cellular components, including lipids, proteins, and nucleic acids (Mittler, 2002). Antioxidant enzymes protect cell membranes from lipid peroxidation, a common consequence of heat stress, by neutralizing free radicals and ROS (Apel and Hirt, 2004). These enzymes are essential in maintaining cellular redox homeostasis, preventing oxidative imbalances that could lead to cellular damage (Foyer and Noctor, 2011). Both osmolytes and antioxidant enzymes are integral components of the plant's cellular defense mechanisms against heat stress.

One of the key physiological and biochemical interventions for enhancing heat tolerance involves the application of plant growth regulators (PGRs), which help modulate osmolyte accumulation and boost antioxidant enzyme activities. For instance, salicylic acid (SA), a phenolic compound, acts as a signaling molecule that induces heat tolerance in plants. SA enhances the activity of antioxidant enzymes, including SOD, catalase (CAT), and ascorbate peroxidase (APOX), which mitigate the oxidative damage caused by ROS generated during heat stress (Khanna *et al.*, 2016). Furthermore, SA increases the accumulation of proline, a compatible solute that stabilizes cell membranes, maintains water potential, and functions as an osmoprotectant under heat stress. SA also modulates stomatal conductance and transpiration rate, thereby regulating leaf temperature and water status, which are critical factors in improving maize performance under heat stress (Iqbal *et al.*, 2020). Collectively, SA enhances the physiological and biochemical attributes of maize plants, improving growth and yield under heat-stress conditions. Another effective mitigation strategy involves calcium chloride ($CaCl_2$), a salt that helps protect plants from heat-induced oxidative damage by modulating calcium and calmodulin signaling pathways (Shen *et al.*, 2022). Calcium and calmodulin are essential for the expression of heat shock proteins (HSPs), which play a protective role in preventing protein denaturation and aggregation under high temperatures (Lin *et al.*, 2019). Silicon (Si) is another beneficial element that improves plant performance under heat stress by activating various physio-biochemical mechanisms. Si enhances antioxidant enzyme activity, including that of catalase, peroxidase, and SOD, which scavenge ROS and prevent oxidative damage to cellular structures (Ahmed *et al.*, 2023). Additionally, Si increases the accumulation of osmolytes, such as proline, soluble sugars, and proteins, thus stabilizing cell membranes, maintaining water potential, and protecting cellular macromolecules under heat stress (Mustafa *et al.*, 2021).

Cytokinins (CK), a group of phytohormones, also play a critical role in helping plants cope with environmental stress, including heat stress. Cytokinins act by interacting with various transcription factors through phosphorylation cascades, which regulate the expression of cytokinin-target genes (Li *et al.*, 2023). Recent studies suggest that heat stress triggers a



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stress-adaptive signaling pathway, involving elements such as proteins that act as transporters, secondary messengers like $Ca^{2+}$ ions, and phytohormones, to regulate the accumulation of beneficial osmolytes and antioxidant enzymes (Sabagh *et al.*, 2022). This pathway helps plants cope with heat stress by scavenging ROS, upregulating antioxidant enzyme activities, and protecting cellular membranes through the accumulation of compatible osmolytes (El-Sappah *et al.*, 2022).

The temperature induction response (TIR) technique is a widely recognized method for screening and identifying thermotolerant genotypes in various crop species. This approach involves subjecting plantlets to a sublethal 'induction temperature,' followed by a lethal 'challenging temperature,' and assessing the survival and growth of the plantlets after a recovery period (Raviteja *et al.*, 2023). The principle behind this technique is that the induction temperature stimulates the production of heat shock proteins (HSPs) and promotes the accumulation of osmolytes, which together help protect the plantlets from the detrimental effects of the challenging temperature (Gangappa *et al.*, 2006; Gomathi *et al.*, 2014; Raghavendra *et al.*, 2017). While previous studies have demonstrated the effectiveness of the TIR technique in identifying thermotolerant genotypes, they often lack a critical analysis of the underlying mechanisms and the specific conditions under which these responses occur. A more in-depth examination of the existing literature, coupled with the identification of knowledge gaps, would further strengthen the rationale for this study.

In this context, we hypothesize that the exogenous application of various plant growth regulators (PGRs) and the use of thermotolerance-induced seeds could improve maize heat tolerance by enhancing the accumulation of osmolytes and increasing antioxidant enzyme activity under high-temperature stress. To test this hypothesis, the present study was designed to evaluate the potential of foliar applications of salicylic acid (SA), calcium chloride ($CaCl_2$), orthosilicic acid (OA), benzyl adenine (BA), and thermotolerance-induced seed treatments in promoting osmolyte accumulation and boosting antioxidant enzyme activities under heat stress conditions. By assessing the combined effects of these treatments, this study aims to provide insights into the physiological and biochemical mechanisms that underpin heat tolerance in maize, ultimately contributing to the development of more resilient maize varieties in the face of global climate change.

## MATERIALS AND METHODS

### Experiment site and weather conditions

The experiment was conducted during the summer seasons of 2022 and 2023 at the Agricultural Research Station in Bheemarayanagudi, which is part of the University of Agricultural Sciences, Raichur, India. This research station is located in the northeastern dry zone of Karnataka, specifically in Region II, Zone II, with geographic coordinates of 16°43' N latitude and 76°51' E longitude, at an elevation of 411.75 meters above mean sea level. The region is characterized by a dry climate, with an average annual rainfall of 774.1 mm. The experiment was carried out during the natural high-temperature period from March to July in both years, which coincides with the flowering and grain-filling stages of maize plants. Throughout the crop growth period in both 2022 and 2023, various weather parameters, including maximum and minimum temperatures, relative humidity, and rainfall, were recorded. These data revealed an increase in both temperature and relative humidity during the study period. The highest weekly average temperature recorded was 40°C in the 8$^{th}$ week of 2022, and 42.14°C in the 11$^{th}$ week of 2023. In contrast, the lowest weekly average temperature was recorded as 20.57°C in the first week of 2022 and 19.3°C in the first week of 2023. Detailed daily weather data changes are provided in the supplementary information. Based on these temperature and humidity patterns, it can be concluded that the maize plants in this study were subjected to natural heat stress during the experimental period.

### Experiment layout

The present study utilized two high-yielding maize hybrids: RCRMH-2, sourced from the University of Agricultural Sciences, Raichur, Karnataka, India, and 900MG, provided by Monsanto Pvt. Ltd. The study was conducted during the summers of 2022 and 2023, employing a split-plot design with six sub-treatments, each replicated three times, and two main treatments. The main plot area measured 25.2 m x 24 m, while the subplot size was 4.2 m x 4 m. Sowing was done on March 26, 2022, and March 19, 2023, following standard maize cultivation practices. The plants were irrigated using drip irrigation to ensure optimal moisture conditions throughout the growing period.

### Treatments

The experiment consisted of two main treatments (M), which were maize hybrids RCRMH-2 and 900MG. To mitigate the effects of heat stress, several sub-treatments (S) were applied, including a control (no foliar spray), foliar sprays of salicylic acid (SA) at 200 ppm, calcium chloride ($CaCl_2$) at 2000 ppm, orthosilicic acid (OA) at 200 ppm, benzyl adenine (BA) at 20 ppm, and thermotolerance-induced seeds (TIR seeds). Foliar applications were made once at 30 days after sowing (DAS), with the concentrations of the sprays



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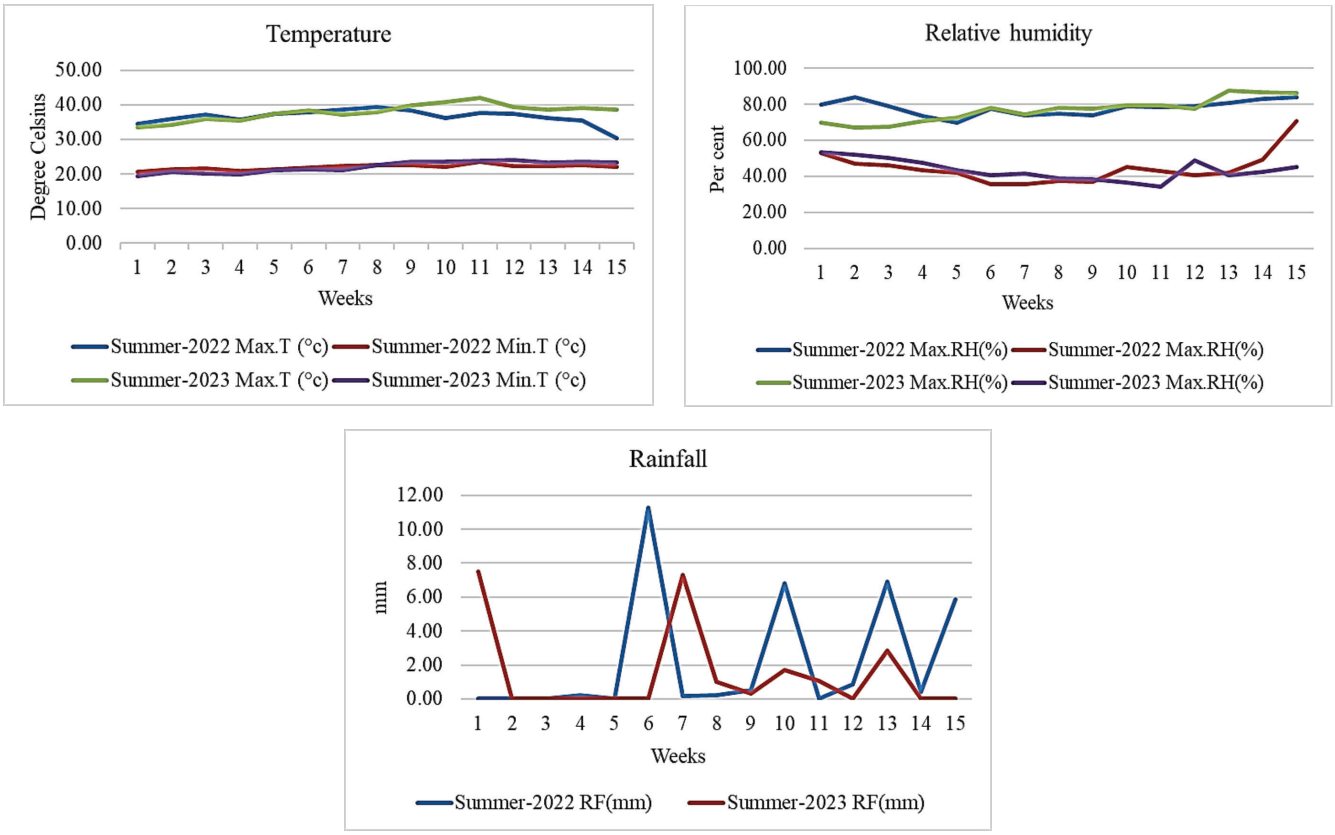



**Fig. 1. Weekly trends of temperature, relative humidity, and rainfall during the crop growth period of 2022 and 2023**

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selected based on a review of the literature. TIR seeds were subjected to a specific heat treatment to induce thermotolerance. The seeds were exposed to a graded temperature range of 32-50°C for 4.5 hours, followed by exposure to a lethal temperature of 56°C for 3 hours. After this thermal treatment, the seeds were allowed to recover for 24 hours under room conditions before being sown in the field (Raviteja *et al.*, 2023).

## Harvesting and collection of experimental data

For each treatment, five plants were randomly selected and marked for observation. The flag leaf was used to analyze various physiological parameters. The experiment was harvested in June and July of 2022 and 2023, respectively, when the husk covers had turned brown and the silks had dried completely, indicating maturity. The average value for each treatment was calculated based on observations made on the five plants during both crop seasons.

## Estimation of osmolytes in leaf sample

For osmolytes estimation, the flag leave samples were used, and estimation was done at 60 DAS.

## Proline content

The free proline content in leaves was determined, as suggested by Bates *et al.* (1973). For calculation purposes, the standard graph was prepared using L-proline.

Proline (µmol $g^{-1}$ F.W.) = [(µg proline/ml) x ml toluene) / 115.5 µg/µmole] / [(g sample)/dilution factor]

## Total protein content

The protein concentration was determined by Lowry's Method (Lowry *et al.*, 1951). The final values were expressed in mg $g^{-1}$ F.W. The mg of protein per gram sample from the standard graph (x value) was calculated, and the final value was expressed in mg $g^{-1}$ F.W.

Total proteins (mg $g^{-1}$ F.W.) = (x value x stock solution / aliquot taken)/leaf fresh weight (g)

## Total free amino acids

The total free amino acids were determined by the method given by Moore and Stein (1954), and the final value was expressed in mg $g^{-1}$ F.W. The concentration of the total free amino acids was calculated from the standard graph drawn using serial dilutions of L-glycine and expressed in mg $g^{-1}$ F.W.

## Reducing sugars

Reducing sugars were determined following the standard procedure of Nelson and Somogyi (1952). The concentration of the reducing sugars was calculated from the standard graph drawn using serial dilutions of D-glucose and expressed in mg $g^{-1}$ F.W.

## Total sugars

Total sugars were determined following the standard procedure of Nelson and Somogyi (1952). The amount of total sugar present per g of the sample was calculated using a standard graph of serial dilutions of D-glucose and expressed in mg $g^{-1}$ F.W.

## Non-reducing sugars

The non-reducing sugars were calculated using the formula below and expressed in mg $g^{-1}$ F.W.

Non-reducing sugars = Total sugars - Reducing sugars

## Antioxidant enzyme activity in leaf samples

Flag leave samples were used to estimate the antioxidant enzyme activity, and the estimated DAS was 60.

### *Extraction procedure*

Phosphate buffer (0.1 M, pH 7.5) containing 0.5 mM EDTA was used as the grinding medium for enzyme extraction. A 1 g leaf sample was homogenized in 10 mL of the extraction buffer using a pre-cooled mortar and pestle. The resulting homogenate was then centrifuged at 15,000 × g for 20 minutes at 4°C. The supernatant was collected and stored in a deep freezer for subsequent enzyme assays. The enzyme extraction procedure was consistent across all measured antioxidant enzymes, except for ascorbate peroxidase, where 1 mM ascorbic acid was added to the extraction buffer to facilitate enzyme extraction (Dhindsa *et al.*, 1981).

### *Superoxide dismutase*

Superoxide dismutase (SOD) activity was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), as outlined by Dhindsa *et al.* (1981). The reaction mixture consisted of phosphate buffer (50 mM, pH 7.8), methionine (13 mM), EDTA (0.1 mM), NBT (25 mM), $Na_2CO_3$ (50 mM), and 0.1 mL of enzyme extract, all prepared in test tubes. Riboflavin (2 µM) was added to initiate the reaction. Three sets of test tubes were prepared: one with enzyme extract, one without enzyme extract, and one reference blank containing enzyme extract but kept in the dark. All test tubes, except the reference blank, were exposed to light for 15 minutes. After incubation, the absorbance of the samples was measured at 560 nm against a blank (phosphate buffer). One unit of enzyme activity was defined as the amount of enzyme that reduced the absorbance of the sample to 50% of the control (test tubes without enzyme). SOD activity was expressed as mmol $mg^{-1}$ protein $min^{-1}$.

Unit of enzyme = Control – (Sample-Reference blank) / Control / 2 x Dilution factor

where,

Control = readings of the set without enzyme extract in light conditions, Sample = readings of set with enzyme extract in light conditions, Reference blank = readings of set with enzyme extract in dark conditions and the dilution factor is the factor by which the stock solution was diluted.

### *Ascorbate peroxidase*

The activity of ascorbate peroxidase (APOX) was measured using the protocol outlined by Nakano and Asada (1981). The enzyme extraction procedure followed the previously described method. For the APOX assay, the reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM $H_2O_2$, and 100 µL of enzyme extract, with a final volume of 3 mL. The reaction was initiated by the addition of $H_2O_2$, and the decrease in absorbance at 290 nm, indicative of ascorbate oxidation, was monitored using a UV-VIS spectrophotometer over a 2-minute period. Enzyme activity was calculated based on the amount of ascorbic acid oxidized, expressed as millimoles of ascorbic acid oxidized per milligram of protein per minute.

m mol ascorbic acid oxidised $mg^{-1}$ protein $min^{-1}$ = (initial reading - final reading)/2.8 x dilution factor

The initial reading was recorded at zero seconds, the final reading was recorded at 60 seconds, 2.8 is the molar extinction coefficient, and the dilution factor is the factor by which the stock solution was diluted.

### *Catalase*

Catalase (CAT) activity was determined following the method described by Aebi (1984). The reaction mixture was prepared by combining 50 mM phosphate buffer (pH 7.0), 12.5 mM $H_2O_2$, and 50 µL of enzyme extract, with a final volume of 3 mL. The reaction was initiated by the addition of $H_2O_2$, and the decrease in absorbance at 240 nm was monitored for 2 minutes. The decomposition of $H_2O_2$ was quantified by comparing the initial and final concentrations of $H_2O_2$, using a standard curve derived from known $H_2O_2$ concentrations. CAT activity was expressed as millimoles of $H_2O_2$ decomposed per minute per milligram of protein.

m mol $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein = (initial $H_2O_2$ - final $H_2O_2$) / 36.5 x dilution factor

where, the initial $H_2O_2$ concentration and the final $H_2O_2$ concentration were calculated from the standard



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graph, 36.5 is the molar extinction coefficient of $H_2O_2$,and the dilution factor is the factor by which the stock solution was diluted.

### *Glutathione reductase*

Glutathione reductase (GR) activity was measured according to the protocol established by Smith *et al.* (1989). The reaction mixture contained 66.67 mM phosphate buffer (pH 7.5), 0.33 mM EDTA, 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 66.67 µM NADPH, 0.66 mM oxidized glutathione (GSSG), 0.1 mL enzyme extract, and distilled water to reach a final volume of 3.0 mL. The reaction was initiated by the addition of 0.66 mM GSSG, and the increase in absorbance was monitored at 412 nm for 2 minutes. GR activity was calculated and expressed as micromoles of GSSG reduced per minute per milligram of protein (µmol $mg^{-1}$ $min^{-1}$).

m mol glutathione reduced $mg^{-1}$protein $min^{-1}$ = (initial reading - final reading) / 6.2 x dilution factor

where, the initial reading was recorded at zero seconds, the final reading was recorded at 60 seconds, 6.2 is the extinction coefficient and the dilution factor is the factor by which the stock solution was diluted.

### Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) to compare the effects of different treatments. Post-hoc comparisons of means were performed using the least significant difference (LSD) method, as outlined by Fisher *et al.* (1992). Statistical significance was assessed at the 5% probability level ($P < 0.05$), following the methodology of Panse and Sukhatme (1967). The analysis was conducted using the OPSTAT software (Sheoran *et al.*, 1998), and the critical difference (CD) and coefficient of variation (CV%) were also calculated. Graphs and tables were generated using Microsoft Excel.

## RESULTS AND DISCUSSION

### Osmolytes accumulation

The osmolytes quantified at 60 days after sowing (DAS) include proline, proteins, free amino acids, and sugars. Significant differences were observed not only between hybrids but also among the various mitigating measures, as well as in their interaction effects.

### Proline content

The RCRMH-2 hybrid exhibited the highest average proline content, measuring 5.09 µmol $g^{-1}$ fresh weight (F.W.) ($P < 0.05$), followed closely by the 900MG hybrid with 4.95 µmol $g^{-1}$ F.W. Foliar spray applications and thermotolerance-induced seeds significantly enhanced proline content ($P < 0.05$), with the BA treatment yielding the most pronounced effect, resulting in a notable increase to 5.71 µmol $g^{-1}$ F.W. in treated plants. In contrast, control plants had the lowest proline content, averaging 3.89 µmol $g^{-1}$ F.W. The interaction effects further highlighted the impact of BA treatment, which produced the highest proline levels within each hybrid, 5.85 µmol $g^{-1}$ F.W. in RCRMH-2 and 5.56 µmol $g^{-1}$ F.W. in 900MG, both significantly higher than their respective controls (Table 1).

**Table 1. Effect of mitigating measures on proline, protein and free amino acid contents in maize hybrids under heat stress conditions**

| Hybrids/mitigation treatment | Proline (µmol $g^{-1}$ F.W.) | | | Protein (mg $g^{-1}$ F.W.) | | | Free amino acids (mg $g^{-1}$ F.W.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean |
| Control | $3.95^{d*}$ | $3.84^{d}$ | $3.89^{c}$ | $0.84^{ab}$ | $0.748^{b}$ | $0.794^{a}$ | $18.57^{d}$ | $17.33^{d}$ | $17.95^{c}$ |
| Salicylic acid @ 200ppm | $4.85^{bc}$ | $5.08^{abc}$ | $4.96^{ab}$ | $1.119^{ab}$ | $1.151^{a}$ | $1.135^{a}$ | $20.84^{c}$ | $21.98^{bc}$ | $21.41^{b}$ |
| $CaCl_2$ @ 2000ppm | $5.45^{ab}$ | $5.16^{abc}$ | $5.31^{ab}$ | $1.192^{a}$ | $1.144^{a}$ | $1.168^{a}$ | $22.83^{ab}$ | $20.71^{c}$ | $21.77^{b}$ |
| Orthosilicic acid @ 200ppm | $4.93^{bc}$ | $4.53^{cd}$ | $4.73^{bc}$ | $1.137^{ab}$ | $1.152^{a}$ | $1.144^{a}$ | $21.03^{c}$ | $23.07^{ab}$ | $22.05^{ab}$ |
| Benzyl adenine @ 20ppm | $5.85^{a}$ | $5.56^{ab}$ | $5.71^{a}$ | $1.167^{a}$ | $1.142^{a}$ | $1.155^{a}$ | $23.5^{a}$ | $23.12^{ab}$ | $23.31^{a}$ |
| Temperature induced response | $5.51^{ab}$ | $5.51^{ab}$ | $5.51^{ab}$ | $1.155^{a}$ | $1.125^{ab}$ | $1.14^{a}$ | $22.93^{ab}$ | $23.97^{a}$ | $23.45^{a}$ |
| Mean | 5.09 | 4.95 | | 1.102 | 1.077 | | 21.61 | 21.70 | |
| SOV | H | T | H x T | H | T | H x T | H | T | H x T |
| S. Em ± | 0.012 | 0.029 | 0.041 | 0.005 | 0.016 | 0.022 | 0.049 | 0.077 | 0.110 |
| CD ($p \leq 0.05$) | 0.074 | 0.085 | 0.120 | NS | 0.047 | NS | NS | 0.229 | 0.323 |

*Within a column values with same alphabet do not differ significantly at $p \leq 0.05$
H=Hybrids, T=Treatments, Interaction=HxT



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## Total protein content

No significant differences in total protein content were observed between the hybrids. However, the mitigating measures had a significant effect ($P < 0.05$) on protein content. The hybrids and their interactions with the treatments, including foliar sprays and thermotolerance induction, did not show any significant variations. Among the foliar sprays, the application of calcium chloride ($CaCl_2$) at 2000 ppm significantly increased protein content in both hybrids, with an average increase to 1.168 mg $g^{-1}$ fresh weight (F.W.). In contrast, control plants exhibited the lowest protein content, averaging 0.748 mg $g^{-1}$ F.W. Interaction effects revealed that $CaCl_2$ was the most effective treatment in both hybrids, resulting in protein levels of 1.192 mg $g^{-1}$ F.W. in RCRMH-2 and 1.144 mg $g^{-1}$ F.W. in 900MG, both significantly higher than their respective control values (Table 1).

## Total free amino acids

The quantity of free amino acids was significantly ($P < 0.05$) influenced by the mitigating treatments and their interactions, but not by the hybrids themselves. The mitigating measures were effective in enhancing the free amino acid content, with the thermotolerance-induced seed treatment resulting in a significant increase to 23.45 mg $g^{-1}$ fresh weight (F.W.). In contrast, control plants exhibited the lowest free amino acid content, averaging 17.95 mg $g^{-1}$ F.W. Interaction effects revealed that the BA treatment had the most pronounced impact on both hybrids, with RCRMH-2 reaching 23.5 mg $g^{-1}$ F.W. and 900MG reaching 23.12 mg $g^{-1}$ F.W., both significantly higher than their respective control values (Table 1).

## Sugars contents

Significant variations were observed in the levels of reducing, non-reducing, and total sugars between the two hybrids and across the different treatments in the present investigation (Table 2).

### *Reducing sugars*

Reducing sugars were significantly ($P < 0.05$) affected by the mitigating measures and their interaction effects, while no significant differences were observed between the hybrids. The mitigating treatments notably increased reducing sugars content, with the thermotolerance-induced seed treatment (TIR) resulting in a significant increase of 1.217 mg $g^{-1}$ fresh weight (F.W.). In contrast, control plants exhibited the lowest levels of reducing sugars, averaging 0.791 mg $g^{-1}$ F.W. Interaction effects showed that the TIR treatment was the most effective for both hybrids, with reducing sugar levels of 1.105 mg $g^{-1}$ F.W. in RCRMH-2 and 1.33 mg $g^{-1}$ F.W. in 900MG, both significantly higher than their respective control values (Table 2).

### *Non-reducing sugars*

The levels of non-reducing sugars were significantly ($P < 0.05$) influenced by the hybrids, mitigating measures, and their interactions. Among the hybrids, RCRMH-2 exhibited significantly higher non-reducing sugars content, with a mean value of 4.928 mg $g^{-1}$ fresh weight (F.W.), compared to 900MG, which recorded 4.785 mg $g^{-1}$ F.W. Similarly, the mitigating measures significantly increased non-reducing sugar levels, with

**Table 2. Effect of mitigating measures on sugar contents in maize hybrids under heat stress conditions**

| Hybrids/mitigation treatment | Reducing sugars (mg $g^{-1}$ F.W.) | | | Non-reducing sugars (mg $g^{-1}$ F.W.) | | | Total sugars (mg $g^{-1}$ F.W.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean |
| Control | $0.806^{cd*}$ | $0.776^{d}$ | $0.791^{a}$ | $4.454^{cd}$ | $4.29^{d}$ | $4.372^{b}$ | $5.224^{c}$ | $5.114^{c}$ | $5.169^{b}$ |
| Salicylic acid @ 200ppm | $0.955^{abcd}$ | $1.238^{abc}$ | $1.096^{a}$ | $5.331^{a}$ | $4.962^{abc}$ | $5.147^{a}$ | $6.21^{a}$ | $5.518^{bc}$ | $5.864^{a}$ |
| $CaCl_2$ @ 2000ppm | $1.018^{abcd}$ | $1.134^{abcd}$ | $1.076^{a}$ | $5.037^{ab}$ | $4.989^{abc}$ | $5.013^{a}$ | $5.936^{ab}$ | $5.57^{bc}$ | $5.753^{ab}$ |
| Orthosilicic acid @ 200ppm | $1.168^{abcd}$ | $0.886^{bcd}$ | $1.027^{a}$ | $4.672^{bcd}$ | $4.717^{bcd}$ | $4.694^{ab}$ | $5.813^{ab}$ | $5.635^{abc}$ | $5.724^{ab}$ |
| Benzyl adenine @ 20ppm | $1.272^{ab}$ | $1.099^{abcd}$ | $1.186^{a}$ | $4.926^{abc}$ | $5.039^{ab}$ | $4.982^{a}$ | $6.059^{ab}$ | $5.806^{ab}$ | $5.933^{a}$ |
| Temperature induced response | $1.105^{abcd}$ | $1.33^{a}$ | $1.217^{a}$ | $5.147^{ab}$ | $4.714^{bcd}$ | $4.931^{ab}$ | $6.032^{ab}$ | $5.962^{ab}$ | $5.997^{a}$ |
| Mean | 1.054 | 1.077 | | 4.928 | 4.785 | | 5.879 | 5.601 | |
| SOV | H | T | H x T | H | T | H x T | H | T | H x T |
| S. Em ± | 0.005 | 0.006 | 0.009 | 0.010 | 0.029 | 0.041 | 0.049 | 0.026 | 0.037 |
| CD ($p \leq 0.05$) | NS | 0.019 | 0.026 | 0.062 | 0.085 | 0.120 | NS | 0.076 | 0.108 |

*Within a column values with same alphabet do not differ significantly at $p \leq 0.05$
H=Hybrids, T=Treatments, Interaction=HxT



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the application of salicylic acid resulting in a significant increase to 5.147 mg $g^{-1}$ F.W. The lowest levels of non-reducing sugars were observed in the control plants, with a mean value of 4.372 mg $g^{-1}$ F.W. Interaction effects revealed that salicylic acid was the most effective treatment for enhancing non-reducing sugar content in both hybrids, with RCRMH-2 reaching 5.331 mg $g^{-1}$ F.W. and 900MG reaching 4.962 mg $g^{-1}$ F.W., both significantly higher than their respective controls (Table 2).

### *Total sugars*

The study found that the levels of total sugars in plants were significantly (*P < 0.05*) influenced by the mitigating measures and their interaction effects, although no significant variations were observed between the different hybrids. The mitigating measures had a notable impact on total sugar content, with the thermotolerance-induced seed treatment yielding the highest mean value of 5.997 mg $g^{-1}$ fresh weight (F.W.). In contrast, control plants exhibited the lowest mean value of 5.169 mg $g^{-1}$ F.W. Interaction effects revealed that BA treatment was the most effective for increasing total sugar content, with RCRMH-2 reaching 6.059 mg $g^{-1}$ F.W. and 900MG reaching 5.806 mg $g^{-1}$ F.W., both significantly higher than their respective control values (Table 2).

### Antioxidant enzymes

Tables 3 and 4 present the results of different antioxidant enzyme activity quantified 60 DAS are significantly affected.

### *Superoxide dismutase*

The study found that superoxide dismutase (SOD) activity was significantly (*P < 0.05*) influenced by both the hybrids and the various mitigating treatments, as well as their interaction effects (Table 3). Among the hybrids, RCRMH-2 exhibited significantly higher SOD activity, with a mean value of 22.75 mmol $mg^{-1}$ protein $min^{-1}$, compared to 900MG, which recorded 22.15 mmol $mg^{-1}$ protein $min^{-1}$. The mitigating measures, particularly the application of calcium chloride ($CaCl_2$), led to a significant increase in SOD activity, with a value of 23.92 mmol $mg^{-1}$ protein $min^{-1}$. In contrast, control plants showed the lowest SOD activity, with a mean of 16.60 mmol $mg^{-1}$ protein $min^{-1}$. Interaction effects further revealed that $CaCl_2$ was the most effective treatment for both hybrids, with RCRMH-2 reaching 24.31 mmol $mg^{-1}$ protein $min^{-1}$ and 900MG reaching 23.52 mmol $mg^{-1}$ protein $min^{-1}$, both significantly higher than their respective controls (Table 3).

### *Ascorbate peroxidase*

The activity of ascorbate peroxidase (APOX) was significantly (*P < 0.05*) influenced by the mitigating treatments and their interactions, although no significant differences were observed between the hybrids. The mitigating treatments had a notable impact on increasing APOX enzyme activity. Among these, the application of orthosilicic acid resulted in the highest APOX activity, with 9.07 mmol ascorbic acid oxidized $mg^{-1}$ protein $min^{-1}$, while control plants exhibited the lowest activity, averaging 3.81 mmol ascorbic acid oxidized $mg^{-1}$ protein $min^{-1}$. Interaction effects further emphasized orthosilicic acid as the most effective treatment for both hybrids, with RCRMH-2 reaching 9.48 mmol $mg^{-1}$ protein $min^{-1}$ and 900MG reaching 8.66 mmol $mg^{-1}$ protein $min^{-1}$, both significantly higher than their respective controls (Table 3).

**Table 3. Effect of foliar sprays and thermotolerance induction on superoxide dismutase and ascorbate peroxidase enzymes in maize hybrids under heat stress conditions**

| Hybrids/mitigation treatment | Superoxide dismutase (mmol $mg^{-1}$ protein $min^{-1}$) | | | Ascorbate Peroxidase (m mol ascorbic acid oxidized $mg^{-1}$ protein $min^{-1}$) | | |
|---|---|---|---|---|---|---|
| | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean |
| Control | $16.82^{b}$ | $16.38^{b}$ | $16.6^{b}$ | $3.82^{f}$ | $3.8^{f}$ | $3.81^{c}$ |
| Salicylic acid @ 200ppm | $24.06^{a}$ | $23.28^{a}$ | $23.67^{a}$ | $6.53^{de}$ | $7.7^{bcd}$ | $7.11^{b}$ |
| $CaCl_2$ @ 2000ppm | $24.31^{a}$ | $23.52^{a}$ | $23.92^{a}$ | $6.09^{e}$ | $6.98^{cde}$ | $6.54^{b}$ |
| Orthosilicic acid @ 200ppm | $24.15^{a}$ | $22.95^{a}$ | $23.55^{a}$ | $9.48^{a}$ | $8.66^{ab}$ | $9.07^{a}$ |
| Benzyl adenine @ 20ppm | $23.6^{a}$ | $23.4^{a}$ | $23.5^{a}$ | $7.51^{bcd}$ | $7.21^{cde}$ | $7.36^{b}$ |
| Temperature induced response | $23.58^{a}$ | $23.38^{a}$ | $23.48^{a}$ | $8.05^{bc}$ | $6.62^{de}$ | $7.33^{b}$ |
| Mean | 22.75 | 22.15 | | 6.91 | 6.83 | |
| SOV | H | T | H x T | H | T | H x T |
| S. Em ± | 0.024 | 0.081 | 0.115 | 0.089 | 0.149 | 0.211 |
| CD (*p≤0.05*) | 0.149 | 0.239 | 0.339 | NS | 0.441 | 0.623 |

*Within a column values with same alphabet do not differ significantly at *p≤0.05*,
H=Hybrids, T=Treatments, Interaction=HxT



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**Table 4. Effect of foliar sprays and thermotolerance induction on catalase and glutathione reductase enzymes in maize hybrids under heat stress conditions**

| Hybrids/mitigation treatment | Catalase (m mol $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein) | | | Glutathione reductase (m mol glutathione reduced $mg^{-1}$ protein $min^{-1}$) | | |
|---|---|---|---|---|---|---|
| | RCRMH-2 | 900MG | Mean | RCRMH-2 | 900MG | Mean |
| Control | 0.458$^{a}$ | 0.519$^{a}$ | 0.489$^{a}$ | 0.465$^{c}$ | 0.48$^{c}$ | 0.473$^{b}$ |
| Salicylic acid @ 200ppm | 0.713$^{a}$ | 0.734$^{a}$ | 0.723$^{a}$ | 0.836$^{abc}$ | 0.719$^{bc}$ | 0.777$^{ab}$ |
| $CaCl_2$ @ 2000ppm | 0.702$^{a}$ | 0.679$^{a}$ | 0.691$^{a}$ | 0.68$^{bc}$ | 0.768$^{abc}$ | 0.724$^{ab}$ |
| Orthosilicic acid @ 200ppm | 0.707$^{a}$ | 0.722$^{a}$ | 0.715$^{a}$ | 0.723$^{bc}$ | 0.902$^{abc}$ | 0.813$^{ab}$ |
| Benzyl adenine @ 20ppm | 0.626$^{a}$ | 0.657$^{a}$ | 0.642$^{a}$ | 0.697$^{bc}$ | 0.823$^{abc}$ | 0.76$^{ab}$ |
| Temperature induced response | 0.66$^{a}$ | 0.708$^{a}$ | 0.684$^{a}$ | 1.181$^{a}$ | 1.033$^{ab}$ | 1.107$^{a}$ |
| Mean | 0.644 | 0.670 | | 0.764 | 0.787 | |
| SOV | H | T | H x T | H | T | H x T |
| S. Em ± | 0.007 | 0.012 | 0.017 | 0.009 | 0.022 | 0.032 |
| CD ($p \leq 0.05$) | NS | 0.025 | 0.042 | NS | 0.066 | 0.093 |

*Within a column values with same alphabet do not differ significantly at $p \leq 0.05$,
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### *Catalase*

The activity of catalase (CAT) was influenced by different mitigating measures and their interaction effects, although no significant variations were observed between the hybrids. The mitigating treatments significantly increased CAT activity compared to the control (Table 4). Among the treatments, salicylic acid application resulted in the highest CAT activity, with a value of 0.723 mmol $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein. In contrast, control plants exhibited the lowest CAT activity, averaging 0.489 mmol $H_2O_2$ decomposed $min^{-1}$ $mg^{-1}$ protein. Interaction effects further highlighted that salicylic acid was the most effective treatment for both hybrids, with RCRMH-2 reaching 0.713 mmol $min^{-1}$ $mg^{-1}$ protein and 900MG reaching 0.734 mmol $min^{-1}$ $mg^{-1}$ protein, both significantly higher than their respective control values (Table 4).

### *Glutathione reductase (GR)*

Grutathione reductase (GR) activity was significantly influenced by the mitigating measures and their interaction effects, although no significant differences were observed between the hybrids (Table 4). The mitigating treatments significantly enhanced GR activity, with the thermotolerance-induced seed treatment (TIR) resulting in a markedly higher GR activity of 1.107 mmol glutathione reduced $mg^{-1}$ protein $min^{-1}$. In contrast, control plants exhibited the lowest GR activity, with a value of 0.473 mmol glutathione reduced $mg^{-1}$ protein $min^{-1}$. Interaction effects highlighted TIR as the most effective treatment for both hybrids, with RCRMH-2 exhibiting a GR activity of 1.181 mmol $min^{-1}$ $mg^{-1}$ protein and 900MG showing a GR activity of 1.033 mmol $min^{-1}$ $mg^{-1}$ protein, both significantly higher than their respective control levels (Table 4).

The current study demonstrates that mitigating measures significantly increased the accumulation of osmolytes and the activities of antioxidant enzymes in maize under natural heat stress conditions. Among the hybrids, RCRMH-2 exhibited higher tolerance to heat stress compared to 900MG, resulting in greater accumulation of osmolytes and enhanced antioxidant enzyme activities. This suggests that RCRMH-2 responds more effectively to mitigating treatments. Proline content, in particular, showed a significant increase across all mitigating measures. Previous studies have highlighted that the exogenous application of benzyl adenine (BA) enhances the expression of key genes involved in proline biosynthesis, such as pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), both of which play critical roles in proline synthesis and accumulation under heat stress conditions (Mishra and Dubey, 2006; Szabados and Savoure, 2010). According to Thomas *et al.* (1992), BA activates a common signalling pathway that leads to the accumulation of phosphoenolpyruvate carboxylase (PEPc) and proline in plant cells. PEPc catalyzes the first step in proline biosynthesis from glutamate, suggesting that BA may regulate proline biosynthesis by modulating the expression and activity of PEPc and other enzymes involved in this pathway. Furthermore, a study by Gomathi *et al.* (2014) demonstrated that sugarcane settlings and calli induced for thermotolerance showed elevated levels of proline, which were associated with the activation of stress-responsive proteins and genes primarily expressed during sub-lethal stress induction. These metabolic alterations are crucial for plant survival under

heat stress, as they help mitigate cellular damage and maintain cellular functions. In line with these findings, Gangappa *et al.* (2006) emphasized the importance of such metabolic changes in enabling plants to cope with heat stress conditions.

Previous studies have demonstrated that sunflower seedlings subjected to sub-lethal heat stress exhibit an increase in protein content, primarily due to the activation of stress-responsive proteins and genes. This increase is associated with the regulation of senescence processes, which influence chlorophyll metabolism and enhance net assimilation, leading to higher sugar and protein contents (Senthil Kumar *et al.*, 2003). Among the mitigating treatments, benzyl adenine (BA) has been shown to play a pivotal role in enhancing protein content by modulating these pathways. Moreover, hormone, proteome, and transcriptome analyses have highlighted the critical role of cytokinins, such as BA, in plant tolerance to heat stress. BA treatment has been found to upregulate heat shock proteins (HSPs), which are essential for maintaining cellular functions under high-temperature stress, including stabilizing proteins and sugars. The upregulation of these stress proteins helps mitigate damage to cellular structures and enzymes, contributing to increased heat tolerance. Supporting this, proteomic profiling of thermotolerance-induced tomato seedlings revealed a significantly higher expression of heat shock proteins, particularly those involved in the plant's physiological and biochemical responses to heat stress. The overexpression of the small heat shock protein sHSP24.4 has been linked to the acquired thermotolerance in groundnuts, as demonstrated in a study by Gangappa *et al.* (2006). These findings emphasize the crucial role of HSPs in facilitating heat adaptation and highlight the potential of cytokinin treatments, such as BA, to enhance thermotolerance by regulating stress-responsive pathways in plants.

Sugars are small organic compounds that play crucial roles as osmolytes in plants under heat stress. In addition to stabilizing cellular structures and maintaining osmotic balance, sugars also function as antioxidants and signalling molecules, influencing the expression of genes related to heat stress tolerance (Ghosh *et al.*, 2021). The current study supports the findings of previous research that mitigating measures significantly increase the levels of reducing sugars, non-reducing sugars, and total sugars under heat stress conditions. Studies have shown that exogenous application of benzyl adenine (BA) at 20 ppm can modulate carbohydrate metabolism in maize during periods of heat stress. This treatment has been linked to an increase in certain organic acids, such as citric and malic acid, as well as sugar alcohols and disaccharides. These changes contribute to maintaining energy supply and osmotic balance in plant cells under heat stress (Jespersen *et al.*, 2015). Similarly, in wheat, BA treatment has been shown to enhance invertase activity, which plays a critical role in regulating sucrose remobilization, a key factor in the stay-green trait. Additionally, there was a noticeable increase in sucrose phosphate synthase (SPS) activity, which is essential for sucrose synthesis under stress conditions (Wang *et al.*, 2015). Recent studies have further demonstrated that BA at 20 ppm can improve starch and sugar quality during drought stress. Treatment with 6-BA improved pasting viscosities, reduced retrogradation percentage, and altered starch granule characteristics, such as increasing granule size, reducing the proportion of short chains, and modifying the average chain length and glucose units (Huo and Yang, 2022). These findings highlight the beneficial effects of BA on carbohydrate metabolism, not only under heat stress but also in other stress conditions such as drought. In line with these observations, thermotolerance-induced seedlings have been reported to exhibit elevated sugar levels due to the increased expression of heat shock proteins (HSPs), which enhance antioxidant activity. For example, improved photosynthetic activity, driven by HSP expression, has been associated with increased sugar accumulation in rice (Vijayalakshmi *et al.*, 2015). These findings suggest that the combination of heat shock protein upregulation and sugar metabolism alterations plays a significant role in enhancing plant resilience to heat stress.

The activity of key antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APOX), catalase (CAT), and glutathione reductase (GR), is significantly enhanced under various mitigating measures aimed at alleviating heat stress. A study by Wang *et al.* (2021) demonstrated that the application of 6-benzyl adenine (BA) increased the activities of ascorbate peroxidase, glutathione reductase, and monodehydroascorbate reductase under waterlogging stress, highlighting its role in boosting antioxidant capacity. Cytokinins (CK), such as BA, function as signalling molecules that regulate the expression of genes encoding antioxidant enzymes, as shown by Kosakivska *et al.* (2022). Moreover, CK modulates antioxidant enzyme activity by influencing their protein stability, subcellular localization, and interactions with other proteins. For example, CK can enhance the stability of SOD and CAT by preventing their degradation via proteolytic pathways, as reported by Li *et al.* (2021).

In addition to modulating antioxidant enzymes directly, CK also regulates the biosynthesis and signalling of other phytohormones, including abscisic acid (ABA), gibberellins, auxins, and ethylene, all of which play crucial roles in plant responses to heat stress. These phytohormones contribute to the modulation of



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antioxidant enzyme activities and pigment production, thereby enhancing plant stress tolerance. Malarkodi and Manonmani (2023) also observed that the application of salicylic acid (SA) significantly increased the activity of SOD, CAT, and peroxidase enzymes, which function to scavenge reactive oxygen species (ROS) and prevent oxidative damage to cells. SA acts as a signal molecule, activating the expression of genes encoding antioxidant enzymes, such as SOD, CAT, APOX, and peroxidase. Additionally, SA modulates antioxidant enzyme activities by affecting substrate availability, cofactor levels, post-translational modifications, and the synthesis of secondary metabolites. For instance, SA has been shown to increase levels of ascorbate and glutathione, which are essential cofactors for APOX and GR, respectively (Ignatenko *et al.*, 2019). Furthermore, Tan *et al.* (2011) reported that calcium chloride ($CaCl_2$) treatment led to increased antioxidant enzyme activity. The mechanism behind this increase is believed to involve the modulation of enzyme activity through changes in protein stability, subcellular localization, and interactions with other proteins. Like CK, $CaCl_2$ also enhances the stability of SOD and CAT by preventing their proteolytic degradation, as noted by Naeem *et al.* (2017) and Khushboo *et al.* (2018). Orthosilicic acid (OSA) has also been shown to increase antioxidant enzyme activity. Previous studies on barley by Naz *et al.* (2021) indicated that OSA treatment enhanced antioxidant enzyme activity by modulating osmolyte content and regulating stress-related endogenous hormones, such as ABA and indole-3-acetic acid (IAA).

This study provides practical strategies for improving maize production under high-temperature stress, a major challenge intensified by global warming. The hybrid RCRMH-2 demonstrated exceptional heat tolerance, with higher osmolyte and antioxidant activity, making it a promising choice for cultivation in heat-prone regions. Key treatments were found to effectively mitigate stress: BA enhanced proline accumulation, boosting stress resilience; calcium chloride increased protein levels, strengthening plant structure; and thermotolerance-induced seed treatment elevated sugar concentrations. Salicylic acid proved to be a cost-effective means of improving plant defenses. However, further research is needed to understand the molecular mechanisms behind thermotolerance-induced resistance using genomics and proteomics, to develop a comprehensive biostimulant combining salicylic acid, BA, orthosilicic acid, and calcium chloride, and to evaluate the feasibility and effectiveness of these methods on a commercial scale. Additionally, potential side effects, such as altered plant growth or unintended interactions with other inputs, should be monitored. Long-term studies are also required to assess any ecological impacts and ensure the sustainability of these practices for future agricultural use.

## CONCLUSION

This study emphasizes the crucial role that both hybrid selection and mitigating treatments play in enhancing maize's resilience to heat stress by modulating osmolyte accumulation and antioxidant enzyme activity. The results highlight that the hybrid RCRMH-2 outperformed 900MG across several physiological parameters, demonstrating its potential as a more heat-tolerant variety. Additionally, specific mitigating treatments, such as BA and calcium chloride, significantly boosted key stress-responsive mechanisms, including proline and protein accumulation. Thermotolerance-induced seed treatments also emerged as a powerful strategy for regulating sugar metabolism and enhancing antioxidant defenses. The practical implications of this research are significant. The study shows that combining targeted treatments with optimal hybrid selection can effectively mitigate the detrimental effects of heat stress-a growing concern in maize production due to climate change. By identifying precise biochemical responses, such as increased proline accumulation and improved enzyme activity, this research offers actionable insights for breeding programs and agronomic practices aimed at improving maize heat tolerance. These findings have the potential to safeguard maize yields in the face of global warming, contributing to both food security and agricultural sustainability.

### Conflicts of interest

The authors declare that they have no conflicts of interest.

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