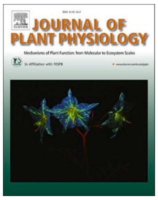
Journal of Plant Physiology 289 (2023) 154096

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph



Brassinosteroid modulates ethylene synthesis and antioxidant metabolism to protect rice (*Oryza sativa*) against heat stress-induced inhibition of source–sink capacity and photosynthetic and growth attributes

Faroza Nazir[a,1], Badar Jahan[b,1], Sarika Kumari[a], Noushina Iqbal[a], Mohammed Albaqami[c], Adriano Sofo[d], M. Iqbal R. Khan[a,*]

[a] *Department of Botany, Jamia Hamdard, New Delhi-110062, India*
[b] *Department of Botany, Aligarh Muslim University, Aligarh-202002, India*
[c] *Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[d] *Department of European and Mediterranean Cultures: Architecture, Environment, Cultural Heritage (DiCEM), University of Basilicata, Via Lanera 20, 75100, Matera, MT, Italy*

ABSTRACT

This study presents an exploration of the efficacy of brassinosteroids (BRs) and ethylene in mediating heat stress tolerance in rice (*Oryza sativa*). Heat is one of the major abiotic factors that prominently deteriorates rice production by influencing photosynthetic efficiency, source–sink capacity, and growth traits. The application of BR (0.5 mM) and ethylene (200 μl l^{-1}) either individually and/or in combination was found to alleviate heat stress-induced toxicity by significantly improving photosynthesis, source–sink capacity and defense systems; additionally, it reduced the levels of oxidative stress markers and ethylene formation. The study revealed the positive influence of BR in promoting plant growth responses under heat stress through its interplay with ethylene biosynthesis and enhanced plant defense systems. Interestingly, treatment with the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) substantiated that BR application to heat-stressed rice plants enhanced ethylene-dependent pathways to counteract the underlying adversities. Thus, BR action was found to be mediated by ethylene to promote heat tolerance in rice. The present study sheds light on the potential tolerance mechanisms which can ensure rice sustainability under heat stress conditions.

1. Introduction

Plants experience various recurring stresses throughout their lifecycle, and heat stress is a prominent environmental factor that triggers cellular, metabolic and molecular reprogramming in plants and adversely affecting their growth and development (Janni et al., 2020). Rice (*Oryza sativa*), a vital food source for over half of the global population, confronts numerous environmental disturbances (Wu et al., 2019). Heat stress is a major hindrance to the growth of rice, especially during its reproductive phase, resulting in substantial crop loss. During heat stress, the cellular integrity of plants is disrupted due to redox imbalance and increased production of reactive oxygen species (ROS). This leads to limitation in photosynthetic efficiency and the activities of enzymes involved in sugar metabolism, ultimately impacting the source-sink dynamics (Hassan et al., 2021; Gautam et al., 2022). Heat stress also exerts a negative influence on the plant–water relationship, dry matter production and photosystem II (PSII) efficiency, which hamper nutrient homeostasis and differentially modify ethylene biosynthesis (Khan et al., 2013; Savada et al., 2017; Buttar et al., 2020).

Heat stress-induced inimical impact could be inferred through the climate models stating the statistical approximation of adverse scenarios concerning agro-economic productivity. For instance, a report suggests that a 1 °C rise in global mean temperature will drastically affect the global yields of staple crops, including rice, by 3.2% (Zhao et al., 2017a). Among all cereal crops, it has been reported that rice could still obtain optimal growth at temperatures between 27 °C and 32 °C with no considerable diminution in yield efficiencies (Aghamolki et al., 2014). However, if the temperature ranges above 32 °C, it could cause detrimental impacts on different phases of rice crop cycles (Aghamolki et al., 2014). Rice has been regarded as a vital food crop for approximately 50% of the world's population and has approximately 167 million hectares of harvesting area with a production of 782 million tons (Xu et al., 2021). In addition, rice provides 76% of the calorific intake worldwide (Fitzgerald et al., 2009). However, it has been estimated that approximately 16% of the rice harvesting area will be prone to at least 5 reproductive days of temperatures above the critical temperature level

---

* Corresponding author.
E-mail address: iqbal.khan@jamiahamdard.ac.in (M.I.R. Khan).

[1] Equally contributing authors.

https://doi.org/10.1016/j.jplph.2023.154096
Received 6 June 2023; Received in revised form 13 September 2023; Accepted 17 September 2023

by 2030, which specifies the physiologically critical temperature in the reproductive stage; this projection is enhanced to 27% by the year 2050 (Gourdji et al., 2013; Xu et al., 2021).

To deal with heat stress-induced adversities, plants undergo various alterations in their physio-biochemical and molecular responses to sustain their survival during stressful cues. This cellular reprogramming mainly involves the activation of antioxidant machinery to maintain redox balance and eliminate excess ROS production to protect the plant cell (Wang et al., 2014, 2019). Brassinosteroids (BRs) are steroidal hormones that play an indispensable role in mitigating the inhibitory effects of heat stress on photosynthesis (Ogweno et al., 2007; Thussagunpanit et al., 2015; Ahammed et al., 2020; Chen et al., 2021). BRs improve ROS scavenging and minimize oxidative damage by inducing the activation of antioxidant genes (Cao et al., 2005; Ahammed et al., 2016). Numerous pieces of evidence suggest that BR interacts with other phytohormones and mediates physio-chemical processes in plants (Choudhary et al., 2012; Zhu et al., 2016; Nazir et al., 2021; Yang et al., 2021). The interaction between BRs and ethylene aids in the regulation of plant growth under optimal and abiotic stresses and has been well established (Wang et al., 2011; Wei et al., 2015; Zhu et al., 2016, 2018; Ohri et al., 2019). For instance, BRs can alleviate the negative impact of salt stress by regulating ethylene production, which helps restore the activity of 1-aminocyclopropane-1-carboxylate oxidase (ACO; Wang et al., 2011). Moreover, BRs have an effect on ethylene biosynthesis by increasing the levels of 1-aminocyclopropane-1-carboxylate synthase (ACS) and stabilizing the ethylene insensitive-3-like (EIL) protein family in tomato (*Solanum lycopersicum*) under salinity stress (Zhu et al., 2016). Additionally, endogenous levels of BR, along with ethylene, play a pivotal role in mediating plant growth and heat stress-induced responses (Ahammed et al., 2016). In addition to BR, ethylene, a gaseous phytohormone, also mediates the modulation of heat tolerance by regulating photosynthesis, source−sink capacity, pollen development, and antioxidant enzyme activities (Jegadeesan et al., 2018; Pan et al., 2019; Poór et al., 2021; Gautam et al., 2022). It has been revealed that ethylene is involved in the BR-induced alternative oxidase (AOX) pathway, which plays important roles in abiotic stress tolerance in cucumber (*Cucumis sativus*) seedlings (Wei et al., 2015). Nonetheless, the mechanism controlling BR-triggered heat stress tolerance and its crosstalk with ethylene in plants during heat stress is largely unknown. With this in mind, the aim of the present study was to examine how BR modulates photosynthesis, source−sink capacity and defense systems, as well as reduced levels of oxidative stress markers and ethylene formation under heat stress conditions. The findings from our research will provide valuable insights for plant experts in understanding the role of BR in heat stress tolerance.

2. Materials and methods

2.1. Plant growth conditions and material used

Healthy rice (*Oryza sativa* L. IR64) seeds of equal size were subjected to a surface disinfection process using a 0.1% sodium hypochlorite solution for a duration of 10 min. Subsequently, they were washed with double-distilled water to ensure thorough cleansing and removal of any potential contaminants. This meticulous procedure aims to maintain the seeds' integrity and quality, making them suitable for experimentation purposes. The seeds were then sown in earthen pots (8 and 6 cm diameter across the top and bottom, respectively, and 11 cm in height) with acid-washed sand that had been distilled in accordance with the protocol given by Hewitt (1952). In the experiment, plants were exposed to heat stress for 6 h during the middle of the 14-h photoperiod to 40 °C daily at 10 days after sowing (DAS) during the leaf emergence stage for two weeks and kept at 25 °C for one week during the experimental period. The experiment was conducted for 30 days). Control plants were kept at a temperature of 27 °C, and sampling was performed at 30 DAS.

In one set of experiments, we examined the response of rice to different concentrations of 24-epibrassinolide (source of BR; 0, 0.1, 0.5, 1.0 and 1.5 mM) on methionine content, ACS activity, maximum efficiency of PSII, net photosynthesis and plant dry mass (PDM; Fig. 1). In another experiment, best concentration of BR (0.5 mM) was applied either alone or in combination with ethephon (source of ethylene; 200 μl l^{-1}; Khan and Khan, 2014). Additionally, a third set of experiments was performed to validate the results with an inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine (AVG; 50 μM) which was applied under heat stress after 20 DAS (Table 2). All treatments were applied to the foliage of plants in no-stress and/or heat-stressed conditions at 15 DAS. The control plants were sprayed with 25 ml water. In addition, 25 ml of 0.5% surfactant teepol was supplemented with the control, BR and ethylene treatments.

2.2. Measurement of hydrogen peroxide content and lipid peroxidation

To quantify the hydrogen peroxide (H_2O_2) content, we implemented the spectrophotometric method described by Okuda et al. (1991), and lipid peroxidation was quantified by determining thiobarbituric acid reactive substances (TBARS) content according to Dhindsa et al. (1981). The comprehensive illustration of these methods is mentioned in Jahan et al. (2020).

2.3. Determination of antioxidant enzymatic activities

Activities of APX (EC 1.11.1.11) and GR (EC 1.6.4.2), were estimated by the method of Nakano and Asada (1981) and Foyer and Halliwell (1976) with slight changes. Dehydroascorbate reductase (DHAR) activity (EC 1.8.5.1) activity was estimated by adopting the method of Foyer et al. (1989). Details of the procedures are given in Jahan et al. (2020) and File S1.

2.4. Estimation of glutathione and ascorbate content

To determine the content of reduced glutathione (GSH), we utilized an enzymatic recycling method outlined by Griffith (1980). For the estimation of ascorbate (AsA) content, we followed the method described by Law et al. (1983) with slight changes. Details are given in Jahan et al. (2020) and File S1.

2.5. Estimation of ATP-sulfurylase activity and sulfur content

Enzymatic activity of adenosine triphosphate sulfurylase (ATP-S) was estimated using molybdate-dependent formation of pyrophosphate according to the method of Lappartient and Touraine (1996).

Sulfur content was estimated in leaf samples homogenized in a digestion tube containing 4 ml of the acid mixture (concentrated nitric oxide (HNO_3) and perchloric acid ($HClO_4$) in the ratio of 85:1, v/v), and 7.5 mg of selenium dioxide (SeO_2), which was used as a catalyst to carry out the reaction. Detailed protocol has been mentioned in Jahan et al. (2020).

2.6. Determination of cysteine and methionine content

Cysteine content in leaves was estimated following the method of Giatonde (1967). Methionine content was estimated by adopting the protocol of Horn et al. (1946). Details are given in File S1.

2.7. Determination of photosynthetic characteristics

Using an infrared gas analyzer (CI-340, Photosynthesis system, CID Bio-Science, USA), photosynthetic traits including net photosynthesis, stomatal conductance, and intercellular carbon dioxide (CO_2) concentration were evaluated in topmost expanded leaves of plants in each treatment on a sunny day between 11:00 and 12:00 h at light saturating intensity. These observations were recorded at an atmospheric CO_2

concentration of 380 ± 5 μmol mol^{-1}, relative humidity of 70%, PAR of 400 μmol m^{-2} s^{-1}, and air temperature of 28 °C.

A chlorophyll fluorometer (Junior-PAM, Heinz Walz, and Germany) was used to measure the PSII photochemical efficiency (variable fluorescence to maximal fluorescence; Fv/Fm) of the fully expanded second leaf from the top of the plant. The minimal fluorescence (Fo) and Fm values were obtained after the plants were dark-adapted for 30 min. A weak measuring pulse (125 μmol m^{-2} s^{-1}) and a saturating pulse (720 μmol m^{-2} s^{-1}) were used to measure the Fo in order to obtain Fm. In addition, the ratio of Fv to Fm was used to calculate the quantum yield efficiency of PS II, and the Fv was calculated from the difference between Fo and Fm.

Using a SPAD chlorophyll metre (SPAD 502 DL PLUS, Spectrum Technologies, USA), the chlorophyll content of intact leaves was determined.

Carboxylation efficiency was calculated with the values of net photosynthesis and intercellular CO_2 concentration as mentioned by Iqbal et al. (2011).

Water use efficiency was calculated from the ratio of net photosynthesis and stomatal conductance, as described by Von Caemmerer and Farquhar (1981).

2.8. Determination of carbohydrate metabolism enzymes activity

The enzyme activity of fructose-1, 6 bisphosphatase (FBPase) was estimated by adopting the method of Rao and Terry (1989). Sucrose synthase (SuSy), sucrose phosphate synthase (SPS), and soluble acid invertase (SAI) activities were determined spectrophotometrically adopting the method of Kalwade and Devarumath (2014). ADP-glucose pyrophosphorylase (AGPase) activity was evaluated following the procedure of Kleczkowski et al. (1993), through the conversion of NADP to NADPH at 340 nm. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity was estimated by following the protocol of Usuda (1985), respectively. Details are given in File S1.

2.9. Determination of carbohydrate metabolism-related attributes

Soluble sugar and sucrose content were estimated by the method of Xu et al. (2015), while starch content was determined by the method of Kuai et al. (2014). The content of total non-structural carbohydrate (NSC) was estimated by combining the soluble sugar content and the starch content (mg g^{-1} DW) evaluated in the present study. Details are given in File S1.

2.10. Determination of water status, plant dry mass accumulation and heat tolerance index

Leaf relative water content (RWC) was determined following the protocol of Barrs and Weatherley (1962). Details are given in File S1.

Plants were carefully uprooted, washed and dried in hot air oven at 80 °C till constant weight, the measurements for PDM was taken using weighing machine.

Heat tolerance index (HTI) was computed with the ratio of dry biomass of treated plants to dry biomass of control plants as per follow:

$$\text{HTI} = (\text{Ratio of dry mass of treated plants} \div \text{Ratio of dry mass of control plants}) \times 100$$

2.11. Measurement of ACS activity and ethylene evolution

ACS activity was determined by following the method of Avni et al. (1994) and Woeste et al. (1999). Leaf tissue (5.0 g) was ground in 100 mM HEPES buffer (pH 8.0) containing 4 mM DTT, 2.5 mM pyridoxal phosphate and 25% PVP. The homogenized preparation was centrifuged at 12, 000×*g* for 15 min. One mL of supernatant was placed in a 30 mL tube and 0.1 mL of 5 mM S-adenosyl methionine (AdoMet) was added and incubated for 2 h at 22 °C. The ACC formed was determined by its conversion to ethylene by addition of 0.1 mL of 20 mM $HgCl_2$ followed by the addition of 0.1 mL of a 1:1 mixture of saturated NaOH/NaCl and placed on ice for 10 min. In the control set, AdoMet was not added.

The ethylene evolution was estimated by using gas chromatograph (Nucon 5700, New Delhi, India). The detailed protocol has been provided by Khan et al. (2014).

2.12. Statistical analysis

Data were analysed statistically using analysis of variance (ANOVA) by RStudio (version 2022.02.3-492), and the results are presented as a treatment mean ± SE (n = 4). Additionally, Sigma Plot (ver. 12.0) was used to create bar graphs, while RStudio (ver. 2022.02.3-492) was used to generate principal component analysis (PCA) and correlation plots. For the significant data at $p < 0.05$, the least significant difference (LSD) was computed. Bars showing the same letter are not significantly different by LSD test at $p < 0.05$.

3. Results

3.1. Differential response of BR concentration on photosynthetic characteristics, plant dry mass accumulation and ethylene evolution

In order to access the standardized value of BR concentration, plants were treated with varying concentrations of BR that differentially affected methionine content and ACS activity. Treatment of BR (0.5 mM) significantly enhanced the content of methionine by 43.33% in comparison to control, however, when the concentration of BR was elevated beyond 0.5 mM, the levels of methionine decreased (Fig. 1). ACS activity maximally enhanced up to 40.75% at 0.5 mM of BR, while further increase in BR concentration reduced the activity of ACS largely (Fig. 1).

BR treatment also affected photosynthesis related attributes such as net photosynthesis, maximum efficiency of PSII and PDM (Fig. 1). Supplementation of BR increased net photosynthesis by 28.81%, maximum efficiency of PSII by 6.04% and PDM by 38.16% at 0.5 mM concentration, in comparison to the control. Beyond the standard BR concentration i.e., 0.5 mM, the net photosynthesis, maximum efficiency of PSII and the PDM were significantly decreased. Keeping in the view that 0.5 mM BR was best dose and taken for further BR related study on rice under heat stress.

3.2. Brassinosteroid and ethylene alleviate heat stress induced oxidative damages

To ascertain the impact of BR and ethylene application on heat stress-triggered oxidative stress, level of H_2O_2 and TBARS were measured with application of BR and ethylene under normal and heat stress environments (Table 1). Heat stress markedly elevated the H_2O_2 and TBARS content by 120.0% and 93.26%, respectively, as compared to control plants. Supplementation of ethylene under heat stress significantly reduced the levels of H_2O_2 and TBARS by 27.49% and 20.79% respectively. While, BR application to heat stressed plants resulted in more efficient reduction of oxidative stress than ethylene by decreasing TBARS and H_2O_2 levels in comparison to heat stressed plants (Table 1). Maximum reduction in H_2O_2 (32.65%) and TBARS (31.63%) levels were observed with the combined BR and ethylene treatment in heat stressed plants, implying the significant role of combined application in heat stress alleviation (Table 1).

3.3. Effect of brassinosteroid and ethylene on antioxidant metabolism under heat stress

The role of BR and ethylene in modulating the antioxidant enzyme

Table 1
Contents of hydrogen peroxide and thiobarbituric acid reactive substances, and ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase activities, ascorbate and glutathione contents in rice (*Oryza sativa*). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress (HT) and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (ET; 200 µl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$).

Traits	Control	HT	BR	ET	HT + BR	HT + ET	HT + BR + ET
Hydrogen peroxide content (µmol g^{-1} FW)	56.54 ± 1.42^{e}	124.30 ± 3.10^{a}	53.36 ± 1.33^{g}	55.05 ± 1.38^{f}	86.23 ± 2.15^{c}	90.14 ± 2.25^{b}	83.72 ± 2.09^{d}
Thiobarbituric acid reactive substances content (nmol g^{-1} FW)	17.82 ± 0.55^{e}	34.44 ± 1.06^{a}	15.14 ± 0.46^{g}	16.20 ± 0.53^{f}	25.42 ± 0.76^{c}	27.28 ± 0.81^{b}	23.64 ± 0.71^{d}
Ascorbate peroxidaseactivity (µmol AsA $min^{-1}mg^{-1}$ protein)	0.67 ± 0.02^{g}	1.01 ± 0.04^{d}	0.93 ± 0.034^{e}	0.87 ± 0.03^{f}	1.59 ± 0.055^{b}	1.48 ± 0.05^{c}	1.66 ± 0.061^{a}
Glutathione reductase activity (nmol NADH $min^{-1}mg^{-1}$ protein)	21.9 ± 1.1^{g}	29.6 ± 1.26^{d}	28.2 ± 1.26^{e}	27 ± 1.27^{f}	40.2 ± 1.64^{b}	38.7 ± 1.64^{c}	42.8 ± 1.72^{a}
Monodehydroascorbate reductase activity (nmol NADPH min^{-1} mg^{-1} protein)	35.7 ± 0.90^{g}	40 ± 0.83^{f}	52.1 ± 1.21^{d}	48.7 ± 1.94^{e}	56.3 ± 1.33^{b}	54.3 ± 1.08^{c}	59.6 ± 1.45^{a}
Dehydroascorbate reductase activity (nmol AsA min^{-1} mg^{-1} protein)	112.4 ± 2.9^{g}	126 ± 1.98^{f}	149 ± 5.9^{c}	139 ± 5.47^{e}	154.5 ± 3.89^{b}	147 ± 3.17^{d}	166 ± 4.067^{a}
Ascorbate content (nmol g^{-1} FW)	4876 ± 122.71^{c}	2321 ± 58.41^{g}	5214 ± 208^{a}	5121 ± 204.8^{b}	4322 ± 86.44^{e}	4287 ± 97^{f}	4567 ± 105^{d}
Glutathione content (nmol g^{-1} FW)	385.23 ± 7.70^{g}	426.53 ± 8.53^{f}	451.02 ± 9.02^{d}	438.13 ± 8.76^{e}	478.22 ± 9.56^{b}	467.33 ± 9.34^{c}	529.24 ± 10.58^{a}

Table 2
Ethylene content, plant dry mass, chlorophyll content and net photosynthesis in rice (*Oryza sativa*). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress (HT) and treated with foliar applications of brassinosteroid (BR; 0.5 mM) and/or ethylene (ET; 200 µl l^{-1}) at 30 days after sowing (DAS). Treatment of 1-aminoethoxyvinylglycine (AVG, ethylene biosynthesis inhibitor; 50 µM) at 20 DAS were also given under heat stress. Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$).

Traits	Control	HT	HT + BR	HT + BR + ET	HT + BR + ET + AVG
Ethylene content (ng kg^{-1} FW s^{-1})	22.1 ± 1.66^{d}	56.5 ± 2.42^{a}	29.1 ± 1.86^{c}	31.3 ± 1.65^{b}	20.23.1 ± 1.81^{e}
Plant dry mass (g $plant^{-1}$)	0.74 ± 0.051^{b}	0.47 ± 0.022^{e}	0.73 ± 0.034^{c}	0.86 ± 0.064^{a}	0.71 ± 0.031^{d}
Chlorophyll content (SPAD value)	23.9 ± 1.65^{b}	18.21 ± 1.43^{e}	22.1 ± 1.57^{c}	27.7 ± 1.72^{a}	21.6 ± 1.46^{d}
Net photosynthesis (µmol CO_2 m^{-2} s^{-1})	18.7 ± 1.87^{a}	14.1 ± 1.64^{d}	17.5 ± 1.72^{b}	19.1 ± 1.92^{a}	16.42 ± 1.53^{c}

activities including APX, GR, MDHAR and DHAR during heat stress were investigated (Table 1). Under non-stress condition, ethylene application effectively elevated the activities of APX, GR, MDHAR and DHAR enzymes by 29.85%, 23.29%, 36.41% and 23.67% respectively, and BR application enhanced the activities of these antioxidant enzymes by 38.80%, 28.77%, 45.94% and 32.56%, as compared to control plants.

In response of heat stress, ethylene treatment substantially enhances APX (46.53%), GR (30.74%), MDHAR (35.75%) and DHAR (16.67%) activities as compared to heat stressed plants. Furthermore, BR enhanced these enzymatic antioxidant activities by 57.43%, 35.82%, 40.75% and 22.62%, respectively compared to heat stressed plants. Interestingly, the cumulative application of BR and ethylene enhanced the activity of these enzymes in heat stressed rice plants to a significant higher level (Table 1).

The activity of non-enzymatic antioxidants such as AsA and GSH in rice plants during heat stress were also measured to determine the plant behavior to heat stress-induced oxidative damage (Table 1). The levels of AsA decreased, while GSH significantly increased under heat stress but ethylene treatment enhanced the levels of both AsA and GSH by 84.71% and 21.32%, as compared to heat stressed plants. Furthermore, application of BR further increased AsA (86.21%) and GSH (24.16%) levels than the heat stressed plants. However, it was the cumulative BR and ethylene treatment that maximally increased the AsA content by 96.77% and GSH content by 37.38% as compared to heat stressed plants (Table 1).

3.4. Brassinosteroid and ethylene improved the sulfur assimilation during heat stress

Impact of BR and ethylene applications were examined on sulfur-assimilation, which is influenced by ATP-S activity, sulfur, methionine and cysteine content in response of heat stress. During heat stress, sulfur, cysteine and methionine contents, along with ATP-S activity were drastically decreased in rice plants as compared to BR and ethylene treated plants (Fig. 2A–D). However, application of BR, ethylene and BR + ethylene during heat stress increased the sulfur content by 88.52%, 44.92% and 103.94% respectively, as compared to heat stressed plants (Fig. 2C). Moreover, both BR and ethylene application individually during heat stress also enhanced the ATP-S activity, cysteine and methionine contents though comparatively higher increase was reported with BR than ethylene (Fig. 2A, B and D). However, it was the combined application of BR and ethylene that maximally enhanced ATP-S activity by 160.11%, cysteine by 110.62% and methionine by 109.30% in comparison to heat stressed plants.

3.5. Brassinosteroid and ethylene improved the photosynthetic traits under heat stress

The influence of BR and ethylene on photosynthetic attributes including net photosynthesis, stomatal conductance, intercellular CO_2 concentration and maximum efficiency of PSII in response of non-stress and heat stress condition were determined (Fig. 3A–D). Heat stress drastically reduced net photosynthesis in rice plants by 32.51%, whereas stomatal conductance, intercellular CO_2 concentration and maximum efficiency of PSII were reduced by 35.64%, 42.69% and 24.04%, as compared to control plants (Fig. 3A–D). However, supplementation of BR and ethylene either alone or in combination ameliorated heat stress and increased the net photosynthesis, stomatal conductance, intercellular CO_2 concentration and maximum efficiency of PSII in comparison to the heat-stressed plants. It was the cumulative treatment of BR and ethylene that maximally alleviated the heat stress-induced adversities and enhanced the above-mentioned photosynthetic traits more efficiently than individual application of these two phytohormones, suggesting of a collaborative interaction between the two in heat tolerance (Fig. 3A–D).

Rice plants exposed to heat stress exhibited reduced leaf chlorophyll

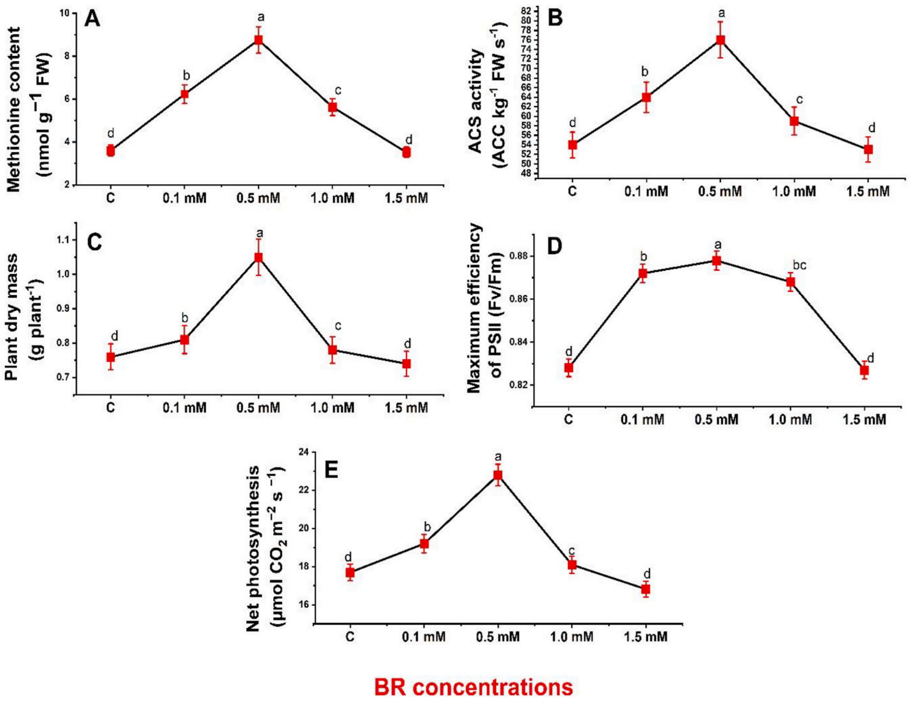


Fig. 1. Response of rice (*Oryza sativa*) to different concentrations of brassinosteroid (BR; 0.1, 0.5, 1.0 and 1.5 mM) as observed by measuring the (A) methionine content (nmol g^{-1} FW), (B) 1-amino cyclopropane1-carboxylic acid synthase activity (ACS; ng ACC kg^{-1} FW s^{-1}), (C) plant dry mass (g $plant^{-1}$), (D) maximum efficiency of PSII (Fv/Fm) and (E) net photosynthesis (µmol CO_2 m^{-2} s^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$). C, control; BR, brassinosteroid.

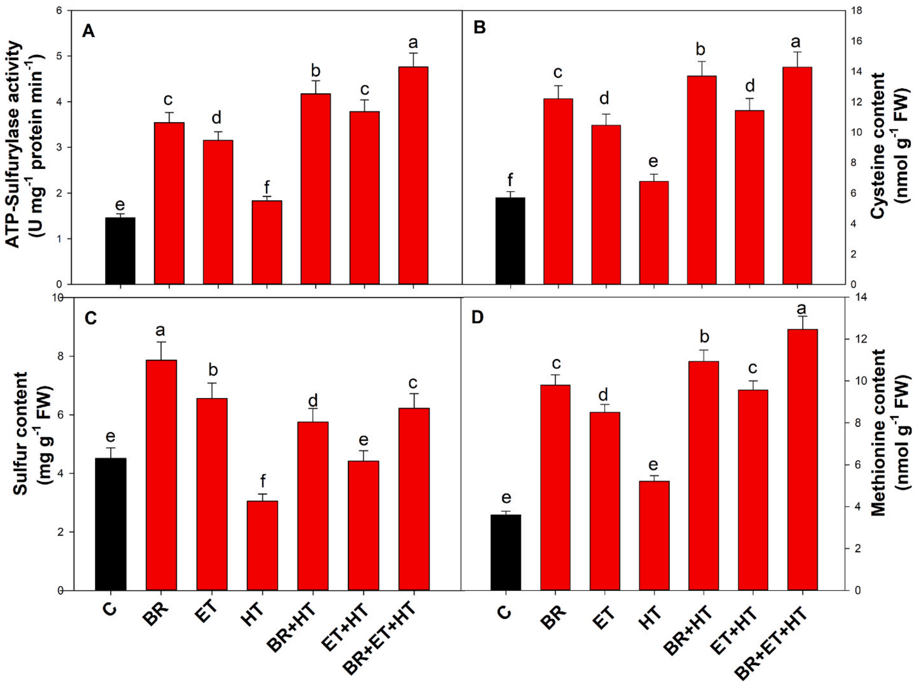


Fig. 2. (A) Adenosine triphosphate sulfurylase (ATP-S; U mg^{-1} protein min^{-1}) activity, contents of (B) cysteine (nmol g^{-1} FW), (C) sulfur (mg g^{-1} DW), and (D) methionine (nmol g^{-1} FW) in rice (*Oryza sativa*). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 µl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

content by 34.83% as compared to control plants. Moreover, the application of BR and ethylene in alone during heat stress alleviated the chlorophyll loss and further increased the chlorophyll content to significantly higher levels by 33.33%, and 20.94% respectively, as compared to heat-stressed plants (Fig. 3E). Similarly, heat stress decreased the carboxylation efficiency and water use efficiency of plants by 34.28% and 29.93%, while the individual application of BR and ethylene during heat stress resulted in enhanced carboxylation

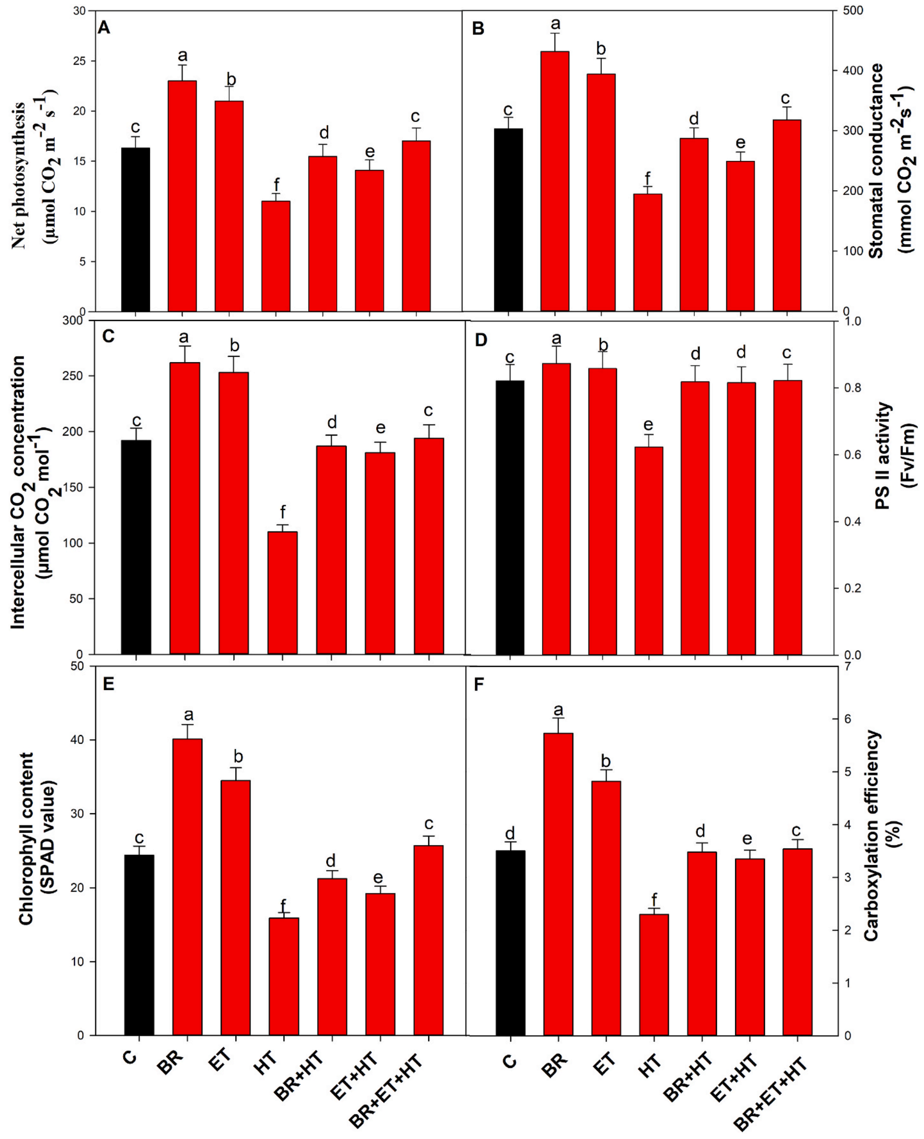


Fig. 3. (A) Net photosynthesis (μmol CO_2 m^{-2} s^{-1}), (B) stomatal conductance (nmol CO_2 m^{-2} s^{-1}), (C) intercellular CO_2 concentration (μmol CO_2 mol^{-1}), (D) maximum efficiency of PSII (Fv/Fm), (E) chlorophyll (SPAD value) and (F) carboxylation efficiency (%) in rice (*Oryza sativa*). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 μl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

efficiency by 51.30% and 45.65% and water use efficiency by 41.66% and 31.87% respectively, as compared to heat stressed plants. Moreover, the cumulative application of BR and ethylene showed maximal increase in carboxylation efficiency by 53.91% and water use efficiency by 51.56% as compared to heat stressed plants. Hence, both carboxylation efficiency (Fig. 3F) and water use efficiency (Fig. 6B) showed maximum alleviation from heat stress with combined BR and ethylene treatment where the increase was above the control as well as heat stress condition.

3.6. Brassinosteroid and ethylene improved the accumulation of carbohydrate metabolism-enzymes and its related attributes under heat stress

Under heat stress, the activity of enzymes involved in carbohydrate metabolism such as FBPase, SPS, AGPase and RuBisCO decreased by 15.0%, 27.77%, 22.72% and 13.58%, while the activity of SuSy and SAI increased 29.76% and 31.40%, respectively compared to control plants. Application of BR and ethylene individually in response to heat stress showed a substantial increase in FBPase (29.41%, 20.58%), SuSy (5.87%, 3.67%), SPS (57.38%, 46.15%), SAI (3.77%, 1.88%), AGPase (42.58%, 30.82%), and RuBisCO activities (27.14%, 21.43%) in comparison to heat stressed plants. However, cumulative application of BR and ethylene under heat stress condition showed a significant increase in these enzymes by 33.82%, 11.92%, 71.0%, 12.58%, 55.47%, and 38.14%, respectively, as compared to heat-exposed plants (Fig. 4A–F).

Under heat stress, total NSC, starch and sucrose content were drastically reduced by 29.31%, 29.63%, and 24.39%, while soluble sugar content significantly increased by 48.72%, as compared to control plants. Moreover, the treatment of BR and ethylene to heat stressed plants led to increased levels of total NSC (36.58%, 31.70%), soluble sugar (10.35%, 3.45%), starch (35.26%, 26.31%) and sucrose (32.47%, 25.16%) contents respectively, compared to heat-treated plants. Furthermore, significant increase in total NSC (51.22%), soluble sugar (18.97%), starch (49.05%) and sucrose (54.62%) contents were observed under the co-application of BR and ethylene as compared to

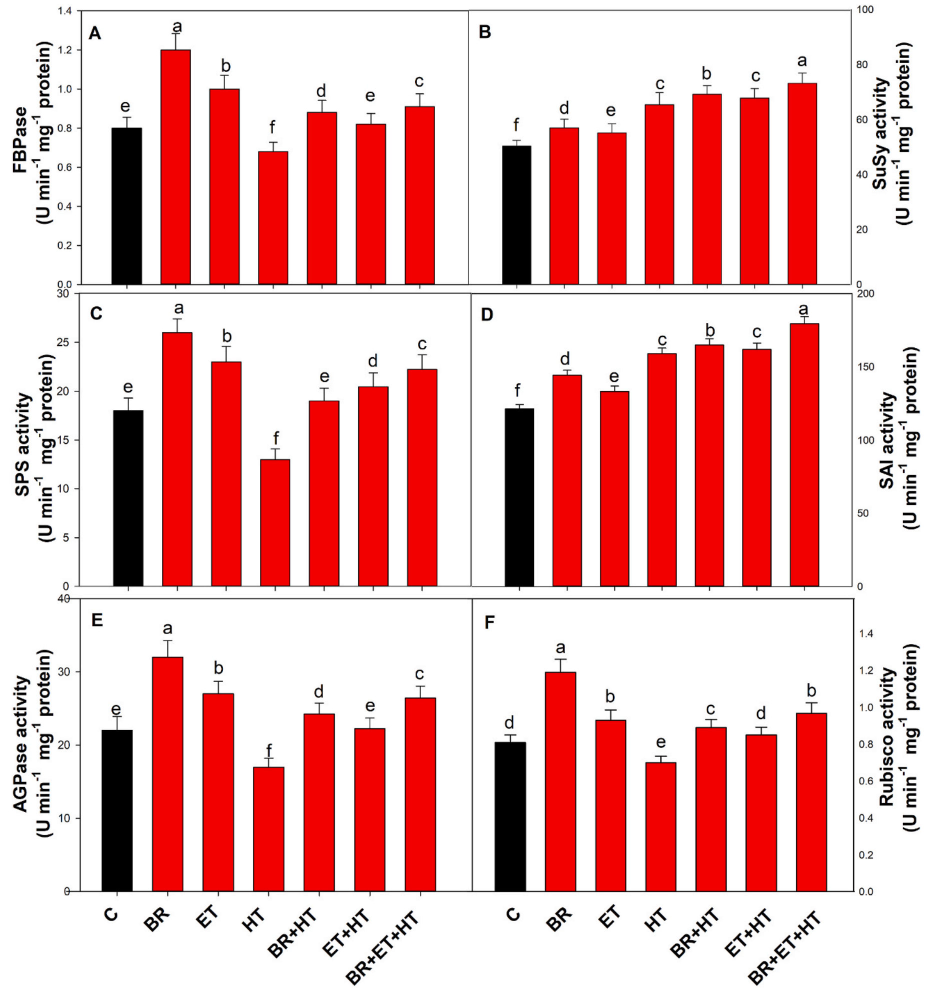


Fig. 4. (A) Fructose bisphosphatase (FBPase; U min^{-1} mg^{-1} protein) activity, (B) sucrose synthase (SuSy; U min^{-1} mg^{-1} protein) activity, (C) sucrose phosphate synthase (SPS; U min^{-1} mg^{-1} protein) activity, (D) soluble acid invertase (SAT; U min^{-1} mg^{-1} protein) activity, (E) ADP-glucose pyrophosphorylase (AGPase; U min^{-1} mg^{-1} protein) activity, and (F) Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; U min^{-1} mg^{-1} protein) activity in rice (*Oryza sativa* L.). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 μl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p <$ 0.05). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

heat stressed plants (Fig. 5A–D).

3.7. Brassinosteroid and ethylene improved the water status, plant dry mass accumulation and heat tolerance index under heat stress

Another growth-related attributes, relative water content and PDM were also found to be negatively influenced in response of heat stress, while the applications of BR and ethylene either alone or in combination during heat stress resulted in increased relative leaf water content and PDM, compared to heat stressed plants. However, cumulative treatment of BR and ethylene were found more efficient as compared to individual treatments in response of heat stress (Fig. 6C–D). Heat stressed plants exerted a notable decrease in heat tolerance index by 53.75% in comparison to control. However, the independent applications of BR and ethylene under heat stress further enhanced the heat tolerance index by 59.39% and 34.11%, respectively, in comparison to heat-treated plants. Co-application of BR and ethylene showed a prominent improvement in heat tolerance index by 87.11% as compared to heat-stressed plants (Fig. 6E).

Various treatments have been shown to induce phenotypic changes in rice plants (Fig. 6A). When subjected to heat stress, plants exhibited reduced root and leaf dimensions, as well as decreased biomass compared to the control plants. However, the application of BR and ethylene, either individually or in combination, during heat stress resulted in enhanced phenotypic characteristics relative to heat-stressed plants(Fig. 6A).

3.8. Brassinosteroid enhances heat stress tolerance in plants in an ethylene-dependent pathway

The combined application of BR and ethylene during heat stress led to a notable improvement in plant physiological traits, indicating an intriguing interaction between these two growth regulators. To explore this interaction further, we examined the effect of exogenous treatments of BR, ethylene, and their combination on ACS activity and ethylene production during heat stress(Fig. 7A and B). Our findings showed that heat stress increased ACS activity and endogenous ethylene levels compared to control plants. However, when BR and ethylene were applied together during heat stress, ethylene levels decreased along with ACS activity. Surprisingly, the combined treatment of BR and ethylene during heat stress resulted in significantly higher ACS activity and endogenous ethylene levels compared to individual treatments (Fig. 7A and B).

Furthermore, to validate the dependency of BR on ethylene biosynthetic pathway we used potential inhibitor of ethylene, AVG, to determine its effect on plant growth attributes during heat stress. The

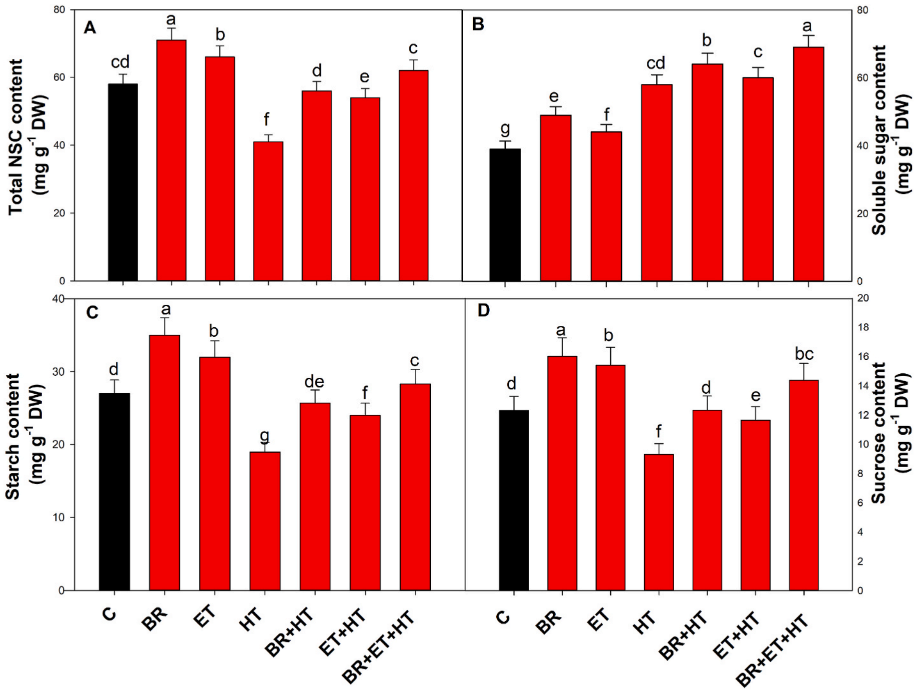


Fig. 5. (A) Total non-structural carbohydrate (NSC; mg g^{-1} DW) content, (B) soluble sugar content (mg g^{-1} DW), (C) starch content (mg g^{-1} DW), and (D) sucrose content (mg g^{-1} DW) in rice (*Oryza sativa* L.) Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 μl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

application of AVG along with the combined BR and ethylene treatment to the plants during heat stress resulted in a significant reduction in endogenous level of ethylene, reduced net photosynthesis, chlorophyll content and PDM accumulation in rice plants (Table 2).

3.9. Principal component analysis

In the conducted analysis on rice, the principal component analysis (PCA) was employed to assess the degree of data variation and the correlation between different treatments and parameters(Fig. 8A). Under different treatments, the two components (PC1 and PC2) accounted for 88.89% of data variability, with PC1 accounting for 64.38% of the total variation and PC2, accounting for 24.51% of the total variation. The oxidative stress indicators like H_2O_2, TBARS were converged together with ACS activity and ethylene, corresponded to heat stress treatment, and were positively correlated with each other suggesting the role of stress ethylene with heat stress induced oxidative stress.

The bi-plot and correlation graph showed a positive relationship between PDM, chlorophyll content, net photosynthesis, stomatal conductance, intercellular CO_2 concentration, maximum efficiency of PSII, carboxylation and water use efficiency and sulfur content which was maximally observed in the individual BR and ethylene treatment under no stress. In addition, enzymatic and non-enzymatic antioxidants, carbohydrate metabolism related traits along with cysteine and methionine are positively correlated but maximally expressed in the ethylene + BR + HT treatment (Fig. 8A and B).

4. Discussion

Rice is highly susceptible to high temperatures, which disrupt plant cellular and developmental processes such as photosynthesis, pollen germination, grain yield and spikelet fertility (Karwa et al., 2020). During the postanthesis stage, heat stress alters carbon metabolism and hormonal regulation in rice, negatively affecting reproductive success, seed setting, and grain filling processes (Shi et al., 2017). To cope with such harsh conditions, the rice plant employs stress avoidance and tolerance mechanisms that strengthen its ability to survive. Phytohormones function as critical switches in a complex and sophisticated regulatory network that maintains plant homeostasis and molecular mechanisms while also allowing adaptation to transmit heat stress resistance in plants (Khan et al., 2019; Yang et al., 2021).

BRs and ethylene play a critical role in modulating plant growth responses and mitigating adversities caused by imposed conditions of heat stress by stimulating several heat shock proteins (HSPs) and other protective enzymes (Thussagunpanit et al., 2015; Anwar et al., 2018). Several studies have shown that hormonal action is dependent on a cascade of interactions and that BR and ethylene both contribute significantly to the amelioration of heat stress (Wu and Yang, 2019; Ahammed et al., 2020; Poór et al., 2021).). Recent studies demonstrated that BZR1 (Brassinazole resistant 1) suppresses HSP-associated inhibitors by directly repressing the expression of *ERF49* (ethylene responsive factors), leading to increased *HSPs* expression, and enhanced heat stress resistance in *Arabidopsis thaliana* (Arabidopsis; Chen et al., 2022). In addition, we aimed to diagnose a mechanistic approach responsible for BR-mediated heat tolerance. BR application affects plant growth-related attributes such as net photosynthesis, stomatal conductance, intercellular CO_2 concentration, maximum efficiency of PSII, carboxylation and water use efficiency, chlorophyll content and PDM under normal or heat stress conditions; it influences ACS activity and methionine content, both of which play a pivotal role in ethylene biosynthesis. In our observation, heat stress markedly lowered RuBisCO and FBPase activities in rice plants. However, the application of BR and ethylene alleviated this effect (Fig. 4). Our observations are consistent with those of Gautam et al. (2022) and Zhao et al. (2017b), who found increased RuBisCO and FBPase activity in BR- and ethylene-treated plants.

Studies have revealed that leaf relative water content is a valuable indicator of hydration levels and can provide benefits for plants under stressful environments (Mullan and Pietragalla, 2012; Vialet-Chabrand

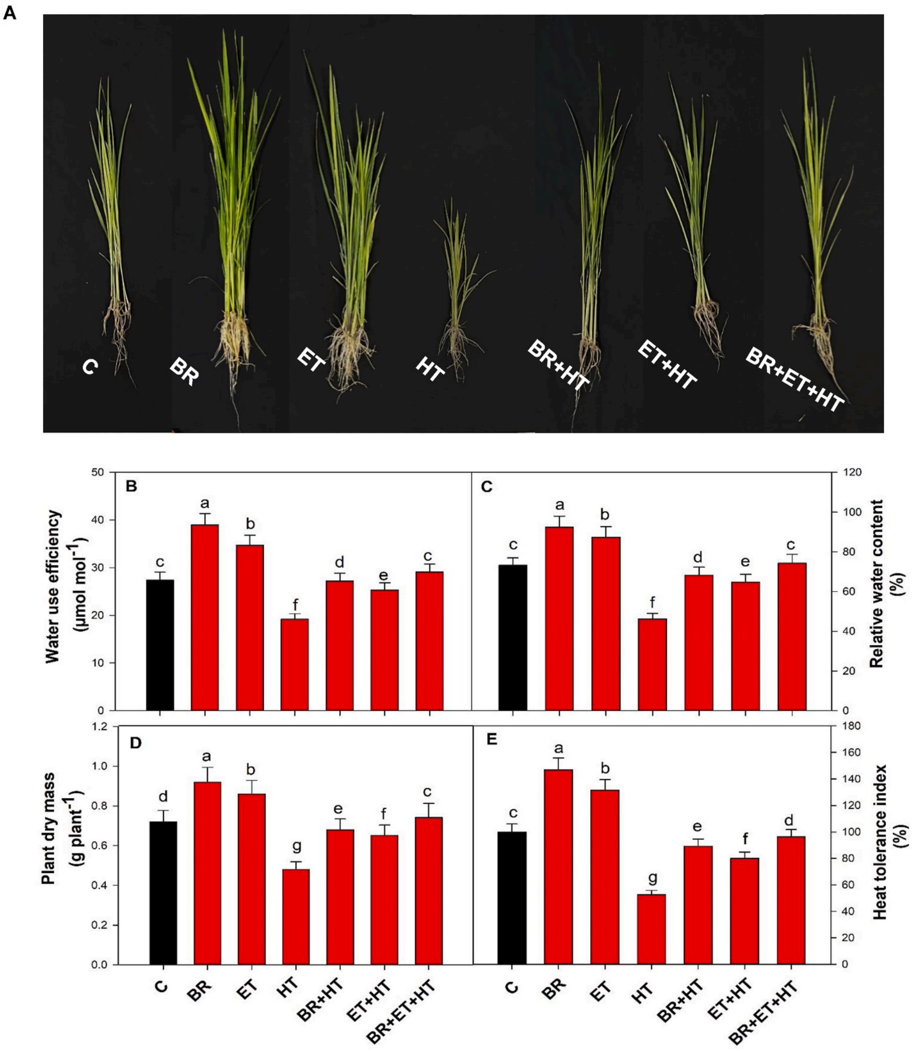


Fig. 6. (A) Phenotypic representation of plant growth subjected with different treatments, (B) water use efficiency (μmol mol^{-1}), (C) relative water content (%), (D) PDM (g plant^{-1}, (E) heat tolerance index (HTI; %) in rice (*Oryza sativa* L.). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 μl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p < 0.05$). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

et al., 2017). Additionally, PDM has emerged as an important growth attribute, accounting for dry matter accumulation in plants and being attributed to key assets of plant growth and survival under abiotic stresses (Steiner et al., 2021). Hence, plasticity in PDM allocation reflects the adaptability of plants under stress, while a decrease in PDM can negatively impact growth and yield. . The prevailing study speculates that an enhancement in relative leaf water content implies a stable plant−water relationship, favoring PDM accumulation in BR- and ethylene-treated plants under heat stress. Our results are consistent with those obtained by Wang et al. (2022), who reported that BR (0.05–1 μM) treatment effectively alleviated the heat stress-induced decrease in relative water content and significantly increased the leaf net photosynthesis rate and plant dry mass production in soybeans (*Glycine max*) under heat stress conditions.

Heat stress-induced ROS accumulation disrupts plant homeostasis and cellular functions (Buttar et al., 2020), and to cope with this, intricate antioxidant defense machinery is activated (Karwa et al., 2020). In agreement with this, in the present study, heat stress-induced oxidative damage was found to be reduced by the supplementation of BR and/or ethylene in rice. The reduction was evident in the decreased levels of TBARS and H_2O_2, along with increased antioxidative metabolism. The ascorbate-glutathione (AsA-GSH) cycle, a major pathway in H_2O_2 scavenging plays a crucial role in mitigating heat stress (Iqbal et al., 2021). In our research, BR and/or ethylene application was found to mitigate heat stress-triggered oxidative damage by decreasing H_2O_2 and TBARS levels through enhancing the activity of APX, GR, DHAR and MDHAR and the nonenzymatic antioxidants AsA and GSH. These mechanisms contribute to the maintenance of redox homeostasis, thereby ensuring optimal cellular functionality under heat stress conditions (Table 1). Notably, previous studies have also highlighted the positive effects of BR and ethylene on AsA-GSH levels, as well as their involvement in enhancing stress tolerance (Mazorra et al., 2014; Kaya et al., 2020; Fatma et al., 2021). Wang et al. (2022) also found that BR had a significant regulatory effect on the antioxidant system in soybeans under heat stress. Previous studies showed that BR could upregulate the expression of genes encoding antioxidant enzymes, such as *Cu/Zn-SOD*, *CAT1*, *cAPX* and *GR1,* in tomato and cucumber seedlings (Xia et al., 2009; Zhou et al., 2014)., This transcriptional regulation suggests that BR plays a crucial role in enhancing antioxidant capacity. Our study, along with previous research, supports the notion that maintaining ROS homeostasis is a key physiological mechanism by which BR improves plant heat stress tolerance. Although there have been indications of crosstalk between BR and ethylene in mediating thermotolerance (Anwar et al., 2018; Jiroutova et al., 2018), the specific mechanisms

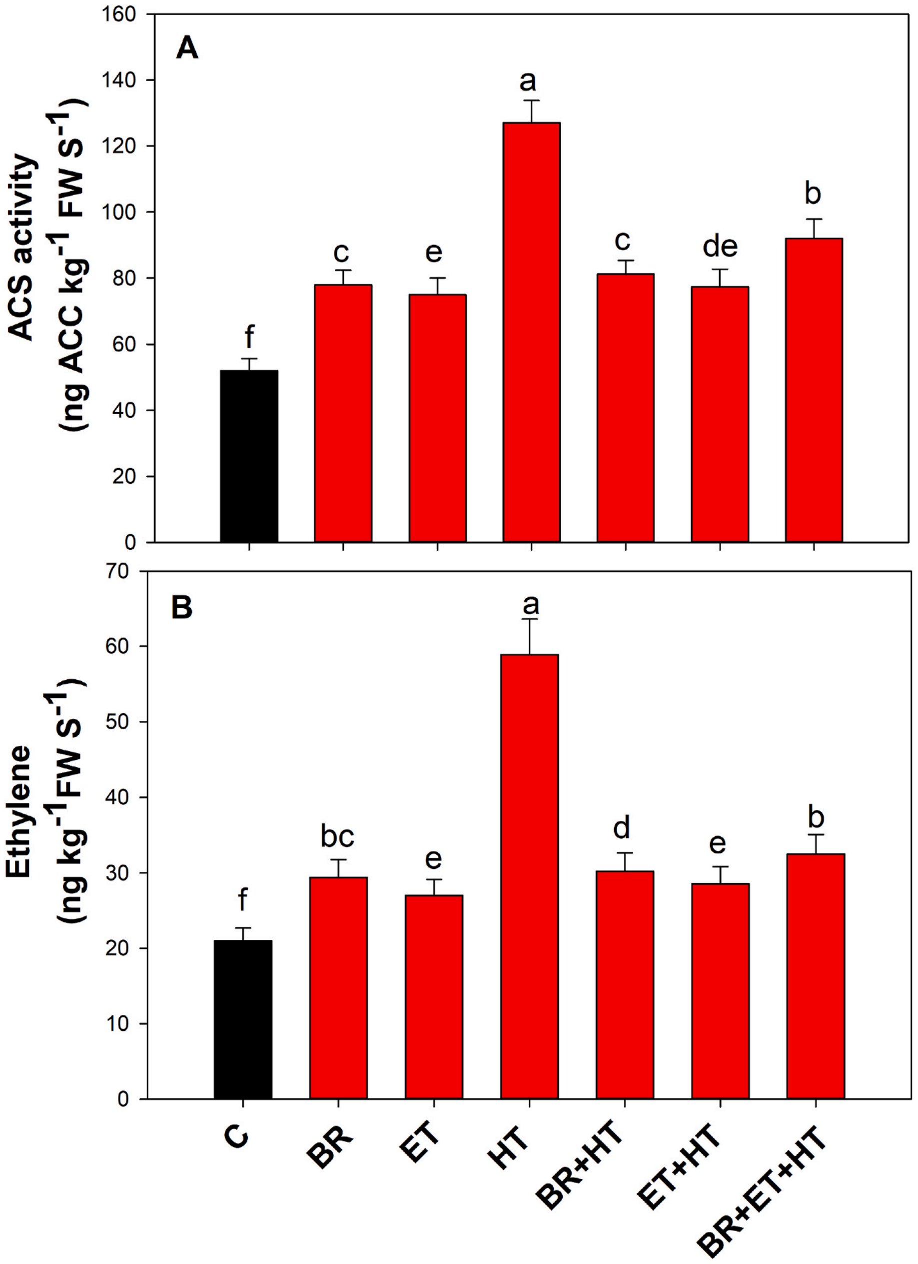


Fig. 7. (A) 1-aminocyclopropane-1-carboxylate synthase activity (ACS; ng ACC kg^{-1} FW s^{-1}) and (B) ethylene content (ng kg^{-1} FW s^{-1}) in rice (*Oryza sativa*). Plants were grown in the presence (40 °C) or absence (25 °C) of heat stress and treated with foliar application of brassinosteroid (BR; 0.5 mM) and/or ethylene (200 μl l^{-1}) at 30 days after sowing (DAS). Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at ($p <$ 0.05). C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress.

underlying this pathway remain to be fully elucidated. Furthermore, there is a research gap in understanding the combined treatment of BR and ethylene and how they jointly modulate stress tolerance. We observed the additive effect of BR and ethylene in reducing heat tolerance in rice compared to their individual applications. The combination of BR and ethylene caused the maximum increase in defense systems, with a greater reduction in oxidative stress markers. This enhancement led to improved RuBisCO activity, carboxylation and water use efficiency of the plants and increased photosynthetic traits over the control. The importance of BR in enhancing photosynthesis in rice under heat stress has been shown (Thussagunpanit et al., 2015). Ethylene also enhances photosynthesis in rice exposed to heat stress (Wu and Yang, 2019; Gautam et al., 2022a).

In addition to the plant defense system, sugars play a critical role in regulating plant growth and adaptation to heat stress (Zhang et al., 2018). Our observations suggest that the combined application of BR and ethylene in rice plants under heat stress leads to significant increase in total NSC and soluble sugar contents. These sugars act as osmotolerant molecules and provide a nutrient source for carbon. By regulating sucrose and starch concentrations, plants maintain source-sink homeostasis and prevent feedback inhibition, enhancing their resilience to heat stress (Wang et al., 2019; Gautam et al., 2022a). These findings emphasize the significance of sugar metabolism in plant responses to heat stress. The interaction between the source and sink in plants plays a vital role in carbon assimilation and distribution, which ultimately affects sink strength and source activity (Yu et al., 2015). In our study, the combined application of BR and ethylene in rice plants under heat stress resulted in increased accumulation of sucrose and starch, along with enhanced AGPase activity. This was achieved by boosting SPS activity and reducing SuSy and SAI activities. As a result, we observed a

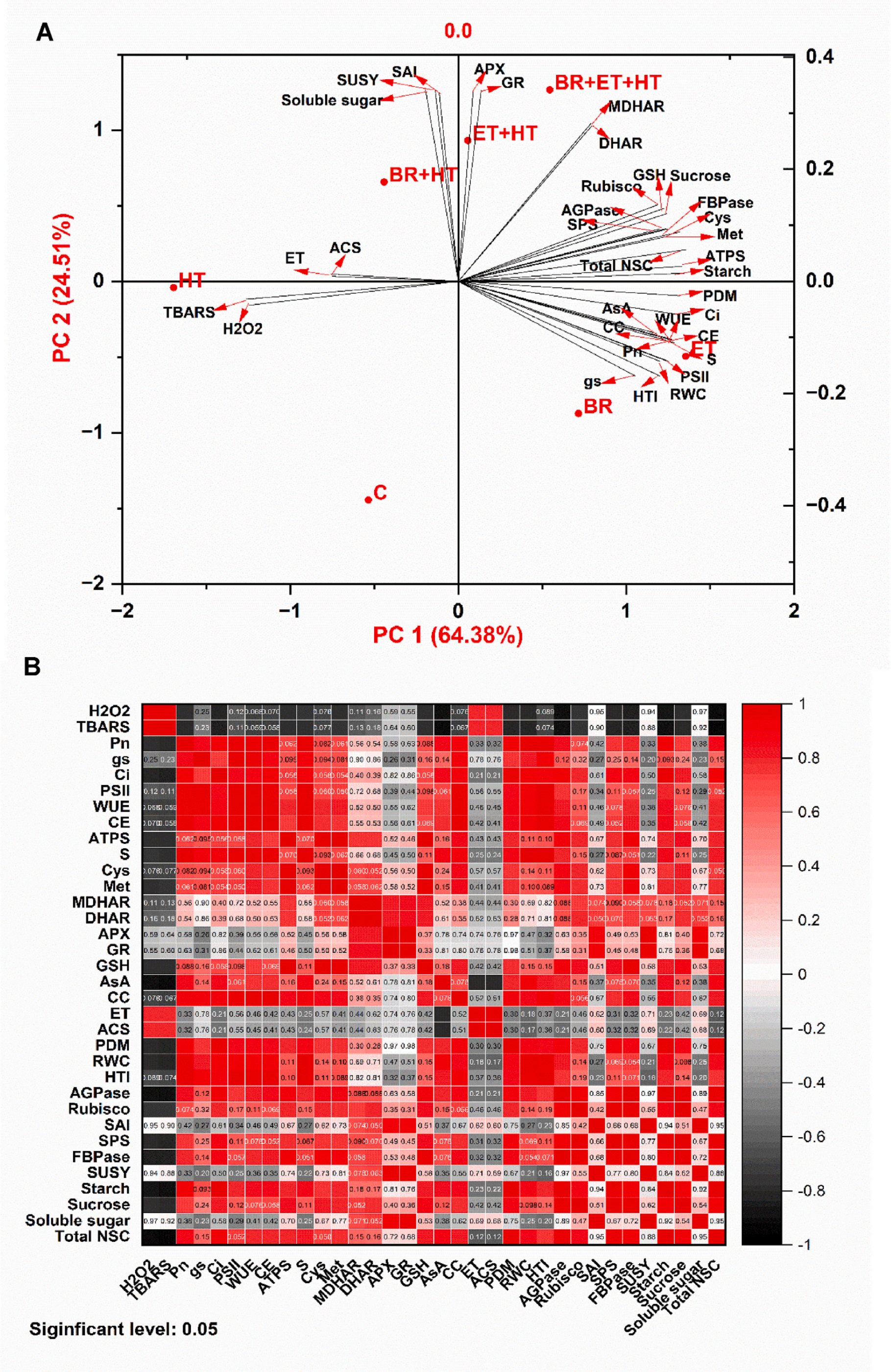


Fig. 8. Principal component analysis: (A) PCA biplot comparing the different studied traits in response of independent applications of BR and ET, and combined application of BR + ET under heat stress. (B) Correlation graph between traits studied. The traits which were correlated in PCA biplot are H_2O_2, TBARS, photosynthetic rate (Pn), stomatal conductance (g_s), intercellular CO_2 concentration (Ci), maximum efficiency of PSII, chlorophyll content, carboxylation efficiency (CE), water use efficiency (WUE), ATP-S, sulfur (S), cysteine (Cys), GSH, AsA, APX, GR, GPX, MDHAR, DHAR, ACS, ET, NSC, soluble sugar, starch, sucrose, FBPase, SuSy, SPS, SAI, AGPase, RuBisCO, relative water content (RWC), PDM (PDM) and heat tolerance index (HTI). The correlation plot is color-coded, indicating the strength of the correlation: Red highlights the positive correlations while black highlights negative correlations. The stronger the color, the larger the correlation magnitude. C, control; BR, brassinosteroid; ET, ethylene; HT, heat stress. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reduction in heat stress-induced oxidative stress and improved photosynthesis-related processes and source-sink capacity. These findings align with previous studies that have highlighted the role of BR and ethylene in maintaining source−sink capacity, carbohydrate metabolism and thermotolerance in rice seedlings confined to high-temperature stress (Sonjaroon et al., 2018; Gautam et al., 2022a).

Heat stress decreases sulfur content while increasing ATP-S activity and the content of cysteine and methionine. However, the application of ethylene and BR, individually and in combination, under heat stress further increases sulfur assimilation through increased ATP-S activity and the content of cysteine, methionine and sulfur, along with reduced GSH production. GSH functions as an antioxidant, detoxifying ROS and changing to oxidized GSH (GSSG), which is then converted back to GSH by GR. The redox balance is maintained by the AsA-GSH cycle, where DHAR, APX, GR and MDHAR increase to balance cellular homeostasis under stress. It has been revealed that increased ATP-S activity with ethylene resulted in increased cysteine and GSH content along with photosynthesis and growth (Iqbal et al., 2013). Manipulation of cysteine and GSH biosynthesis enhances resilience to Cd stress (Masood et al., 2016). With an increase in sulfur assimilation and cysteine, GSH and methionine content, we can predict an increase in ethylene content. Although excess stress ethylene is formed in response to heat stress, when this is supplemented with a BR and ethylene combination, a decrease in ethylene level was observed, although it was still above the control level. However, individual BR and ethylene treatment under heat stress resulted in ethylene evolution that was below the control and caused less stress alleviation. These results show that if stress ethylene signifies stress conditions, then its reduction should be beneficial to plants. However, the combined ethylene and BR under heat stress exhibited a higher pool of cysteine and methionine, resulting in an optimum ethylene level, which mediated higher GSH synthesis and thus greater ROS reduction and better photosynthesis and growth. Reports suggest that when there is sufficient ethylene that binds to receptors, only ethylene signaling is initiated; otherwise, the signaling is inhibited. Ethylene functions as an inverse agonist by blocking its receptors, causing a reduction in constitutive triple response (CTR1) activity, which inhibits ethylene insensitive 2 (EIN2), and EIN2 affects intracellular signaling, resulting in the majority of ethylene responses (Binder, 2020). The relationship between ethylene, GSH and sulfur has also been explored in wheat (*Triticum aestivum*) for Cd tolerance (Khan et al., 2015). Ethylene production was associated with salt tolerance in wheat, leading to decreased oxidative stress markers and increased polyamine and proline accumulation (El-Bassiouny and Bekheta, 2005). Ethylene was found to optimize GSH production through its influence on the AsA-GSH cycle for the modulation of cellular redox homeostasis under salt stress (Sehar et al., 2021). GSH triggers ethylene biosynthesis by regulating the transcription and translation of its key biosynthesis enzymes, ACS and ACO, thereby enhancing tolerance. Exogenous GSH supplementation improved stress tolerance in wild-type plants but not in the ethylene-*insensitive 2.1 mutant*, suggesting an ethylene-mediated pathway for GSH-triggered tolerance (Datta et al., 2015). In rice, ethylene-mediated signaling reduced oxidative damage, maintained chlorophyll content, and improved thermotolerance in rice seedlings under heat stress (Wu and Yang, 2019).

In the current study, it was found that exogenous supplementation of BR or ethylene reduced internal ethylene levels and ACS activity, with BR having a more prominent effect than ethylene. It is known that ethylene has a dual nature. It acts as a signaling molecule, alerting plants to initiate stress responses by sensing stress and increasing its concentration. However, its optimal conditions directly influence photosynthesis and growth. Both BR and ethylene reduced ethylene production, which specifies that stress is reduced but the level of ethylene is sufficiently below control and does not cause an alleviation effect. In contrast, the application of BR and ethylene in combination not only maximally reduces oxidative stress but also enhances the photosynthetic and growth characteristics of the plant.

The research conducted by Hansen et al. (2009) demonstrated that BR exert their effects on plant physiology by enhancing the stability of the ACS5 protein through post-transcriptional mechanisms. In a study by Arteca et al. (1988), it was observed that BR-induced ethylene production can be partially inhibited by the scavenging of ROS, highlighting the connection between BR, ethylene, and ROS signaling pathways. Furthermore, Zhu et al. (2016) established a coordinated response between BR and ethylene in plant salt stress tolerance, with both growth regulators influencing each other's effects. In the present study, it was observed that the application of AVG to ethylene and BR treated plants, resulted in a decrease in thermotolerance, chlorophyll content, net photosynthesis and PDM, indicating the dependence of BR action on the ethylene pathway. These findings contribute to our understanding of the interplay between BR and ethylene in plant stress responses.

5. Conclusion and future prospects

Conclusively, our findings demonstrate that the exogenous application of BR can effectively mitigate the negative impacts of heat stress in rice plants. Through its interaction with ethylene biosynthesis and enhancement of plant defense systems, BR improves photosynthetic efficiency, source−sink capacity and growth traits. This was confirmed by the application of AVG to BR and ethylene-treated plants. Overall, BR acts as a significant plant hormone not only alleviating heat stress but also enhancing physiological efficiency in rice plants by modulating ethylene production. Further research is needed to unravel the intricate crosstalk between BR and other phytohormones in counteracting heat stress-induced adversities. These findings call for additional physiological investigations to elucidate the mechanisms underlying BR- and ethylene-mediated heat tolerance and the associated signaling pathways. By deciphering the molecular machinery and identifying key genes and signaling molecules involved, we can contribute to the development of heat-tolerant rice varieties and promote sustainable agriculture practices.

CRediT authorship contribution statement

Faroza Nazir: Software, Data curation, Formal analysis, Writing – original draft. **Badar Jahan:** Software, Data curation. **Sarika Kumari:** Software, Writing – review & editing. **Noushina Iqbal:** Software, Writing – original draft. **Mohammed Albaqami:** Software, Writing – review & editing. **Adriano Sofo:** Software. **M. Iqbal R. Khan:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

Acknowledgement

F. N. acknowledges DBT-Research Associateship, India. The authors extend their appreciation to the Deputyship for Research and Innovation, “Ministry of Education” in Saudi Arabia for funding this research (IFKSUOR3-098-2).

References

Aghamolki, M.T.K., Yusop, M.K., Oad, F.C., Zakikhani, H., Jaafar, H.Z., Kharidah, S., Musa, M.H., 2014. Heat stress effects on yield parameters of selected rice cultivars at reproductive growth stages. J. Food Agric. Environ. 12 (2), 741–746.

Ahammed, G.J., Li, X., Zhou, J., Zhou, Y.H., Yu, J.Q., 2016. Role of hormones in plant adaptation to heat stress. In: Plant Hormones under Challenging Environmental Factors. Springer, Dordrecht, pp. 1–21.

Ahammed, G.J., Li, X., Liu, A., 2020. Brassinosteroids in plant tolerance to abiotic stress. J. Plant Growth Regul. 39, 1451–1464.

Anwar, A., Liu, Y., Dong, R., Bai, L., Yu, X., Li, Y., 2018. The physiological and molecular mechanism of brassinosteroid in response to stress: a review. Biol. Res. 51, 46.

Arteca, R.N., Bachman, J.M., Mandava, N.B., 1988. Effects of indole-3-acetic acid and brassinosteroid on ethylene biosynthesis in etiolated mung bean hypocotyl segments. J. Plant Physiol. 133, 430–435.

Avni, A., Bailey, B.A., Mattoo, A.K., Anderson, J.D., 1994. Induction of ethylene biosynthesis in *Nicotiana tabacum* by a Trichoderma viride xylanase is correlated to the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase transcripts. Plant Physiol. 106, 1049–1055.

Barrs, H.D., Weatherley, P.E., 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust. J. Biol. Sci. 15 (3), 413–428.

Binder, B.M., 2020. Ethylene signaling in plants. J. Biol. Chem. 295 (22), 7710–7725.

Buttar, Z.A., Wu, S.N., Arnao, M.B., Wang, C., Ullah, I., Wang, C., 2020. Melatonin suppressed the heat stress-induced damage in wheat seedlings by modulating the antioxidant machinery. Plants 9, 809.

Cao, S., Xu, Q., Cao, Y., Qian, K., An, K., Zhu, Y., Binzeng, H., Zhao, H., Kuai, B., 2005. Loss of function mutations in *DET2* gene lead to an enhanced resistance to oxidative stress in Arabidopsis. Physiol. Plantarum 123, 57–66.

Chen, J., Fei, K., Zhang, W., Wang, Z., Zhang, J., Yang, J., 2021. Brassinosteroids mediate the effect of high temperature during anthesis on the pistil activity of photo-thermosensitive genetic male-sterile rice lines. Crop J 9 (1), 109–119.

Chen, X., Xue, H., Zhu, L., Wang, H., Long, H., Zhao, J., Zhu, S., 2022. ERF49 mediates brassinosteroid regulation of heat stress tolerance in *Arabidopsis thaliana*. BMC Biol. 20 (1), 1–17.

Choudhary, S.P., Oral, H.V., Bhardwaj, R., Yu, J.Q., Tran, L.S.P., 2012. Interaction of brassinosteroids and polyamines enhances copper stress tolerance in *Raphanus sativus*. J. Exp. Bot. 63 (15), 5659–5675.

Datta, R., Kumar, D., Sultana, A., Hazra, S., Bhattacharyya, D., Chattopadhyay, S., 2015. Glutathione regulates1-aminocyclopropane-1-carboxylate synthase transcription via WRKY33 and 1-aminocyclopropane-1-carboxylate oxidase by modulating messenger RNA stability to induce ethylene synthesis during stress. Plant Physiol. 169, 2963–2981.

Dhindsa, R.H., Plumb-Dhindsa, P., Thorpe, T.A., 1981. Leaf senescence correlated with increased level of membrane permeability, lipid peroxidation and decreased level of SOD and CAT. J. Exp. Bot. 32, 93–101.

El-Bassiouny, H.M.S., Bekheta, M.A., 2005. Effect of salt stress on relative water content, lipid peroxidation, polyamines, amino acids and ethylene of two wheat cultivars. Int. J. Agric. Biol. 7, 363–368.

Fatma, M., Iqbal, N., Gautam, H., Sehar, Z., Sofo, A., D'Ippolito, I., Khan, N.A., 2021. Ethylene and sulfur coordinately modulate the antioxidant system and ABA accumulation in mustard plants under salt stress. Plants 10 (1), 180.

Fitzgerald, M.A., McCouch, S.R., Hall, R.D., 2009. Not just a grain of rice: the quest for quality. Trend. Plant Sci. 14 (3), 133–139.

Foyer, C.H., Dujardyn, M., Lemoine, Y., 1989. Responses of photosynthesis and the xanthophyll and ascorbate-glutathione cycles to changes in irradiance, photoinhibition and recovery. Plant Physiol. Biochem. 27, 751–760.

Foyer, C.H., Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplast: a proposed role in ascorbic acid metabolism. Planta 133, 21–25.

Gautam, H., Fatma, M., Sehar, Z., Iqbal, N., Albaqami, M., Khan, N.A., 2022. Exogenously- sourced ethylene positively modulates photosynthesis, carbohydrate metabolism, and antioxidant defense to enhance heat tolerance in rice. Int. J. Mol. Sci. 23 (3), 1031.

Giatonde, M.K., 1967. A spectrophotometric method for direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem. J. 104, 627–633.

Gourdji, S.M., Sibley, A.M., Lobell, D.B., 2013. Global crop exposure to critical high temperatures in the reproductive period: historical trends and future projections. Environ. Res. Lett. 8 (2), 024041.

Griffith, O.W., 1980. Determination of GSH disulphide using GSH reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–211.

Hansen, M., Chae, H.S., Kieber, J.J., 2009. Regulation of ACS protein stability by cytokinin and brassinosteroid. Plant J. 57, 606–614.

Hassan, M.U., Chattha, M.U., Khan, I., Chattha, M.B., Barbanti, L., Aamer, M., Iqbal, M. M., Nawaz, M., Mahmood, A., Ali, A., Aslam, M.T., 2021. Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. Plant Biosyst.- Int. J. Deal. Aspects Plant Biol. 155 (2), 211–234.

Hewitt, E.J., 1952. Sand and Water Culture Methods Used in the Study of Plant Nutrition. Sand and Water Culture Methods Used in the Study of Plant Nutrition. Tech. Commun. East Malling, Common. Bur. Hort. Plant 241, 202-32.

Horn, M.J., Jones, D.B., Blum, A.E., 1946. Colorimetric determination of methionine in proteins and foods. J. Biol. Chem. 166, 313–320.

Iqbal, N., Nazar, R., Syeed, S., Masood, A., Khan, N.A., 2011. Exogenously-sourced ethylene increases stomatal conductance, photosynthesis, and growth under optimal and deficient nitrogen fertilization in mustard. J. Exp. Bot. 62 (14), 4955–4963.

Iqbal, N., Umar, S., Khan, N.A., Corpas, F.J., 2021. Nitric oxide and hydrogen sulfide coordinately reduce glucose sensitivity and decrease oxidative stress via ascorbate-glutathione cycle in heat-stressed wheat (*Triticum aestivum* L.) plants. Antioxidants 10, 108.

Iqbal, N., Masood, A., Khan, M.I.R., Asgher, M., Fatma, M., Khan, N.A., 2013. Cross-talk between sulfur assimilation and ethylene signaling in plants. Plant Signal. Behav. 8 (1), e22478.

Jahan, B., AlAjmi, M.F., Rehman, M.T., Khan, N.A., 2020. Treatment of nitric oxide supplemented with nitrogen and sulfur regulates photosynthetic performance and stomatal behavior in mustard under salt stress. Physiol. Plantarum 168, 490–510.

Janni, M., Gullì, M., Maestri, E., Marmiroli, M., Valliyodan, B., Nguyen, H.T., Marmiroli, N., 2020. Molecular and genetic bases of heat stress responses in crop plants and breeding for increased resilience and productivity. J. Exp. Bot. 71, 3780–3802.

Jegadeesan, S., Chaturvedi, P., Ghatak, A., Pressman, E., Meir, S., Faigenboim, A., Firon, N., 2018. Proteomics of heat-stress and ethylene-mediated thermotolerance mechanisms in tomato pollen grains. Front. Plant Sci. 9.

Jiroutova, P., Oklestkova, J., Strnad, M., 2018. Crosstalk between brassinosteroids and ethylene during plant growth and under abiotic stress conditions. Int. J. Mol. Sci. 19 (10), 3283.

Kalwade, S.B., Devarumath, R.M., 2014. Functional analysis of the potential enzymes involved in sugar modulation in high and low sugarcane cultivars. Appl. Biochem. Biotechnol. 172 (4), 1982–1998.

Karwa, S., Bahuguna, R.N., Chaturvedi, A.K., 2020. Phenotyping and characterization of heat stress tolerance at reproductive stage in rice (*Oryza sativa* L.). Acta Physiol. Plant. 42, 29.

Kaya, C., Ashraf, M., Alyemeni, M.N., Ahmad, P., 2020. The role of nitrate reductase in brassinosteroid-induced endogenous nitric oxide generation to improve cadmium stress tolerance of pepper plants by upregulating the ascorbate-glutathione cycle. Ecotoxicol. Environ. Saf. 196, 110483.

Khan, M.I.R., Khan, N.A., 2014. Ethylene reverses photosynthetic inhibition by nickel and zinc in mustard through changes in PS II activity, photosynthetic nitrogen use efficiency, and antioxidant metabolism. Protoplasma 251 (5), 1007–1019.

Khan, M.I.R., Iqbal, N., Masood, A., Per, T.S., Khan, N.A., 2013. Salicylic acid alleviates adverse effects of heat stress on photosynthesis through changes in proline production and ethylene formation. Plant Signal. Behav. 8 (11), e26374.

Khan, M.I.R., Asgher, M., Khan, N.A., 2014. Alleviation of salt-induced photosynthesis and growth inhibition by salicylic acid involves glycinebetaine and ethylene in mungbean (*Vigna radiata* L.). Plant Physiol. Biochem. 80, 67–74.

Khan, M.I.R., Nazir, F., Asgher, M., Per, T.S., Khan, N.A., 2015. Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. J. Plant Physiol. 173, 9–18.

Khan, S., Anwar, S., Ashraf, M.Y., Khaliq, B., Sun, M., Hussain, S., Gao, Z.-q., Noor, H., Alam, S., 2019. Mechanisms and adaptation strategies to improve heat tolerance in rice. Plants 8, 508.

Kleczkowski, L.A., Villand, P., Luthi, E., Olsen, O.A., Preiss, J., 1993. Insensitivity of barley endosperm ADP-glucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation. Plant Physiol. 101 (1), 179–186.

Kuai, J., Liu, Z., Wang, Y., Meng, Y., Chen, B., Zhao, W., Zhou, Z., Oosterhuis, D.M., 2014. Waterlogging during flowering and boll forming stages affects sucrose metabolism in the leaves subtending the cotton boll and its relationship with boll weight. Plant Sci. 223, 79–98.

Lappartient, A.G., Touraine, B., 1996. Demand-driven control of root ATP-sulfurylase activity and SO_4^{2-} uptake in intact canola. Plant Physiol. 111, 147–157.

Law, M.E., Charles, S.A., Halliwell, B., 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts: the effect of hydrogen peroxide and of paraquat. Biochem. J. 210, 899–903.

Masood, A., Khan, M.I.R., Fatma, M., Asgher, M., Per, T.S., Khan, N.A., 2016. Involvement of ethylene in gibberellic acid-induced sulfur assimilation, photosynthetic responses, and alleviation of cadmium stress in mustard. Plant Physiol. Biochem. 104, 1–10.

Mazorra, L.M.M., Senn, M.E., Grozeff, G.E.G., Fanello, D.D., Carrión, C.A., Núñez, M., Bishop, G.J., Bartoli, C.G., 2014. Impact of brassinosteroids and ethylene on ascorbic acid accumulation in tomato leaves. Plant Physiol. Biochem. 74, 315–322.

Mullan, D., Pietragalla, J., 2012. Leaf Relative Water Content. Physiol. Breed II: A field guide to wheat phenotyping, pp. 25–27.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

Nazir, F., Fariduddin, Q., Hussain, A., Khan, T.A., 2021. Brassinosteroid and hydrogen peroxide improve photosynthetic machinery, stomatal movement, root morphology and cell viability and reduce Cu-triggered oxidative burst in tomato. Ecotoxicol. Environ. Saf. 207, 111081.

Ogweno, J.O., Song, X.S., Shi, K., Hu, W.H., Mao, W.H., Zhou, Y.H., Yu, J.Q., Nogués, S., 2007. Brassinosteroids alleviate heat-induced inhibition of photosynthesis by increasing carboxylation efficiency and enhancing antioxidant systems in *Lycopersicon esculentum*. J. Plant Growth Regul. 27, 49–57.

Okuda, T., Matsuda, Y., Yamanaka, A., Sagisaka, S., 1991. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. Plant Physiol. 97 (3), 1265–1267.

Ohri, P., Bhardwaj, R., Kaur, R., Jasrotia, S., Parihar, R.D., Khajuria, A., Sharma, N., 2019. Emerging Trends on Crosstalk of BRs with Other Phytohormones. Brassinosteroids: Plant Growth and Development. Springer, Singapore, pp. 425–441.

Pan, C., Zhang, H., Ma, Q., 2019. Role of ethylene biosynthesis and signaling in elevated CO_2- induced heat stress response in tomato. Planta 250, 563–572.

Poór, P., Nawaz, K., Gupta, R., Ashfaque, F., Khan, M.I.R., 2021. Ethylene involvement in the regulation of heat stress tolerance in plants. Plant Cell Rep. 1–24.

Rao, I.M., Terry, N., 1989. Leaf phosphate status, photosynthesis, and carbon partitioning in sugar beet: I. Changes in growth, gas exchange, and Calvin cycle enzymes. Plant Physiol. 90 (3), 814–819.

Savada, R.P., Ozga, J.A., Jayasinghege, C., Waduthanthri, K.D., Reinecke, D.M., 2017. Heat stress differentially modifies ethylene biosynthesis and signaling in pea floral and fruit tissues. Plant Mol. Biol. 95 (3), 313–331.

Sehar, Z., Iqbal, N., Khan, M.I.R., Masood, A., Rehman, M., Hussain, A., AlAjmi, M.F., Ahmad, A., Khan, N.A., 2021. Ethylene reduces glucose sensitivity and reverses photosynthetic repression through optimization of glutathione production in salt-stressed wheat (*Triticum aestivum* L.). Sci. Rep. 11 (1), 1–12.

Shi, W., Yin, X., Struik, P.C., Solis, C., Xie, F., Schmidt, R.C., Huang, M., Zou, Y., Ye, C., Jagadish, S.V.K., 2017. High day- and night-time temperatures affect grain growth dynamics in contrasting rice genotypes. J. Exp. Bot. 68, 5233–5245.

Sonjaroon, W., Jutamanee, K., Khamsuk, O., Thussagunpanit, J., Kaveeta, L., Suksamrarn, A., 2018. Impact of brassinosteroid mimic on photosynthesis, carbohydrate content and rice seed set at reproductive stage under heat stress. Agric. Nat. Resour. 52 (3), 234–240.

Steiner, F., Zuffo, A.M., Teodoro, P.E., Aguilera, J.G., Teodoro, L.P.R., 2021. Multivariate adaptability and stability of soya bean genotypes for abiotic stresses. J. Agron. Crop Sci. 207 (2), 354–361.

Thussagunpanit, J., Jutamanee, K., Sonjaroon, W., Kaveeta, L., Chai-Arree, W., Pankean, P., Suksamrarn, A., 2015. Effects of brassinosteroid and brassinosteroid mimic on photosynthetic efficiency and rice yield under heat stress. Photosynthetica 53, 312–320.

Usuda, H., 1985. The activation state of ribulose 1, 5-bisphosphate carboxylase in maize leaves in dark and light. Plant Cell Physiol. 26 (8), 1455–1463.

Vialet-Chabrand, S.R., Matthews, J.S., McAusland, L., Blatt, M.R., Griffiths, H., Lawson, T., 2017. Temporal dynamics of stomatal behavior: modeling and implications for photosynthesis and water use. Plant Physiol. 174 (2), 603–613.

Von Caemmerer, S.V., Farquhar, G.D., 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153 (4), 376–387.

Wang, B., Zhang, J., Xia, X., Zhang, W.H., 2011. Ameliorative effect of brassinosteroid and ethylene on germination of cucumber seeds in the presence of sodium chloride. Plant Growth Regul. 65 (2), 407–413.

Wang, X., Cai, J., Liu, F., Dai, T., Cao, W., Wollenweber, B., Jiang, D., 2014. Multiple heat priming enhances thermo-tolerance to a later high temperature stress via improving subcellular antioxidant activities in wheat seedlings. Plant Physiol. Biochem. 74, 185–192.

Wang, W., Xie, Y., Liu, C., Jiang, H., 2022. The exogenous application of brassinosteroids confers tolerance to heat stress by increasing antioxidant capacity in soybeans. Agriculture 12 (8), 1095.

Wang, Y., Zhang, Y., Zhang, Q., Cui, Y., Xiang, J., Chen, H., Hu, G., Chen, Y., Wang, X., Zhu, D., Zhang, Y., 2019. Comparative transcriptome analysis of panicle development under heat stress in two rice (*Oryza sativa* L.) cultivars differing in heat tolerance. PeerJ 7, e7595.

Wei, L.J., Deng, X.G., Zhu, T., Zheng, T., Li, P.X., Wu, J.Q., Zhang, D.W., Lin, H.H., 2015. Ethylene is involved in brassinosteroids induced alternative respiratory pathway in cucumber (*Cucumis sativus* L.) seedlings response to abiotic stress. Front. Plant Sci. 6, 982.

Woeste, K.E., Ye, C., Kieber, J.J., 1999. Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiol. 119, 521–530.

Wu, Y.S., Yang, C.Y., 2019. Ethylene-mediated signaling confers thermotolerance and regulates transcript levels of heat shock factors in rice seedlings under heat stress. Bot. Stud. 60 (1), 23.

Wu, C., Tang, S., Li, G., Wang, S., Fahad, S., Ding, Y., 2019. Roles of phytohormone changes in the grain yield of rice plants exposed to heat: a review. PeerJ 7, e7792.

Xia, X.J., Wang, Y.J., Zhou, Y.H., Tao, Y., Mao, W.H., Shi, K., Yu, J.Q., 2009. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol. 150 (2), 801–814.

Xu, W., Cui, K., Xu, A., Nie, L., Huang, J., Peng, S., 2015. Drought stress condition increases root to shoot ratio via alteration of carbohydrate partitioning and enzymatic activity in rice seedlings. Acta Physiol. Plant. 37 (2), 1-1.

Xu, Y., Chu, C., Yao, S., 2021. The impact of high-temperature stress on rice: challenges and solutions. The Crop J. 9 (5), 963–976.

Yang, J., Miao, W., Chen, J., 2021. Roles of jasmonates and brassinosteroids in rice responses to high temperature stress–A review. Crop J 9 (5), 977–985.

Yu, S.M., Lo, S.F., Ho, T.H.D., 2015. Source–sink communication: regulated by hormone, nutrient, and stress cross-signaling. Trends Plant Sci. 20 (12), 844–857.

Zhang, C.X., Feng, B.H., Chen, T.T., Fu, W.M., Li, H.B., Li, G.Y., Jin, Q.Y., Tao, L.X., Fu, G. F., 2018. Heat stress-reduced kernel weight in rice at anthesis is associated with impaired source-sink relationship and sugars allocation. Environ. Exp. Bot. 155, 718–733.

Zhao, C., Liu, B., Piao, S., Wang, X., Lobell, D.B., Huang, Y., Huang, M., Yao, Y., Bassu, S., Ciais, P., Durand, J.L., 2017a. Temperature increase reduces global yields of major crops in four independent estimates. Proc. Natl. Acad. Sci. USA 114 (35), 9326–9331.

Zhao, G., Xu, H., Zhang, P., Su, X., Zhao, H., 2017b. Effects of 24-epibrassinolide on photosynthesis and Rubisco activase gene expression in *Triticum aestivum* L. seedlings under a combination of drought and heat stress. Plant Growth Regul. 81 (3), 377–384.

Zhou, J., Wang, J., Li, X., Xia, X.J., Zhou, Y.H., Shi, K., Yu, J.Q., 2014. H_2O_2 mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses. J. Exp. Bot. 65 (15), 4371–4383.

Zhu, T., Deng, X., Zhou, X., Zhu, L., Zou, L., Li, P., Zhang, D., Lin, H., 2016. Ethylene and hydrogen peroxide are involved in brassinosteroid induced salt tolerance in tomato. Sci. Rep. 6, 35392.