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Ethylene- and Proline-Dependent Regulation of Antioxidant Enzymes to Mitigate Heat Stress and Boost Photosynthetic Efficacy in Wheat Plants

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Abstract

Ethylene regulates the photosynthetic efficiency of plants grown under challenging environments by the regulation of the antioxidant system and other biomolecules, such as osmolytes (proline). The role of ethylene in modulating proline biosynthesis and subsequent changes in antioxidant system to protect wheat (*Triticum aestivum* L. cv. WH-711) against heat stress was studied. The effects of exogenously sourced ethylene (as 200 µL L^{-1} ethephon: 2-chloroethylphosphonic acid) and proline (50 mM) were studied in the protection of photosynthetic performance and heat stress tolerance by studying mechanisms of proline biosynthesis, activity and gene expression of antioxidants, and ethylene evolution. The cultivars WH-711, RAJ-3765, PBW-373, HD-2967, PBW-550, DBW-17, PBW-343, and UP-2338 were screened for their proline accumulation capacity and tolerance to heat stress. Plants of the cultivar WH-711 with higher proline accumulation and heat tolerance capacity were subjected to a temperature of 40 °C for 6 h per day over 15 days and then allowed to recover at 28 °C. These plants showed increased H_2O_2 and TBARS (thiobarbituric acid reactive substances), proline accumulation, and ethylene evolution, activity of antioxidant enzymes, and reduced photosynthetic characteristics. Ethephon plus proline supplementation under heat stress upregulated the antioxidant defense system, reduced oxidative stress, and upregulated *psb*A and *psb*B expression and photosynthesis. The study's outcome may be taken to improve photosynthetic performance and heat stress tolerance through ethylene-enhanced proline accumulation and antioxidant defense system.

Keywords Antioxidants · Ethylene · Proline · Photosynthesis · Heat stress

Introduction

Climate change has been a significant threat to the environment for decades. In the long run, temperature shifts have been a significant concern experienced globally (Marsicek et al. 2018). It is gradually rising due to the unorganized and unmethodical anthropogenic activities, which unavoidably affect plants. Plants are affected by the progressively increasing temperatures above the threshold at every stage of their life cycle and exhibit metabolically and physiologically irreversible alterations. High-temperature stress has been understood as an exposure to heat that is sufficiently higher than the threshold limit (usually 10–15 °C) for a specified period to induce irreversible alterations (Wahid et al. 2007). Heat stress impairs the growth and development of plants through disruption of the photosynthetic apparatus, which causes the functioning of pigment system II (PSII) to be compromised (Crafts-Brandner et al. 2000; Hasanuzzaman et al. 2013; Wang et al. 2018). Studies have shown that the photosynthetic attributes, stomatal conductance (Haworth et al. 2018; Zhou et al. 2018), intercellular CO_2 levels (Wang et al. 2010), and the activity of ribulose 1,5-bisphosphate carboxylase (Rubisco) (Crafts-Brandner et al. 2000; Kumar et al. 2016; Perdomo et al. 2017) were adversely affected by heat stress resulting in significant yield suppression in cereals, oilseed and other cultivated crops (Zhang et al. 2012; Hussain et al. 2019). Recently, Hu et al. (2020) have reported that heat stress affected processes of photophosphorylation, thylakoid membrane fluidity, chlorophyll biosynthesis, and CO_2 assimilation. The expression of heat stress-responsive

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nuclear genes for the plastid transcription machinery affected the transcript accumulation of plastid-encoded genes in *A. thaliana*, at least in part (Danilova et al. 2018). In wheat leaves, heat stress decreased the expression of the *psbA*, *psbB*, and *psbC* genes, which encode D1, CP47, and CP43 proteins, respectively (Fatma et al. 2021b).

When exposed to heat stress, plants respond in several ways to sustain their growth and production under stressful environments. The response of stress-exposed plant is affected by its developmental stage and the severity of the stress component (Brestic et al. 2012; Yamori et al. 2014). The uncontrolled generation of reactive oxygen species (ROS) under heat stress disrupts cellular functions, causes denaturation of DNA and protein and lipid peroxidation, and eventually cell death (Chaudhary et al. 2021). Medina et al. (2021) recently reviewed and summarized the physiological role and signaling of ROS produced in plants during heat stress. It has been shown that ROS help in heat stress signaling, upregulate the synthesis of heat shock proteins (HSPs) and coordinate several different signaling pathways and transcription factors for thermotolerance (Kotak et al. 2007; Liu et al. 2018; Argosubekti 2020).

The inorganic solutes act as osmoprotectants under stressful environments. Heat stress disturbs the osmotic homeostasis, and proline accumulation has been an adaptive strategy to counter the osmotic crisis (Wang et al. 2020). To reduce physiological and biochemical alterations due to heat stress, plants activate the signaling cascades and transcriptional factors (Hasanuzzaman et al. 2013). To minimize the adversity of heat shock, proline (osmolyte) production, and accumulation have been found crucially significant for the plants' survival and sustenance. In heat-stressed sugarcane leaves, proline was responsible for the increased pressure potential (Wahid et al. 2007). Plants with increased proline content were less affected by stress conditions than wild-type plants with lower proline concentration. Proline synthesis upregulation under stressful conditions has proved as an adaptive criterion for stress tolerance and growth (Kishor et al. 2005). Proline accounts for maintaining cellular osmotic potential under stressful environments. It regulates photosynthesis by improving PSII electron transport and maintaining cellular redox potential, detoxifying ROS, and stabilizing proteins (Ashraf and Foolad 2007; Naliwajski and Sklodowska 2014; Iqbal et al. 2015).

Phytohormones behave as one of the significant compounds responsible for heat mitigation. Upregulation of several phytohormones has been observed in plant species under heat stress. Some plant hormones showed accelerated production, while others were comparatively suppressed in response to the stress stimuli. Plant hormones maintain cellular homeostasis for heat stress tolerance (Alonso-Ramírez et al. 2009; Nazar et al. 2011; Hasanuzzaman et al. 2012; Kwon et al. 2015; reviewed by Li et al. 2021). The proline and other compatible solutes have been shown to be regulated by phytohormones (Per et al. 2017; Iqbal et al. 2019). There is evidence of combined regulation of heat stress signaling by ethylene, salicylic acid, abscisic acid, and jasmonic acid (Larkindale et al. 2005; Muller and Munné-Bosch 2015). Ethylene regulates proline accumulation and osmotic adjustment (Per et al. 2017) and increases abiotic stress tolerance, such as heat (Khan et al. 2013; Gautam et al. 2022), salt stress (Jahan et al. 2021), and metal stress (Khan et al. 2020).

Ethylene, a gaseous signaling molecule, has been shown to be imperative for heat stress tolerance (Gautam et al. 2022). Ethylene-dependent responses in heat prone plants are dose and time determined; a low dose promotes plant defense signaling, while a higher dose inhibits (Chang et al. 2010; Khan et al. 2013; Riyazuddin et al. 2020). Under heat stress, plant cells tend to upregulate various cascades of ethylene signaling, among which the ethylene response factor (ERF) plays a significant role (Xu et al. 2019; Riyazuddin et al. 2020). Ethylene-regulated heat tolerance in plants is mediated by several mechanisms and procedures to acquire cellular integrity, protect photosynthetic setup, and reverse oxidative damage. Savada et al. (2017) have shown that the reproductive physiology of the plant was compromised in response to heat stress in *Pisum sativum*. The biosynthesis of ethylene occurred in a tissue-specific pattern that was influenced by the environment and developmental stage of the tissue. However, the information on ethylene and proline coordination and the mechanism as to how the interaction of these mitigates heat stress are less studied. Ethylene's influence on proline production may affect heat stress tolerance. There could be a link between ethylene synthesis and the modulation of osmolyte (proline) accumulation to protect plants against heat stress. Iqbal et al. (2015) showed that ethylene has a role in regulating salinity stress by influencing the accumulation of osmoprotectants. Studies on *ein2-5* and *ein3-1* (ethylene insensitive) mutants validated the role of ethylene in osmolyte biosynthesis (Cui et al. 2015). The study was undertaken to understand the mechanisms induced by ethylene and proline individually or together to protect photosynthesis under heat stress.

Materials and Methods

Healthy seeds of wheat (*Triticum aestivum* L.) cultivars, WH-711, RAJ-3765, PBW-373, HD-2967, PBW-550, DBW-17, PBW-343, and UP-2338, were surface sterilized with 0.01% $HgCl_2$ and repeatedly washed with deionized water before sowing in 15-cm-diameter earthen pots filled with acid-washed sand. Two plants per pot were maintained and were placed in the net house of the Department of Botany, Aligarh Muslim University, Aligarh (India),

where day/night temperatures were 25/18 ± 3 °C, photoperiod (12 h, 680 µmol m^{-2} s^{-1}), and relative humidity of 65 ± 5%. The plants were saturated with 300 ml of full-strength Hoagland's nutrition solution on alternate days. In the experimentation, one set of plants was maintained at 25 °C (no stress). In contrast, the other set was subjected to 40 °C for 6 h daily for 15 days (heat stress) in the environmental growth chamber (Khera KI-261, New Delhi, India) and was allowed to recover at 25 °C and grown for the experimental period.

In the first experiment, screening of the cultivars was done to select heat-tolerant cultivar with high-proline accumulation capacity. The cultivar WH-711 and UP-2338 emerged as high and low proline accumulation with heat-tolerant and heat susceptible capacity. In the further experiment to study ethylene's influence in the presence or absence of proline in mitigating heat stress, plants were treated with 200 µl L^{-1} ethephon (ETH, 2-chloroethylphosphonic acid) and/or 50 mM proline grown under normal and heat stress. A control set was also maintained. The ethephon concentration was based on our earlier experience (Gautam et al. 2022), while the concentration of proline was selected from a preliminary screening of the effect of proline (0, 50, and 100 mM) on photosynthesis and proline biosynthesis (unpublished). A surfactant teepol (0.5%) was added with the control and ethephon treatments. The treatments were arranged in a randomly blocked design. There were four replicates ($n = 4$) for each treatment. Plants were sampled 30 days after germination (DAG), and different parameters were recorded.

Determination of Photosynthetic and Growth Attributes

Net photosynthesis, stomatal conductance, and intercellular CO_2 concentration were measured in fully expanded topmost intact leaves of plants in each treatment using Infrared Gas Analyzer (CID-340, photosynthesis system, Bioscience, Camas, WA, USA). The measurements were taken between 11 and 12 h at a light saturating intensity, a temperature of 22 °C and a relative humidity of about 60%. The content of chlorophyll was measured in the plants' intact second top leaves with the help of SPAD Chlorophyll meter (502 DL PLUS, Spectrum Technologies, Plainfield, IL, USA).

To determine plant dry mass, plants were gently uprooted, cleaned under running tap water to eliminate dust, and dried in a hot air oven at 80 °C until constant weight. Leaf area was calculated using a leaf area meter (LA211, Systronic, New Delhi, India).

Measurement of Chlorophyll Fluorescence

Junior-PAM chlorophyll fluorometer (Heinz Walz, GmbH, Effeltrich, Germany) was used to measure chlorophyll fluorescence. Supplementary File S1 included details of the measurements.

Estimation of Proline and Glycine Betaine Content

The procedure described by Bates et al. (1973) was used to measure proline content in leaves. The details of the method are given in Supplementary File S1.

The method of Grieve and Grattan's (1983) was followed to estimate glycine betaine (GB) content in leaves by monitoring the production of betaine-periodite complex. The details of the procedure are given in Syeed et al. (2021).

Contents of H_2O_2 and TBARS

The method of Okuda et al. (1991) was used to determine the content of H_2O_2 in leaves, and the content of thiobarbituric acid reactive substances (TBARS) as a measure of the status of lipid peroxidation in leaves was determined by the method given by Dhindsa et al. (1981). The details of the procedure are given in Supplementary File S1.

Assay of Antioxidants Enzyme Activities

Fresh leaves were homogenized in a chilled mortar and pestle with an extraction solution comprising 0.05% (v/v) Triton X-100 and 1% (w/v) PVP in potassium phosphate buffer (100 mM, pH 7.0). The supernatant obtained after centrifugation was used for the assay of superoxide dismutase (SOD, EC; 1.15.1.1) and glutathione reductase (GR, EC; 1.6.4.2) enzymes. For the assay of ascorbate peroxidase (APX, EC; 1.11.1.11), 2.0 mM ascorbate was supplemented with extraction buffer. The activity of SOD was measured using the methods of Beyer and Fridovich (1987) and Giannopolitis and Ries (1977). The activity of APX was assessed using the Nakano and Asada (1981) method, which involved recording the decrease in ascorbate absorbance at 290 nm. The activity of GR was determined using Foyer and Halliwell (1976) method, which involved measure of glutathione-dependent oxidation of NADPH at 340 nm. The details of the methods are given in Supplementary File S1.

Membrane Stability Index

Membrane stability index was determined by adopting the method of Das and Uprety (2006). Fresh leaf samples were cut into discs of small size. The samples (0.2 g) were collected in test tubes with 10 ml of double distilled water. The electrical conductivity (C1) of the samples was measured after 30 min of

incubation at 40 °C in a water bath. The samples were transferred to other test tubes and incubated in a boiling water bath at 100 °C for 15 min, after which electrical conductivity (C2) was measured as described before, and the membrane stability index is computed and reported in percentage using the formula.

$$\text{Membrane Stability Index} = \left[1 - (C1/C2)\right] \times 100.$$

RNA Isolation, cDNA Synthesis, and Real-Time RT-PCR

TRIzol reagent (Ambion, Life Technologies, Austin, TX, USA) was used to extract total RNA from the leaves of treated and control plants according to the manufacturer's instructions. The Nanodrop spectrophotometer was used to measure the amount of isolated RNA (Thermo Scientific, Waltham, MA, USA). Each sample was run on an agarose formaldehyde gel (Turano et al. 1997). Real-time PCR (RT-PCR) was performed in 96-well reaction plates (Roche, Mannheim, Germany) containing a 20-µl reaction mixture of 10X reaction buffer, 2 mM dNTPs, 1 mM $MgCl_2$, 0.35 µM each of forward and reverse primers, 1 µl SYBR Green (10X), 10 µg cDNA template, and 5 U Taq polymerase on a thermal cycler (Lightcycler 480 II, Roche, Germany). Table S1 lists the primer pairs used in quantitative RT-PCR. The process is detailed out in Supplementary File S1.

Estimation of ACS Activity and Ethylene Evolution

Activity of 1-aminocyclopropanecarboxylic acid synthase (ACS; EC, 4.4.1.14) was determined by adopting the methods of Avni et al. (1994) and Woeste et al. (1999). Leaf tissue (5.0 g) was homogenized in a solution containing 100 mM HEPES (pH 8.0), 4 mM DTT, 2.5 mM pyridoxal phosphate, and 25% PVP. The homogenized material was centrifuged for 15 min at 12,000 × *g*. One milliliter of the supernatant was transferred to a 30 ml tube, along with 0.1 ml of 5 mM S-adenosyl methionine (AdoMet) and the mixture was incubated at 22 °C for 2 h. The amount of ACC produced was quantified by converting it to ethylene using 0.1 ml of 20 mM $HgCl_2$ followed by addition of 0.1 ml of a 1:1 ratio of saturated NaOH/NaCl in an ice bath for 10 min. AdoMet was not included in the control group. A gas chromatograph was used to determine the amount of ethylene present. The details of the procedure are given earlier by Fatma et al. (2021a) and presented in Supplementary File S1.

Statistical Analysis

The data were statistically analyzed using analysis of variance (ANOVA) in SPSS 17.0 for Windows and reported as mean ± SE ($n = 4$). For the significant data, the least significant difference was calculated at $p < 0.05$. The data bars with the same letter were not significantly different by the least significant difference (LSD) test at $p < 0.05$.

Results

Screening of Cultivars for Heat Stress Tolerance: Plant Growth and Physiological Parameters

Plants grown under heat stress exhibited decrease in growth and photosynthetic characteristics as compared to control plant (Table 1). The cultivar WH-711 showed minimum reduction in leaf area and plant dry mass by 53.8% and 57%, chlorophyll content, net photosynthesis, and Fv/Fm by 57.2%, 61.7%, and 29.1%, respectively, and maximum proline accumulation of 93.8% compared to control plants under heat stress. Contrarily, the cultivar UP-2338 exhibited maximum decrease in the aforementioned parameters and less accumulation of proline compared to control plants under heat stress. On this basis, WH-711 was selected as the most heat-tolerant and UP-2338 as heat-sensitive cultivars. The screened cultivars showed heat stress tolerance in the order: WH-711 > RAJ-3765 > PBW-373 > HD-2967 > PBW-550 > DBW-17 > PBW-343 > UP-2338.

Ethephon in the Presence of Proline Increases Photosynthesis and Growth Under Heat Stress

Heat stress had a considerable impact on leaf gas exchange parameters and chlorophyll content (SPAD value). In particular, high-temperature stress decreased net photosynthesis, stomatal conductance, intercellular CO_2 concentration, and SPAD value, Fv/Fm, and Rubisco activity by 47.5%, 28.5%, 47.2%, 58.5%, 19.4%, and 36.7%, respectively, in comparison to control. Under no stress condition, ethephon or proline application increased the above photosynthetic parameters; however, their combined application maximally increased these parameters in comparison to control. In addition, plants supplemented with ethephon under heat stress demonstrated increments in net photosynthesis, stomatal conductance, intercellular CO_2 concentration, SPAD value, Fv/Fm, and Rubisco activity by 96.8%, 38.3%, 58.9%, 71.3%, 22.4%, and 63.0%, respectively, compared to the heat-stressed plants. Similarly, proline application to plants under heat stress showed increment in the above-mentioned parameters by 68.8%, 25.8%, 67.5%, 50.0%, 15.5%, and 32.6%, respectively, compared to the heat-stressed plants. Finally, the combined application of ethephon and proline maximally mitigated the negative effects of heat stress and significantly increased photosynthetic attributes by 156.2%,

Table 1 Effect of heat stress (40 °C for 6 h daily for 15 days) on Physiological and growth parameters in wheat (*Triticum aestivum* L.) cultivars WH-711, RAJ-3765, PBW-373, HD-2967, PBW-550, DBW-17, PBW-343, and UP-2338 at 30 days after sowing

Cultivar	Treatment	Leaf area (cm^2 $plant^{-1}$)	Plant dry mass (g $plant^{-1}$)	SPAD value	Net photosynthesis (μmol CO_2 m^{-2} s^{-1})	Maximum quantum yield efficiency of PSII	Proline content (μmol g^{-1} DW)
WH-711	Control	36.4 ± 1.81a	1.21 ± 0.06a	28.6 ± 1.43a	19.6 ± 0.98a	0.93 ± 0.047a	1.2 ± 0.06cd
	Heat stress	16.8 ± 0.82d	0.52 ± 0.026d	13.4 ± 0.67d	7.5 ± 0.38c	0.66 ± 0.033bc	2.3 ± 0.11a
RAJ-3765	Control	34.3 ± 1.71ab	1.12 ± 0.056ab	26.5 ± 1.32ab	18.9 ± 0.94a	0.91 ± 0.046a	1.1 ± 0.06cd
	Heat stress	13.4 ± 0.67de	0.44 ± 0.008de	11.6 ± 0.58de	6.8 ± 0.34cd	0.62 ± 0.031c	2.1 ± 0.11ab
PBW-373	Control	33.6 ± 1.68ab	1.06 ± 0.053ab	25.8 ± 1.29ab	17.8 ± 0.89ab	0.87 ± 0.043a	1.1 ± 0.06cd
	Heat stress	12.6 ± 0.63de	0.39 ± 0.019de	10.4 ± 0.52de	6.2 ± 0.31cd	0.56 ± 0.028cd	2.0 ± 0.1ab
HD-2967	Control	31.5 ± 1.57b	0.96 ± 0.048b	24.2 ± 1.21b	16.6 ± 0.83ab	0.82 ± 0.041ab	0.95 ± 0.05d
	Heat stress	10.8 ± 0.54e	0.32 ± 0.016e	9.7 ± 0.48e	5.7 ± 0.28d	0.51 ± 0.01cd	1.82 ± 0.09b
PBW-550	Control	30.4 ± 1.52bc	0.94 ± 0.047bc	23.8 ± 1.19bc	16.4 ± 0.82ab	0.81 ± 0.04ab	0.9 ± 0.05de
	Heat stress	9.2 ± 0.46ef	0.27 ± 0.013ef	8.1 ± 0.40ef	4.9 ± 0.24de	0.45 ± 0.022d	1.6 ± 0.08bc
DBW-17	Control	29.6 ± 1.48bc	0.91 ± 0.045bc	22.9 ± 1.14bc	15.9 ± 0.79b	0.77 ± 0.039ab	0.62 ± 0.17de
	Heat stress	8.3 ± 0.415f	0.19 ± 0.009f	7.3 ± 0.36f	3.4 ± 0.17de	0.38 ± 0.019de	1.45 ± 0.07c
PBW-343	Control	28.9 ± 1.44bc	0.88 ± 0.044bc	21.8 ± 1.09c	15.5 ± 0.77b	0.73 ± 0.036b	0.8 ± 0.04e
	Heat stress	6.8 ± 0.34fg	0.13 ± 0.006fg	6.2 ± 0.31fg	2.6 ± 0.13e	0.27 ± 0.013e	1.17 ± 0.06cd
UP-2338	Control	27.5 ± 1.37c	0.84 ± 0.042c	20.6 ± 1.03cd	14.6 ± 0.73bc	0.67 ± 0.34bc	0.8 ± 0.04e
	Heat stress	4.6 ± 0.23g	0.07 ± 0.004h	4.4 ± 0.22h	1.2 ± 0.06f	0.18 ± 0.009ef	0.97 ± 0.05d

Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. DW, dry weight

62.5%, 193%, 106%, 53.4%, and 92.7%, respectively, compared to the plants exposed to heat stress (Table 2).

Notably, heat stress more severely hampered plant growth relative to control plants. Individual application of ethephon and proline resulted in increased leaf area and plant dry mass under both normal and heat stress conditions. In compared to heat-stressed plants, ethephon or proline treatment minimized the deleterious effects of heat stress and increased leaf area and plant dry mass. However, application of both ethephon and proline under heat stress maximally alleviated the negative effects caused by heat stress and showed significant increase in leaf area and plant dry mass by 76.1% and 177.0%, respectively, compared to heat-stressed plants (Fig. 1).

Table 2 Net photosynthesis, stomatal conductance, intercellular CO_2 concentration, chlorophyll content (SPAD value), maximum quantum yield efficiency of PSII, and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity of wheat (*Triticum aestivum* L cv. WH-711) leaves at 30 days after sowing

Treatments	Net photosynthesis (μmol CO_2 m^{-2} s^{-1})	Stomatal conductance (mmol CO_2 m^{-2} s^{1})	Intercellular CO_2 concentration (μmol mol^{-1})	SPAD value	Maximum quantum yield efficiency of PSII	Rubisco Activity (μmol CO_2 mg^{-1} protein min^{-1})
Control	12.2 ± 0.78d	358 ± 16.1d	216 ± 10.7d	29.8 ± 1.6d	0.72 ± 0.040d	39.2 ± 2.1d
HT	6.4 ± 0.64g	256 ± 14.3g	114 ± 6.47g	18.8 ± 1.4g	0.58 ± 0.030g	24.8 ± 1.6g
ETH	17.2 ± 1.09b	436 ± 18.9b	352 ± 16.9b	43.8 ± 2.8b	0.86 ± 0.050b	54.4 ± 2.6b
Pro	16.5 ± 0.84c	421 ± 17.8c	338 ± 14.8c	39.6 ± 2.1c	0.78 ± 0.480c	48.8 ± 2.4c
ETH + Pro	18.2 ± 0.99a	464 ± 20.6a	378 ± 17.19a	48.8 ± 3.4a	0.98 ± 0.060a	56.8 ± 2.8a
ETH + HT	12.6 ± 0.87d	354 ± 16.8d	232 ± 11.7d	32.2 ± 1.8d	0.71 ± 0.041d	40.4 ± 2.3d
Pro + HT	10.8 ± 0.86e	322 ± 15.2e	191 ± 10.8e	28.2 ± 2.1e	0.67 ± 0.036e	32.9 ± 2.2e
ETH + Pro + HT	16.4 ± 0.94c	416 ± 18.2c	334 ± 13.3c	38.8 ± 1.9c	0.89 ± 0.050c	47.8 ± 2.4c

Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 μl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$

ETH ethephon, *HT* heat stress, *Pro* proline

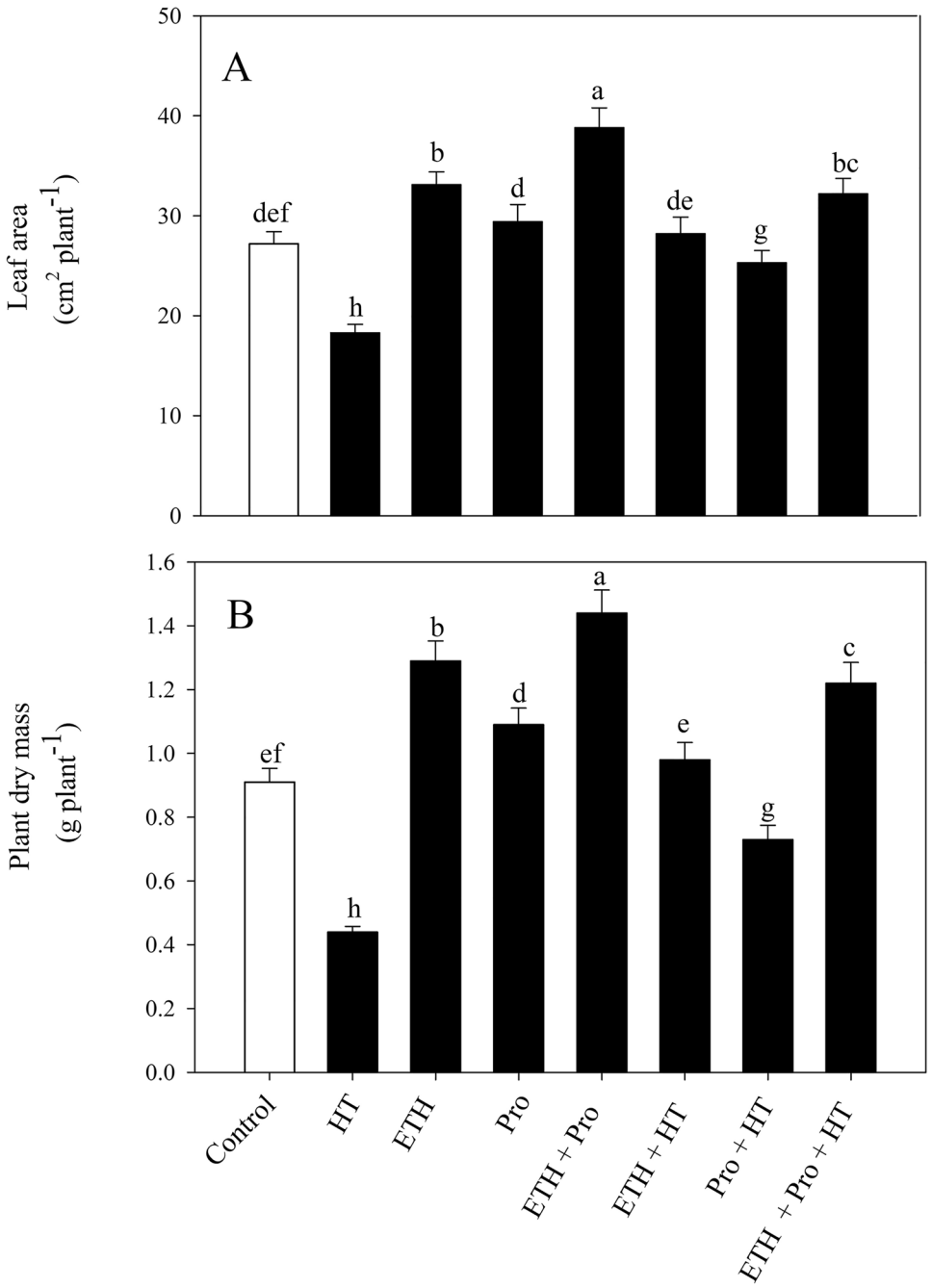

Fig. 1 Leaf area (**a**) and plant dry mass (**b**) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

Effect of Ethephon and Proline on the Expression of Genes Encoding Core PSII Proteins

The expression of two photosynthetic genes, *psbA* and *psbB*, which encode D1 protein and CP47, respectively, was studied to know about the protective role of ethylene/proline under heat stress. Under normal condition, individual application of ethephon and proline equally raised *psbA* expression by 7.5 times and *psbB* expression by 9 times compared to control; however, heat stress decreased the expression by 0.5 times and 0.6 times. Under heat stress, plants supplemented with either ethephon or proline elevated the expression of *psbA* and *psbB* by 9 times and 13 times compared to control. Meanwhile, expression of *psbA* and *psbB* was maximally raised in plants treated with ethephon plus proline under heat stress by 11.2 times and 15 times compared to control (Fig. 2).

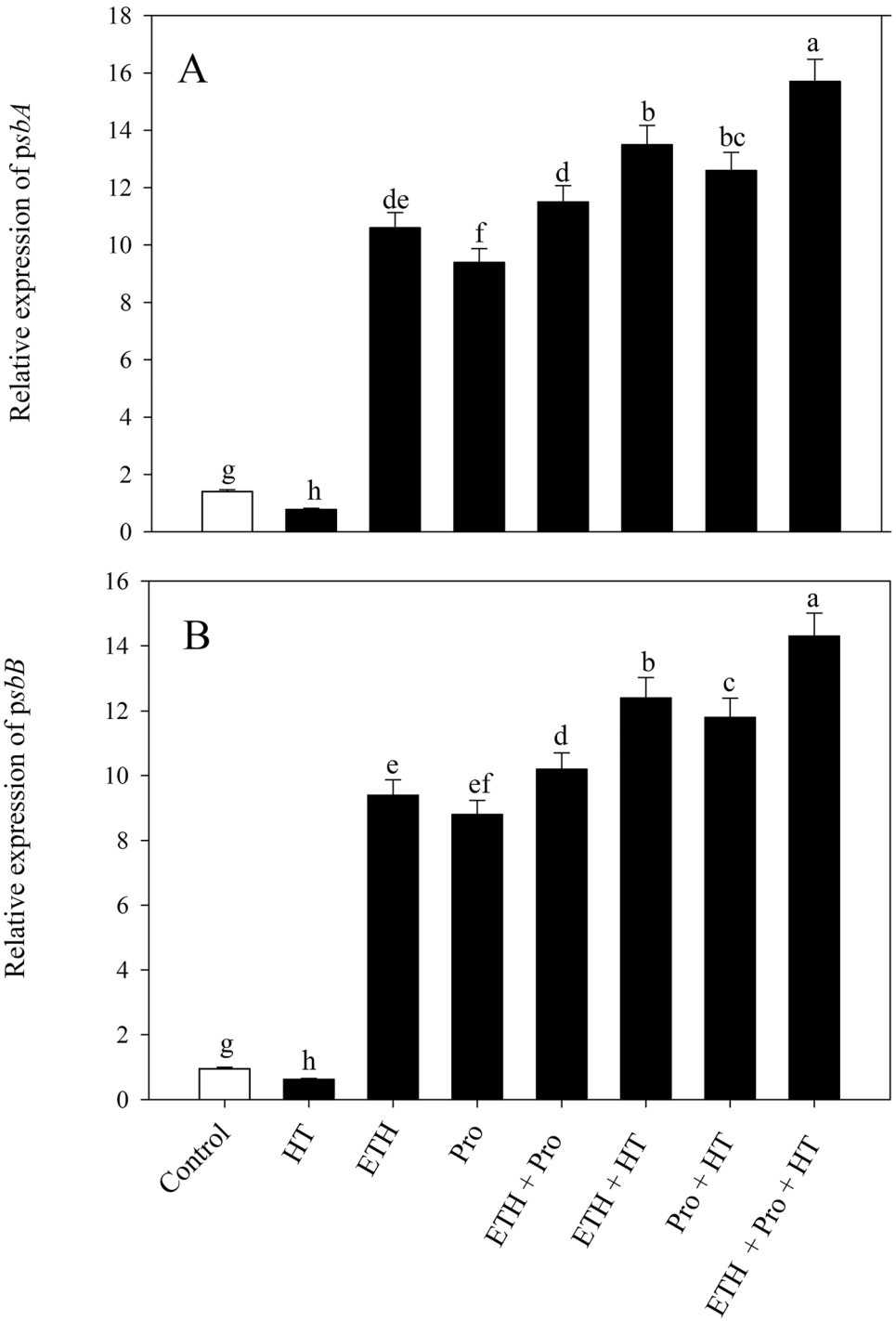

Fig. 2 Relative expression of *psbA* (**a**) and *psbB* (**b**) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

Ethephon and Proline Reduce the Oxidative Stress and Maintain Membrane Stability Index Under Heat Stress

The extent of cellular damage caused by heat stress-induced oxidative stress was determined in terms of H_2O_2 content and membrane damage as TBARS content (Fig. 3). Relative to the control plants, heat stress significantly enhanced H_2O_2 and TBARS content by 138.8% and 169.1% in comparison to control plants. The individual application of ethephon and proline proved effective in mitigating the heat stress-induced oxidative stress and significantly reduced H_2O_2 content by 48.3% and 45% and TBARS content by 25.4% and 12.7%, respectively, in comparison to control plants. However, combined treatment of ethephon and proline maximally reduced heat stress-induced oxidative stress resulting in reduced

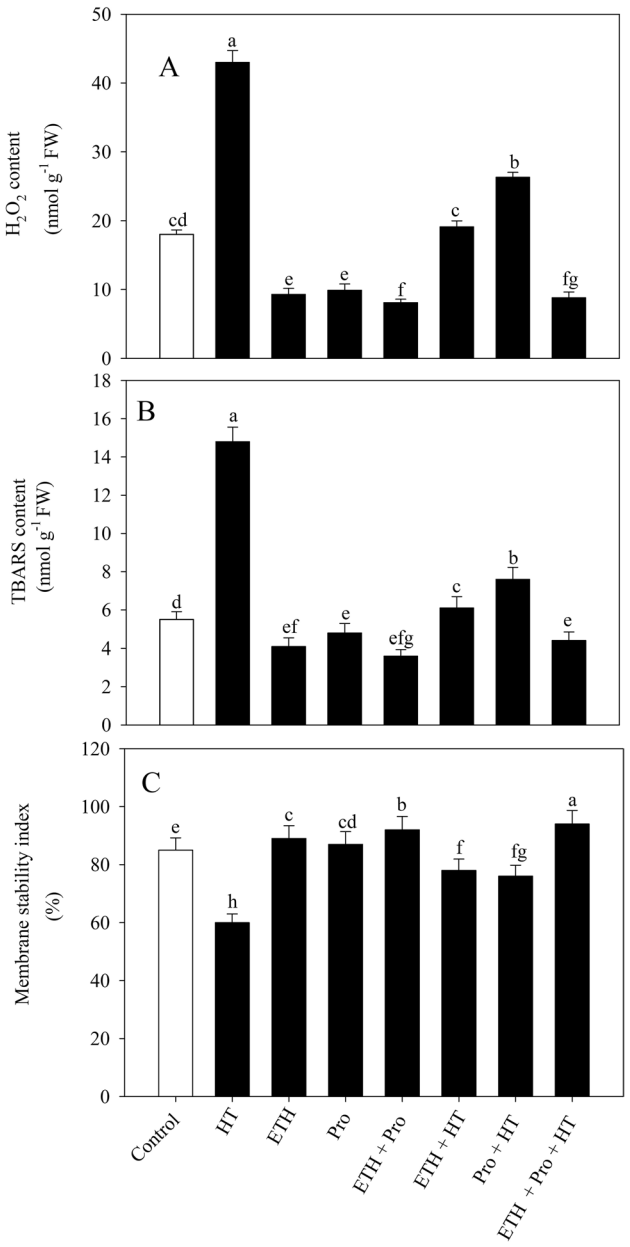

Fig. 3 Content of H_2O_2 (**a**), TBARS (**b**), and membrane stability index (C) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline, *TBARS* thiobarbituric acid reactive substances

H_2O_2 and TBARS content by 55.0% and 34.1%, respectively, relative to control plants.

High-temperature stress significantly decreased membrane stability index by 29.4% in comparison to control plants. Under no stress, the individual application of ethephon and proline increased membrane stability index by 4.1% and 2.2%, respectively, compared to control plants, but the combined application of ethephon and proline increased it by 7.1%, compared to control plants. Supplementation of ethephon together with proline under heat stress showed maximum enhancement in membrane stability index by 9.1%, compared to control plants (Fig. 3).

Ethephon and Proline Accelerate Antioxidant Enzymes Activity Under Heat Stress

The activity of antioxidant enzymes increased in response to heat stress compared to control plants. Relative to the control plants, heat stress stimulated the activity of antioxidant enzymes, SOD, APX, and GR by 30.7%, 43.7%, and 62.1%, respectively. The individual application of ethephon and proline increased the activity of SOD by 62.2% and 57.1%, APX by 111.1% and 106.2%, and GR by 82.4% and 62.5%, respectively, compared to control plants. Under heat stress, supplementation of both ethephon and proline maximally increased activity of these antioxidant enzymes by 74.5%, 120.6%, and 68.5%, respectively, compared to heat-stressed plants (Fig. 4).

Ethephon and Proline Increase Proline and Glycine Betaine Content Under Heat Stress

Figure 5 shows the content of proline and GB in plants grown under no stress or heat stress conditions and subjected to ethephon and proline treatments. Heat stress increased proline and GB content by 127.6% and 45.4%, respectively, compared to control. Ethephon and proline applied individually under no stress increased proline content by 145.2% and 124.2% and GB content by 81.8% and 54.6%, respectively, compared to the control. Plants supplemented with both ethephon and proline together under no stress condition exhibited more increase in proline and GB content by 172.5% and 110.1%, respectively, compared to control. Under heat stress, application of ethephon/proline manifested increase in proline and GB content, compared to the plants exposed to heat stress. The maximum increase in proline and GB content was observed with the combined application of ethephon and proline under heat stress by 34.1% and 56.2%, respectively, relative to the heat-stressed plants (Fig. 5).

Application of Ethephon and Proline on Gene Expression of Glutathione Reductase Under Heat Stress

Exogenous application of ethephon with proline increased the activity of antioxidant enzymes under heat stress, so we tested the changes in the expression level of GR genes by the application of ethephon with proline under heat

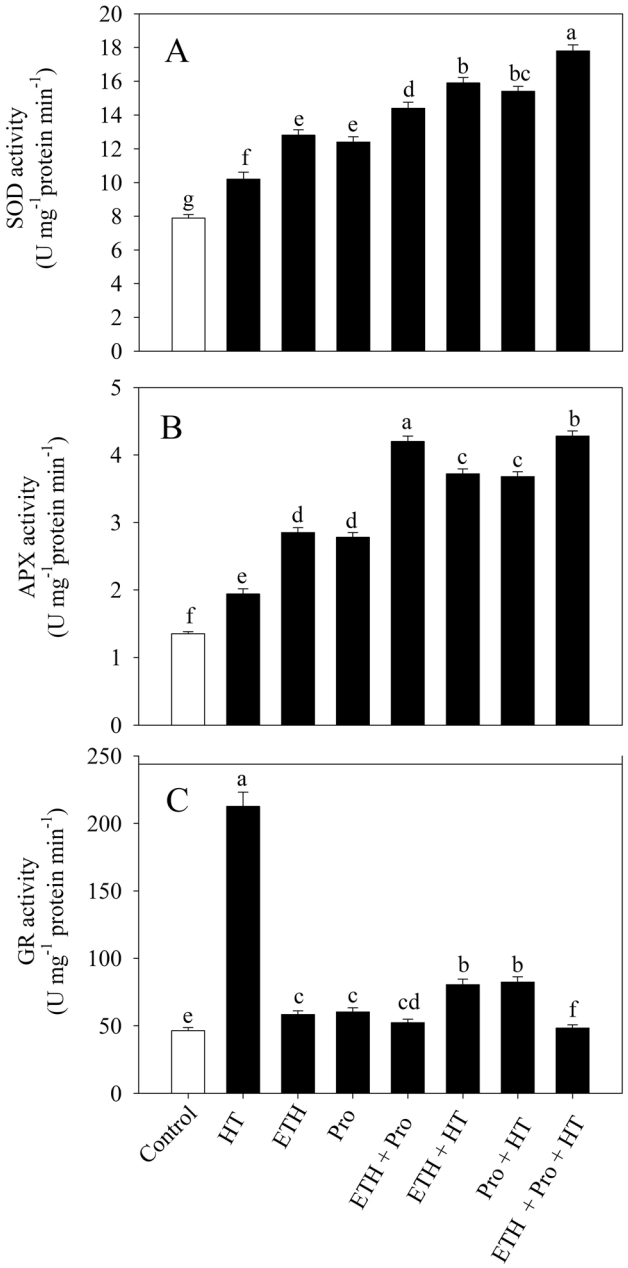

Fig. 4 Activity of superoxide dismutase (SOD, **a**), ascorbate peroxidase (APX, **b**), and glutathione reductase (GR, **c**) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

stress (Fig. 6). Plants treated with combined application of ethephon and proline decreased GR expression by 70% and 97% compared to the control and heat-stressed plants, respectively.

Effect of Ethephon and Proline on ACS Activity and Ethylene Production Under Heat Stress

Plants exposed to heat stress showed higher ACS activity and ethylene production, and ethephon or proline application also increased these in comparison to control plants (Fig. 7). Under heat stress, ethephon/proline supplementation decreased ACS activity and ethylene production equally by about 62.0% and 46.7%, respectively, compared to control plants. Moreover, plants supplemented with ethephon and proline together under heat stress exhibited a maximum decrease in ACS activity and ethylene production by 77.3% and 67.7%, respectively, compared to plants exposed to heat stress.

Discussion

A temperature rise beyond the threshold level for a long time is enough to harm agricultural plants and reduce global plant yield. On the other hand, the temperature threshold varies from species to species and between compartments within the cell (Hasanuzzaman et al. 2013; Asseng et al. 2015; Poór et al. 2021). High-temperature stress in plants is described as a temperature increase that exceeds a critical threshold for a sustained period, causing irreversible harm to plant growth and development processes (Xalxo et al. 2020; Gautam et al. 2021). The integrity of membranes, proteins, and cytoskeleton structure and the efficacy of cellular enzymatic activities are all disrupted by heat stress, impeding important physiological processes and producing metabolic imbalances (Suzuki et al. 2012). One of the principal impacts of high-temperature stress is the excessive generation of ROS, which leads to oxidative stress in the cells (Hemantaranjan et al. 2014; Gautam et al. 2021, 2022). Heat stress also causes loss of photosynthetic pigments and efficiency, disrupts thylakoid structure and electron transport chains in mitochondria and chloroplasts, reduces photoassimilate synthesis, and depletion of carbohydrate stores (Cortleven et al. 2019; Paul et al. 2020; Gautam et al. 2022). Plants enhance their inherent capacity to withstand heat stress (Song et al. 2012). The overproduction of ROS due to heat stress results in lipid peroxidation, DNA damage, protein oxidation, and cell death (Choudhury et al. 2017; Sarwar et al. 2018; Chaudhary et al. 2021). They increase the functions of the antioxidant system, as the ROS function as signaling molecule that confers plants to acclimate and adapt to abiotic stresses (Gautam et al. 2022). However, the effectiveness of these antioxidants is insufficient to minimize oxidative stress, necessitating more research into mechanisms that might enhance antioxidative metabolism. Also, osmolytes are another essential component that maintains the cell redox state by acting as an antioxidant and maintaining osmotic equilibrium.

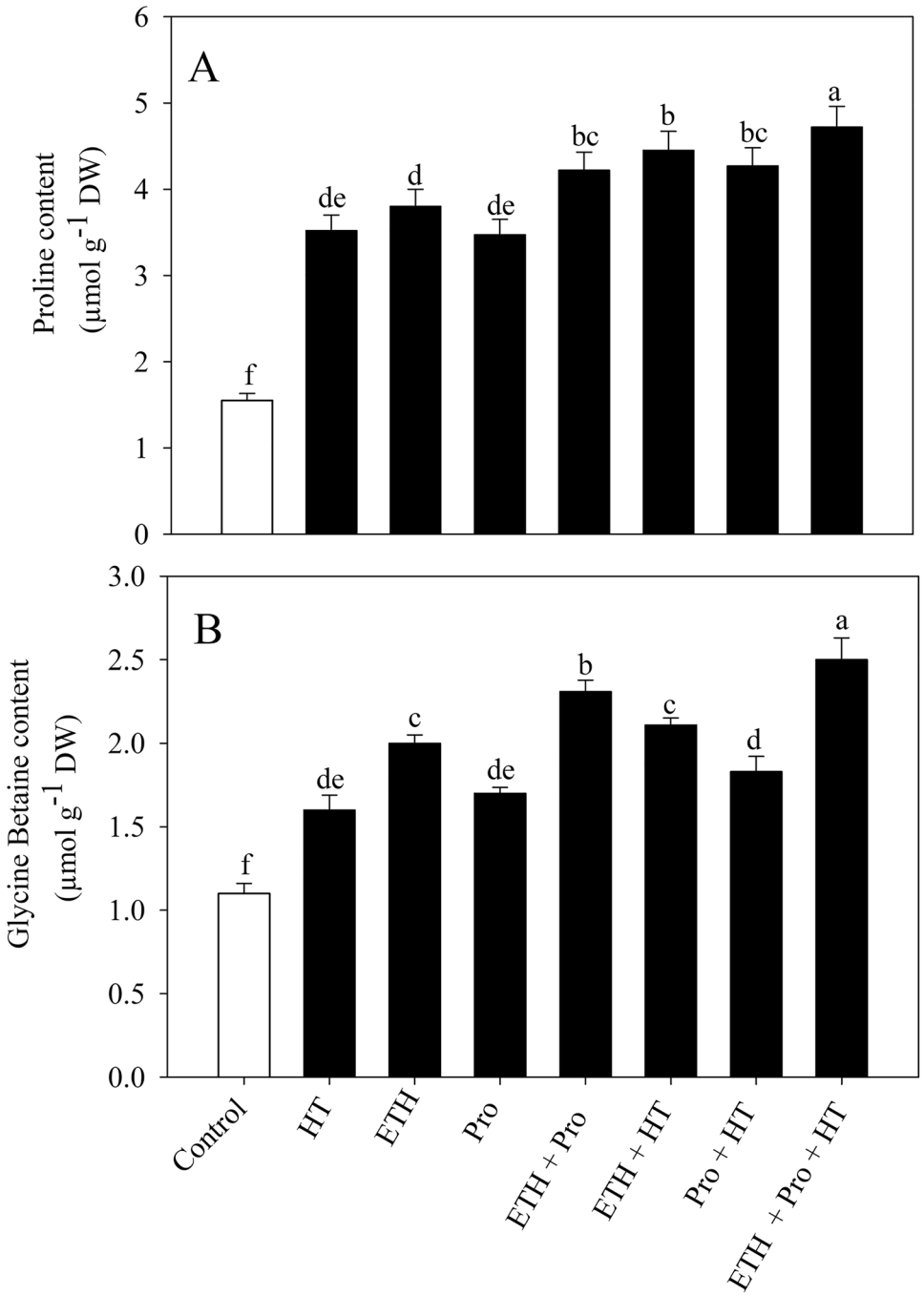

Fig. 5 Content of proline (**a**) and glycine betaine (**b**) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

Phytohormones trigger antioxidant defense and osmolytes accumulation signals which might help to reduce ROS levels. In the present report, the screened cultivars had different potentials for heat stress tolerance. The cultivar WH-711 was most heat tolerant because of its higher capacity to accumulate proline and thus showed higher photosynthetic capacity under heat stress.

Ethylene, a gaseous hormone, is essential for plant growth and development as well as abiotic stress tolerance, such as high temperatures (Gautam et al. 2022). Indeed, ethylene is a fundamental regulator of abiotic stress responses in plants; that is, abiotic stress responses are connected to ethylene build-up at varied concentrations, affecting growth and development (Khan et al. 2014; Thao et al. 2015). Ethylene signaling also aids in the decrease of oxidative stress and the improvement of thermotolerance in plants (Wu and Yang 2019). In addition, the phytohormone and proline are important components that keep the cell redox status by acting as an antioxidant while also maintaining osmotic equilibrium. However, there is little information on

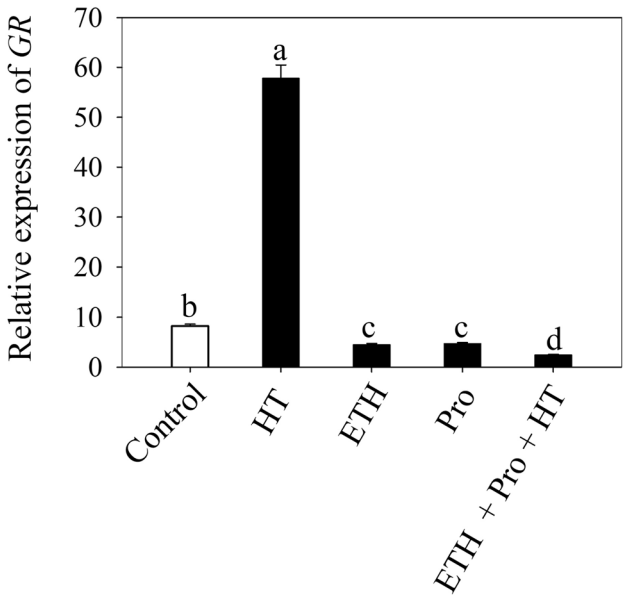

Fig. 6 Relative expression of *GR* in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

how exogenous supplementation of ethephon and proline alters antioxidant metabolism and maintains photosynthesis of plants under heat stress. Both ethephon and proline alleviated heat stress impacts, and the effect of proline depended on ethylene. High-temperature stress increased oxidative stress as observed by increased H_2O_2 and TBARS levels; however, the same measurements were reduced in plants treated with ethephon and proline. Moreover, ethephon and proline increased the membrane stability index in plants, thus reducing oxidative stress. Abid et al. (2018) and Dwivedi et al. (2018) have also shown that supplementation of plant growth regulators positively affected membrane stability index and photosynthetic and growth attributes under water stress. However, there is no report available on ethylene- and proline-mediated regulation of membrane stability index. Further, plants' build-up capacity of antioxidant enzymes upregulates under heat stress to reduce levels of H_2O_2 and TBARS. The present study demonstrated that the activity of antioxidant enzymes and GR gene expression was enhanced by ethephon and proline under heat stress and confirmed that the ethylene signaling system could modulate scavenger enzymes allowing plants to respond to heat-induced oxidative stress. Sharma et al. (2019) have reported that ethylene triggers an antioxidant defense system, reduces oxidative stress, and restores plant growth and photosynthetic efficiency. In addition to this, Zhang et al. (2011) showed that ERF95 known as ESE1 is a direct target of EIN3 and is involved in Arabidopsis' salt stress response. Wu and Yang (2019) showed that ethylene signaling confers thermotolerance by decreasing MDA content and electrolyte leakage and regulating the heat shock factor's transcript level in rice seedlings under heat stress. Huang et al. (2021) demonstrated that transcriptional cascade EIN3-ERF95/ERF97-HSFA2 might play a vital role in the heat stress response, indicating a link between ethylene and its downstream regulation in plant thermotolerance. The study of Wu and Yang (2019) emphasized that transcript levels of ethylene biosynthesis genes and signal transduction-related genes were upregulated under heat stress, resulting in an expansion of the ethylene signal response. They showed that the heat shock factors (Hsfs) activated the antioxidant system, lowering oxidative damage during heat stress. In the present study, ethephon and proline improved photosynthesis and growth under heat stress through the increased accumulation of osmolytes, such as proline and glycine betaine, and optimized ethylene levels under heat stress. Ethylene increases proline biosynthesis, which has a role in the regulation of abiotic stress tolerance (Iqbal et al. 2015). The increased proline metabolism was found to be related to ethylene levels in plants and salt tolerance (Alvarez et al. 2003; Szabados and Savoure 2010; Iqbal et al. 2015). Moreover, the inhibition of ethylene biosynthesis was shown to reduce proline accumulation under heat stress (Lv et al. 2011). In the present study, **e**thephon treatment under heat stress showed increased ACS activity and ethylene production. Notably, heat-stressed plants produced maximum ethylene; this was stress ethylene that hampered plant growth and the overall functioning. Previous research has described the importance of optimal ethylene levels and stress-induced ethylene generation (Fatma et al. 2021a; Sehar et al. 2021; Gautam et al. 2022). The physiological and metabolic alterations associated with high-temperature stress were altered by ethephon administration, which raised ethylene levels to an optimum and regulated antioxidant system and positively influenced the physiological and metabolic changes. The findings of Poór et al. (2021) also showed that supplementation of ethephon modulated osmoprotectants and the antioxidant defense system to control ROS and RNS metabolism and impart heat stress tolerance to plants. The ethylene and proline provided higher concentrations of cellular metabolites and antioxidant activity, increased quantum yield efficiency of PSII, and expression of *psbA* and *psbB* genes for improved photosynthetic performance under heat stress. The ethylene-stimulated transcription and activity of photosynthetic enzymes have been shown (Azoulay Shemer et al. 2008; Zhang et al. 2011). The balance of chlorophyll breakdown and biosynthesis and contribution of antioxidant activity are critical for the photosynthetic apparatus integrity under heat stress.

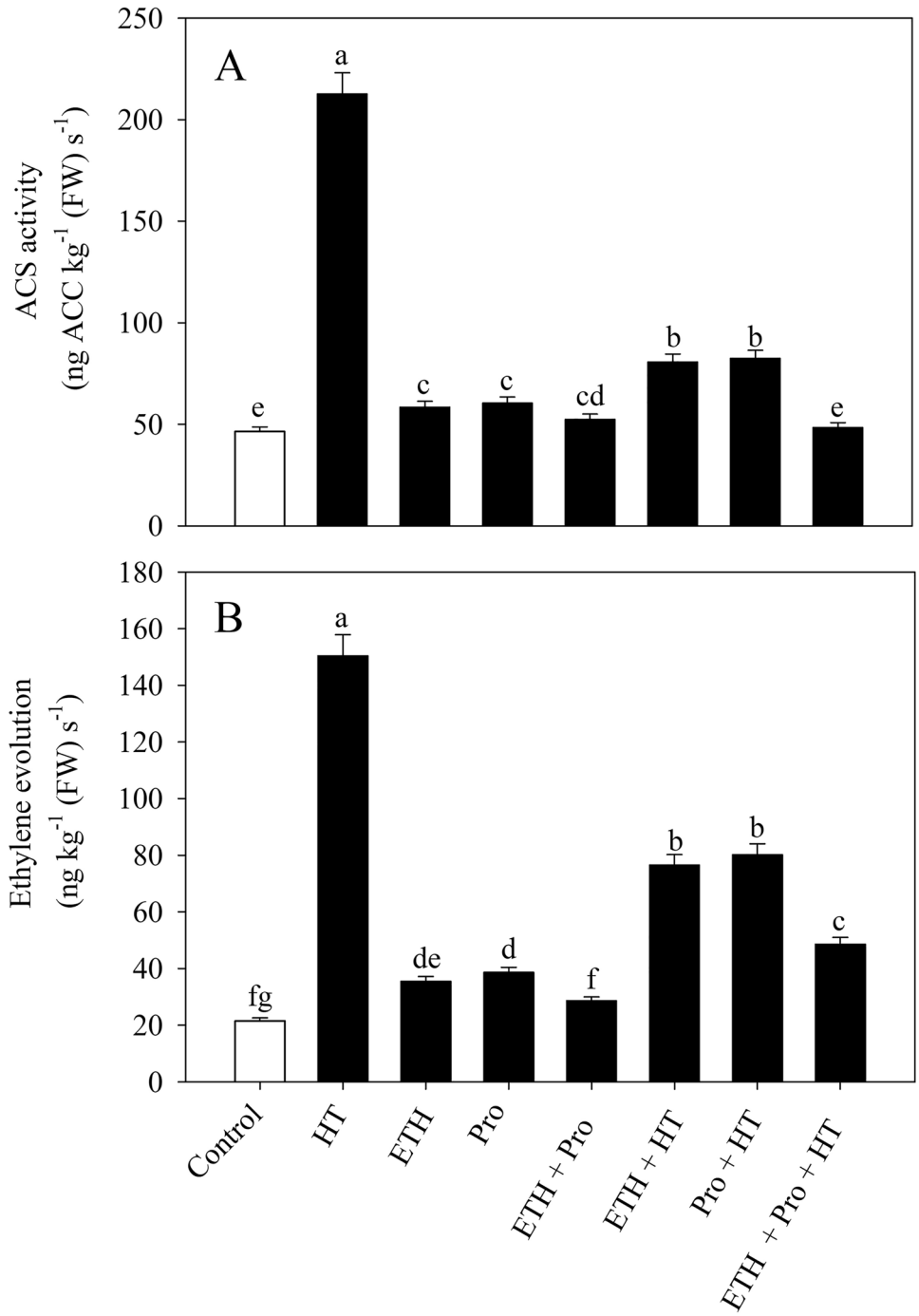

Fig. 7 ACS activity (**a**) and ethylene evolution (**b**) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing. Plants were treated with/without heat stress (40 °C for 6 h daily for 15 days) and 200 µl L^{-1} ETH and/or 50 mM proline. Data are presented as treatment mean ± SE ($n = 4$). Data followed by same letter are not significantly different by the LSD test at $p < 0.05$. *ETH* ethephon, *HT* heat stress, *Pro* proline

Conclusion

Conclusively, ethylene influences photosynthetic efficiency, proline, and antioxidant metabolism during heat stress. The simultaneous exogenous application of ethylene and proline considerably alleviates the harmful effects of heat stress by augmenting the antioxidant enzymes' activity and glutathione reductase expression. It boosted photosynthesis by regulating gas exchange parameters and *psb*A and *psb*B expression. As a result, it may be said that ethylene-mediated proline accumulation and antioxidant system promoted photosynthesis under heat stress.

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Author Contributions ZS conducted the experiment, data collection and analysis, and writing and preparation of original draft, HG

performed data analysis and manuscript editing, AM assisted in manuscript writing, NAK performed conceptualization, supervision, and manuscript editing.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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