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 photosynthetic efficiency of plants grown under challenged environment by interacting with antioxidant system and other biomolecules. The role of ethylene in modulation of proline biosynthesis and subsequent changes in antioxidant machinery to protect heat stress grown wheat was studied. The effects of exogenously-sourced ethylene (as 200 µL L⁻¹ ethephon: 2-chloroethyl phosphonic acid) and proline (50 mM) was studied in the protection of photosynthetic performance and heat stress tolerance of wheat (Triticum aestivum L.) cv. WH-711 by studying mechanisms of proline biosynthesis, activity and gene expression of antioxidants, ethylene evolution. The cultivars, 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 40°C for 6 h per day over 15 days, then allowed to recover at 28°C showed increased levels of H₂O₂ and TBARS (thiobarbituric acid reactive substances), proline accumulation, ACS activity and ethylene evolution, activity of antioxidant enzymes together with reduced photosynthetic characteristics. Ethephon plus proline supplementation under heat stress upregulated antioxidant defense system, reduced oxidative stress and improved psbA and psbB expression and photosynthesis. The outcome of the study may be taken to improve photosynthetic performance and heat stress tolerance through ethylene-enhanced proline accumulation and antioxidant defense system.

Introduction

Climate change has been a significant threat to the environment for decades. Temperature shifts, in the long run, have been a major concern experienced globally (Marsicek et al. 2018). It is gradually rising due to the unorganized and unmethodical anthropogenic activities, which affect plants in an unavoidable manner. Plants are affected by the progressively rising temperatures above the threshold at every stage of their life cycle and exhibit alterations that are metabolically and physiologically irreversible. 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 time 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). Different studies showed that the functioning of essential photosynthetic attributes viz. stomatal conductance (Haworth et al. 2018), intercellular CO₂ levels (Wang et al. 2010), the activity of ribulose 1,5-bisphosphate carboxylase (Rubisco) (Crafts-Brandner et al. 2000; Kumar et al. 2016) were negatively affected by heat stress, resulting in significant yield suppression in many cereals, oil and other cultivated crops (Zhang et al. 2012; Hussain et al. 2019). Recently, Hu et al. (2020) have shown that heat stress affected photosynthesis-related processes, electron transport, CO₂ assimilation, photophosphorylation, chlorophyll biosynthesis, thylakoid membrane fluidity, and photochemical reactions. The expression of heat stress-responsive 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 protein, CP47, and CP43, respectively (Fatma et al. 2021).

Plants respond in several ways when exposed to heat stress to sustain their growth and production under stressful environments. The response of a stress exposed plant is affected by its developmental stage as well as the severity of the stress component (Brestic et al. 2012; Yamori et al. 2014). The uncontrolled generation of reactive oxygen species (ROS) under heat exposure causes disruption of cellular and membranous integrities, DNA and protein denaturation, 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 and upregulates the synthesis of heat shock proteins (HSPs), responsible for thermotolerance, by coordinating the actions of a number of different signaling pathways and transcription factors (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 found as an adaptive strategy to counter the osmotic crisis (Wang et al. 2020). In order to reduce physiological and biochemical alterations, plants activate their signaling cascades and transcriptional machinery in response to heat stress (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 found to be responsible for increased pressure potential (Wahid et al. 2007). Plants with increased proline content were less affected under stress conditions than wild-type plants with lower proline concentration. Proline upregulation under stressful conditions has proved to be 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 adaptive for heat mitigation. Upregulation of several phytohormones has been observed in plant species under heat stress. Some plant hormones show accelerated production, while others are observed as 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 status of proline and other compatible solutes has been shown 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; salt stress (Jahan et al. 2021) and metal stress (Khan et al. 2020).
Ethylene, a gaseous signaling molecule, has been shown 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, plants cells tend to upregulate various cascade of ethylene signaling, among which 12ET response factor (ERF) plays a major role (Xu et al. 2019; Riyazuddin et al. 2020). Ethylene regulated heat tolerance in plants is mediated through several mechanisms and procedures to acquire cellular integrity, protection of photosynthetic setup, and reverse oxidative damage. In a study on Pisum sativum by Savada et al. (2017), it was demonstrated that in response to heat stress, the reproductive physiology of the plant is compromised, and 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 various osmolytes may affect heat stress tolerance. There could be a link between ethylene synthesis and the modulation of osmolyte accumulation for protection of plants against 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 ethylene’s role in osmolyte biosynthesis (Cui et al. 2015). The study was undertaken with the idea that ethylene and proline work together to regulate antioxidant enzyme activity and expression, as well as proline production, in order to maintain the cellular redox status and reverse the negative effects of heat stress on photosynthesis.

Materials And Methods

“Healthy seeds of wheat (Triticum aestivum L.) cultivars, RAJ-3765, PBW-550, WH-711, HD-2967, PBW-343, DBW-17, UP-2338, PBW-343 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 were maintained in the pots placed in the net house of the Department of Botany, Aligarh Muslim University, Aligarh (India); where day/night temperatures was 25/18 ± 3°C, photoperiod (12 h, 680 µmol m\(^{-2}\) s\(^{-1}\)) and relative humidity of 65 ± 5%. On alternate days, the plants were saturated with 300 ml of full-strength Hoagland’s nutrition solution. In the experimentation, one set of plants was maintained at 25°C (no stress), whereas 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 were 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, respectively. In the further experiment for studying the impact of ethylene in the presence or absence of proline in mitigating heat stress, plants were treated with 200 µL\(^{-1}\) ethephon (ETH, 2-chloroethyl phosphonic acid) and/or 50 mM proline grown under normal and high temperature stress. A control set was also maintained. The ethephon concentration was based on our earlier experience (Gautam et al. 2022), while proline concentration was selected from a preliminary
screening of 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 with four replicates (n = 4) for each treatment. Plants were sampled at 30 days after germination (DAG) and different parameters were recorded.

**Determination of Photosynthetic and Growth Attributes**

Using an infrared gas analyzer, net photosynthesis, stomatal conductance, and intercellular CO₂ concentration were measured in fully expanded topmost intact leaves of plants in each treatment (CID-340, photosynthesis system, Bioscience, Camas, WA, USA). The measurements were taken between 11 and 12 hours at a light saturating intensity, a temperature of 22 °C, and a relative humidity of about 60%. With the use of a SPAD Chlorophyll metre, the content of chlorophyll was measured in the plants' intact second top leaves (502 DL PLUS, Spectrum Technologies, Plainfield, IL, USA).

**Measurement of Chlorophyll Fluorescence**

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

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

**Estimation of Proline and Glycine Betaine Content**

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

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

**Contents of H₂O₂ and TBARS**

The method of Okuda et al. was used to determine leaf H₂O₂ (1991). The content of thiobarbituric acid reactive substances (TBARS) was used to determine the status of lipid peroxidation in leaves, as described by Dhindsa et al. (1981). The process is detailed 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 percent (v/v) Triton X-100 and 1 percent (w/v) PVP in potassium-phosphate buffer (100 mM, pH 7.0). The supernatant obtained after centrifugation was used for the assay of SOD (EC; 1.15.1.1) and GR (EC; 1.6.4.2) enzymes. For the assay of APX (EC; 1.11.1.11), 2.0 mM ascorbate was supplemented with
extraction buffer. The activity of SOD was measured using the 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 measured using Foyer and Halliwell's (1976) method, which involved measuring the glutathione-dependent oxidation of NADPH at 340 nm. The method's specifics are detailed 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 (C_i) of the samples was measured after 30 minutes 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 minutes, after which their electrical conductivity (C_2) was measured as described before, and the membrane stability index was computed and reported in percentage using the formula.

\[ \text{MSI} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100 \]

RNA Isolation, cDNA Synthesis and Real Time RT-PCR

TRIzol (Ambion, Life Technologies, 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, USA). Turano et al. (1997). described running 1 μg RNA from each sample on gel electrophoresis in formaldehyde gels to test RNA integrity Table S1 lists the primer pairs utilised in quantitative RT-PCR. The process is described in full in Supplementary file S1.

Estimation of ACS Activity and Ethylene Evolution

Activity of 1-aminocyclopropane-carboxylic 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 minutes at 12,000 x g. One millilitre 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 two hours. The amount of ACC produced was quantified by converting it to ethylene using 0.1 ml of 20 mM HgCl2 followed by 0.1 mL of a 1:1 ratio of saturated NaOH/NaCl in an ice bath for 10 minutes. 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 have been given earlier by Fatma et al. (2021) and presented in the Supplementary File S1.

Statistical analysis
The data was statistically analysed 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 estimated at p<0.05. The least significant difference was calculated for the significant data at bars showing the same letter are 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 the 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, PSII activity by 57.2%, 61.7% and 29.1%, respectively, and maximum proline accumulation of 93.8% compared to control plants under heat stress condition. Contrarily, the cultivar UP-2338 exhibited maximum decrease in the aforementioned parameters and less accumulation of proline compared to the control plants under heat stress condition. On this basis, WH-711 was selected as the most heat-tolerant and UP-2338 as heat-sensitive cultivars. The cultivars showed heat stress tolerance in the order: WH-711 > RAJ-3765 > PBW-373 > HD-2967 > PBW-550 > DBW-17 > PBW-343 > UP-2338.
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. FW, fresh weight; DW, dry weight

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Leaf area (cm² plant⁻¹)</th>
<th>Plant dry mass (g plant⁻¹)</th>
<th>SPAD value</th>
<th>Net photosynthesis (µmol CO₂ m⁻² s⁻¹)</th>
<th>Maximum quantum yield efficiency of PSII</th>
<th>Proline content (µmol g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>36.4 ± 1.81a</td>
<td>1.21 ± 0.06a</td>
<td>28.6 ± 1.43a</td>
<td>19.6 ± 0.98a</td>
<td>0.93 ± 0.047a</td>
<td>1.2 ± 0.06cd</td>
</tr>
<tr>
<td>WH-711</td>
<td>Heat stress</td>
<td>16.8 ± 0.82d</td>
<td>0.52 ± 0.026d</td>
<td>13.4 ± 0.67d</td>
<td>7.5 ± 0.38c</td>
<td>0.66 ± 0.033bc</td>
<td>2.3 ± 0.11a</td>
</tr>
<tr>
<td>RAJ-3765</td>
<td>Control</td>
<td>34.3 ± 1.71ab</td>
<td>1.12 ± 0.056ab</td>
<td>26.5 ± 1.32ab</td>
<td>18.9 ± 0.94a</td>
<td>0.91 ± 0.046a</td>
<td>1.1 ± 0.06cd</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>13.4 ± 0.67de</td>
<td>0.44 ± 0.008de</td>
<td>11.6 ± 0.58de</td>
<td>6.8 ± 0.34cd</td>
<td>0.62 ± 0.031c</td>
<td>2.1 ± 0.11ab</td>
</tr>
<tr>
<td>PBW-373</td>
<td>Control</td>
<td>33.6 ± 1.68ab</td>
<td>1.06 ± 0.053ab</td>
<td>25.8 ± 1.29ab</td>
<td>17.8 ± 0.89ab</td>
<td>0.87 ± 0.043a</td>
<td>1.1 ± 0.06cd</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>12.6 ± 0.63de</td>
<td>0.39 ± 0.019de</td>
<td>10.4 ± 0.52de</td>
<td>6.2 ± 0.31cd</td>
<td>0.56 ± 0.028cd</td>
<td>2.0 ± 0.1ab</td>
</tr>
<tr>
<td>HD-2967</td>
<td>Control</td>
<td>31.5 ± 1.57b</td>
<td>0.96 ± 0.048b</td>
<td>24.2 ± 1.21b</td>
<td>16.6 ± 0.83ab</td>
<td>0.82 ± 0.041ab</td>
<td>0.95 ± 0.05d</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>10.8 ± 0.54e</td>
<td>0.32 ± 0.016e</td>
<td>9.7 ± 0.48e</td>
<td>5.7 ± 0.28d</td>
<td>0.51 ± 0.01cd</td>
<td>1.82 ± 0.09b</td>
</tr>
<tr>
<td>PBW-550</td>
<td>Control</td>
<td>30.4 ± 1.52bc</td>
<td>0.94 ± 0.047bc</td>
<td>23.8 ± 1.19bc</td>
<td>16.4 ± 0.82ab</td>
<td>0.81 ± 0.04ab</td>
<td>0.9 ± 0.05de</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>9.2 ± 0.46ef</td>
<td>0.27 ± 0.013ef</td>
<td>8.1 ± 0.40ef</td>
<td>4.9 ± 0.24de</td>
<td>0.45 ± 0.022d</td>
<td>1.6 ± 0.08bc</td>
</tr>
<tr>
<td>DBW-17</td>
<td>Control</td>
<td>29.6 ± 1.48bc</td>
<td>0.91 ± 0.045bc</td>
<td>22.9 ± 1.14bc</td>
<td>15.9 ± 0.79b</td>
<td>0.77 ± 0.039ab</td>
<td>0.62 ± 0.17de</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>8.3 ± 0.415f</td>
<td>0.19 ± 0.009f</td>
<td>7.3 ± 0.36f</td>
<td>3.4 ± 0.17de</td>
<td>0.38 ± 0.019de</td>
<td>1.45 ± 0.07c</td>
</tr>
<tr>
<td>PBW-343</td>
<td>Control</td>
<td>28.9 ± 1.44bc</td>
<td>0.88 ± 0.044bc</td>
<td>21.8 ± 1.09c</td>
<td>15.5 ± 0.77b</td>
<td>0.73 ± 0.036b</td>
<td>0.8 ± 0.04e</td>
</tr>
<tr>
<td></td>
<td>Heat stress</td>
<td>6.8 ± 0.34fg</td>
<td>0.13 ± 0.006fg</td>
<td>6.2 ± 0.31fg</td>
<td>2.6 ± 0.13e</td>
<td>0.27 ± 0.013e</td>
<td>1.17 ± 0.06cd</td>
</tr>
<tr>
<td>UP-2338</td>
<td>Control</td>
<td>27.5 ± 1.37c</td>
<td>0.84 ± 0.042c</td>
<td>20.6 ± 1.03cd</td>
<td>14.6 ± 0.73bc</td>
<td>0.67 ± 0.34bc</td>
<td>0.8 ± 0.04e</td>
</tr>
</tbody>
</table>
Heat stress had a considerable impact on leaf gas exchange characteristics and chlorophyll content (SPAD value). In particular, high temperature stress decreased net photosynthesis (Pn), stomatal conductance (Gs), intercellular CO₂ concentration (Ci), 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 Pn, Gs, Ci, SPAD, Fv/Fm and Rubisco activity by 96.8%, 38.3%, 58.9%, 71.3%, 22.4% and 63%, respectively, compared to the heat stressed plants. Similarly, proline application to plants under heat stress showed increment in above mentioned parameters by 68.8%, 25.8%, 67.5%, 50%, 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%, 62.5%, 193%, 106%, 53.4% and 92.7%, respectively, compared to the plants exposed to heat stress (Table 2).
Table 2

Net photosynthesis, stomatal conductance, intercellular CO2 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 var. WH 711) leaves treated with 200 µL L⁻¹ ethephon (ETH) and/or 50 mM proline (Pro) in the presence (40°C) (HT) or absence (25°C) of heat stress at 30 DAS. DAS, days after sowing, HT, heat stress.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Net photosynthesis (µmol CO₂ m⁻² s⁻¹)</th>
<th>Stomatal conductance (mmol CO₂ m⁻² s⁻¹)</th>
<th>Intercellular CO₂ concentration (µmol mol⁻¹)</th>
<th>SPAD value</th>
<th>Maximum quantum yield efficiency of PSII (Fv/Fm)</th>
<th>Rubisco Activity (µmol CO₂ mg⁻¹ protein min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.2 ± 0.78d</td>
<td>358 ± 16.1d</td>
<td>216 ± 10.7d</td>
<td>29.8 ± 1.6d</td>
<td>0.72 ± 0.040d</td>
<td>39.2 ± 2.1d</td>
</tr>
<tr>
<td>HT</td>
<td>6.4 ± 0.64g</td>
<td>256 ± 14.3g</td>
<td>114 ± 6.47g</td>
<td>18.8 ± 1.4g</td>
<td>0.58 ± 0.030g</td>
<td>24.8 ± 1.6g</td>
</tr>
<tr>
<td>ETH</td>
<td>17.2 ± 1.09b</td>
<td>436 ± 18.9b</td>
<td>352 ± 16.9b</td>
<td>43.8 ± 2.8b</td>
<td>0.86 ± 0.050b</td>
<td>54.4 ± 2.6b</td>
</tr>
<tr>
<td>Pro</td>
<td>16.5 ± 0.84c</td>
<td>421 ± 17.8c</td>
<td>338 ± 14.8c</td>
<td>39.6 ± 2.1c</td>
<td>0.78 ± 0.480c</td>
<td>48.8 ± 2.4c</td>
</tr>
<tr>
<td>ETH + Pro</td>
<td>18.2 ± 0.99a</td>
<td>464 ± 20.6a</td>
<td>378 ± 17.19a</td>
<td>48.8 ± 3.4a</td>
<td>0.98 ± 0.060a</td>
<td>56.8 ± 2.8a</td>
</tr>
<tr>
<td>ETH + HT</td>
<td>12.6 ± 0.87d</td>
<td>354 ± 16.8d</td>
<td>232 ± 11.7d</td>
<td>32.2 ± 1.8d</td>
<td>0.71 ± 0.041d</td>
<td>40.4 ± 2.3d</td>
</tr>
<tr>
<td>Pro + HT</td>
<td>10.8 ± 0.86e</td>
<td>322 ± 15.2e</td>
<td>191 ± 10.8e</td>
<td>28.2 ± 2.1e</td>
<td>0.67 ± 0.036e</td>
<td>32.9 ± 2.2e</td>
</tr>
<tr>
<td>ETH + Pro + HT</td>
<td>16.4 ± 0.94c</td>
<td>416 ± 18.2c</td>
<td>334 ± 13.3c</td>
<td>38.8 ± 1.9c</td>
<td>0.89 ± 0.050c</td>
<td>47.8 ± 2.4c</td>
</tr>
</tbody>
</table>

Notably, heat stress more severely hampered plant growth relative to the control plants. Individual ethephon and proline applications increased leaf area and plant dry mass under both normal and high temperature 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% and 177%, respectively, compared to heat-stressed plants (Fig. 1).

**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 investigated to know about the protective role of ethylene under heat stress. Under normal condition, individual application of ethephon and proline application raised *psbA* and *psbB* expression approximately by 7.5 times and 9 times compared to control; however, heat stress decreased the expression by 0.5 and 0.6 times. Under heat stress, expression of *psbA* and *psbB* was significantly elevated in plants subjected to ethephon and proline by 9 times and 13 times compared to the control. Meanwhile, plants expression of *psbA* and *psbB* was maximally raised in plants treated with ethephon and proline under heat stress by 11.2 times and 15 times compared to the control (Fig. 2).

**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 

\[ \text{H}_2\text{O}_2 \]

content, and membrane damage as TBARS content (Fig. 3). Relative to the control plants, heat stress significantly enhanced 

\[ \text{H}_2\text{O}_2 \]

and TBARS content by 138.8% and 169% in comparison to control plants. Exogenously applied ethephon and proline proved effective in mitigating the heat stress-induced oxidative stress, and significantly reduced 

\[ \text{H}_2\text{O}_2 \]

content by 48.3% and 45%, and TBARS content by 25.4% and 12.7%, respectively, in comparison to control plants. However, treatment of ethephon together with proline maximally reduced heat stress induced oxidative stress as reduction in 

\[ \text{H}_2\text{O}_2 \]

and TBARS content by 55% and 34%, 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 or 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. In addition, 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 3 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 or 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 normal 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 ethephon and proline under heat stress by 34.1% and 56.2%, respectively, relative to the heat-stressed plants (Figure 5).

Application of ethephon and proline on gene expression of GR 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 stress (Figure 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.

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 evolution, and ethephon or proline application also increased ACS activity and ethylene evolution in comparison to control plants (Figure 7). In contrast, ethephon/proline supplementation decreased ACS activity and ethylene production equally by about 62% and 46.7%, respectively, compared to the control plants. Moreover, plants supplemented with ethephon and proline under heat stress exhibited maximum decrease in ACS activity and ethylene production by 77.3% and 67.7%, respectively, compared to the 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 afflicted cells (Hemantaranjan et al. 2014; Gautam et al. 2021, 2022). Heat stress also causes a loss of photosynthetic pigments and efficiency, aberrant thylakoid structure, disruption of electron transport
chains in mitochondria and chloroplasts, reduced photo-assimilate synthesis, and depletion of carbohydrate stores (Cortleven et al. 2019; Paul et al. 2020; Gautam et al. 2022). Plants have developed various ways to cope with heat stress, including intrinsic heat stress tolerance, which refers to a plant's inherent capacity to withstand heat stress (Song et al. 2012). ROS overproduction due to heat stress causes 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 antioxidative 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. Phytohormones, which trigger antioxidant defense and osmolytes accumulation signals, might also 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 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 buildup at varied concentrations, which affects growth and development (Khan et al. 2014; Thao et al. 2015). Ethylene signaling in plants also aids in the decrease of oxidative stress and the improvement of thermo-tolerance 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 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 was dependent on ethylene. High-temperature stress increased oxidative stress as observed by increased H$_2$O$_2$ and TBARS levels; however, the same measurements were reduced in plants treated with ethephon and proline. Moreover, ethephon, together with 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 the maintenance of 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' buildup capacity of antioxidant enzymes upregulates under heat stress to reduce levels of H$_2$O$_2$ and TBARS. The present study demonstrated that the activity of SOD, APX, GR and their 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) reported that ethylene triggers an antioxidative defense system, reducing oxidative stress and restoring 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 thermo-tolerance by decreasing MDA content and electrolyte leakage and regulates the transcript level of heat shock factor 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. The present study reported that ethephon and proline protected plants from heat stress and increased photosynthesis and growth by increasing the accumulation of osmolytes, such as proline and glycine betaine and optimizing the ethylene level under heat stress. Ethylene increases proline biosynthesis, which has a role in the regulation of abiotic stress tolerance (Iqbal et al. 2014). The increased proline metabolism was found related ethylene level 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, ethephon 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 level and stress-induced ethylene generation (Fatma et al. 2021; 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 is critical for the photosynthetic apparatus’ integrity and optimal operation. However, the involvement of ethylene in these activities under heat stress is less well understood.

**Conclusion**

Conclusively, ethylene influences photosynthetic efficiency, proline, and antioxidant metabolism during heat stress. The simultaneous exogenous application of ethylene and proline considerably alleviates the deleterious effects of heat stress by augmenting the antioxidant defense system. It also boosted photosynthesis and \( psbA \) and \( psbB \) expression. As a result, it is possible to say that ethylene-mediated proline accumulation promoted photosynthesis under heat stress, and that their combined application is beneficial in combating heat stress.
Declarations

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Conflict of Interest

Authors declare that they have no conflict of interest.

Author Contributions

ZS: conducted the experiment, data collection and analysis, writing original draft preparation, HG: data analysis, manuscript editing, AM: assistance in manuscript writing, NAK: conceptualization, supervision, manuscript editing.

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Figures

Figure 1
Leaf area (A) and plant dry mass (B) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L\(^{-1}\) ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at \(p < 0.05\).

**Figure 2**

Relative expression of PSBA (A) and PSBB (B) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L\(^{-1}\) ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at \(p < 0.05\).

**Figure 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 treated with/without high temperature and 50mM proline and/or 200µl L\(^{-1}\) ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at \(p < 0.05\).

**Figure 4**

Activity of SOD (A), APX (B) and GR (C) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L\(^{-1}\) ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at \(p < 0.05\).
Figure 5

Content of proline (A) and glycine betaine (B) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L⁻¹ ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at $p < 0.05$. 
Figure 6

Relative expression of GR in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L⁻¹ ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at p < 0.05.

Figure 7

ACS activity (A) and ethylene evolution (B) in wheat (*Triticum aestivum* L.) leaves at 30 days after sowing treated with/without high temperature and 50mM proline and/or 200µl L⁻¹ ETH. Data are presented as treatment mean ± SE (n=4). Data followed by same letter are not significantly different by LSD test at p < 0.05.

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