Involvement of Ethylene in Reversal of Salt Stress by Salicylic Acid in the Presence of Sulfur in Mustard (Brassica juncea L.)

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Abstract
The involvement of ethylene in reversal of salt (NaCl; 50 mM) stress on photosynthetic activity and growth by salicylic acid (SA; 0.5 mM) together with sulfur (S; 2.0 mM) was studied in mustard (Brassica juncea L. cv. Pusa Vijay). Application of SA plus SO4\(^{2-}\) improved photosynthetic activity through markedly increased S-assimilation, strengthening antioxidant defense system and limiting NaCl-accrued oxidative consequences more conspicuously than their individual effect in B. juncea under 50 mM NaCl stress. Since SA acts as an inhibitor of ethylene and S-assimilation is associated with ethylene synthesis, we tried to figure out the interaction of ethylene in SA and SO4\(^{2-}\)-mediated salt tolerance. The involvement of ethylene was studied by supplementing salt-treated plants with 200 µL L\(^{-1}\) ethephon (an ethylene-releasing compound) or 100 µM norbornadiene (NBD, ethylene action inhibitor) to SA and SO4\(^{2-}\) treatments. Ethephon supplemented to NaCl-treated plants showed optimal ethylene formation, increasing ethylene sensitivity to enhance photosynthesis by affecting antioxidative capacity of plants. SA plus SO4\(^{2-}\) treatment decreased stress ethylene production and optimized ethylene formation under NaCl stress that contributed in the maintenance of high photosynthetic rate, which was reversed with NBD. NaCl-treated plants receiving SA plus SO4\(^{2-}\) and supplemented with NBD showed inhibited photosynthetic characteristics and growth, with minimal S-assimilation capacity, activity of antioxidant enzymes and GSH content. This showed that the reversal of salt stress by SA plus SO4\(^{2-}\) was through ethylene involvement. Overall, ethylene intervened the effect of SA in the presence of SO4\(^{2-}\) to induce S-assimilation, upregulated the enzymatic and non-enzymatic antioxidants, and imparted NaCl tolerance in plants.

Keywords Ethylene · Salicylic acid · Sulfur · Salt stress · Mustard

Introduction
Soil salinity has been the most damaging abiotic stress known for causing the major reductions in the cultivated land area, crop productivity, and quality (Yamaguchi and Blumwald 2005; Shahbaz and Ashraf 2013; Jahan et al. 2021b). The increasing salinization of world cultivable lands at an annual rate of 10% have been projected to be culminated into more than 50% of the arable lands by the year 2050 (Jamil et al. 2011; Jahan et al. 2021b). Salinity-exposed plants exhibit impaired growth and development mainly via oxidative stress as a result of an elevated generation of reactive oxygen species (ROS) (Isayenkov and Maathuis 2019). On the other hand, stressed plants tend to strengthen their antioxidant defense system comprising enzymatic and non-enzymatic antioxidants in order to control the cellular ROS level and minimize ROS-accrued consequences including lipid and protein oxidation (Anjum et al. 2010). Major antioxidant enzymes (such as ascorbate peroxidase, APX; catalase, CAT) and non-enzymatic antioxidants (such as ascorbate, AsA; reduced glutathione, GSH; and \(\alpha\)-tocopherol, flavonoids, and proline) individually and/or cumulatively scavenge varied ROS and therefore counteract ROS-accrued consequences in plants (Ashraf 2009; Sharma and Dietz 2009; Mittal et al. 2012).
Markedly, three major components namely mineral nutrients, phytohormones, and/or their interaction outcomes have been widely reported to significantly modulate and improve the defense machinery in plants against stress impacts on their growth and metabolism, ROS-led oxidative stress, and alterations in antioxidant defense system (Khan et al. 2013, 2015; Jahan et al. 2019, 2021b). Among plant nutrients, sulfur (S) is the essential element, which has been the topic of plant and agricultural research in recent years (Anjum et al. 2015; Asgher et al. 2014; Fatma et al. 2013, 2014, 2021; Kopriva et al. 2016). Sulfur is a major constituent of methionine (Met) and cysteine (Cys), Fe-S clusters, sulfolipids, S-methylmethionine (SMM), S-adenosylmethionine (SAM), glucosinolates, vitamins (biotin and thiamine), coenzyme A, thiolredoxin system, and GSH, a major antioxidant metabolite and non-protein thiol (Fatma et al. 2013; Anjum et al. 2015; Kopriva et al. 2016). Applied S improves the photosynthetic capacity of the plants in salt stress by improving the cellular GSH level, modulating the major components of the ascorbate–glutathione (AsA-GSH) cycle, enhancing ROS metabolism, and thus minimizing oxidative stress and its consequences (Fatma et al. 2013, 2021). Interplay between the reduced S-compound GSH and nitrogenous osmolyte proline was also hypothesized to be involved in the protection of plants against salinity (and metal) stress (Anjum et al. 2014; Kopriva et al. 2016).

Among the major phytohormones, salicylic acid (SA) and ethylene are of great significance in context with their numerous functions in plants (Rivas-San Vicente and Plasencia 2011; Khan et al. 2012; Kazan 2015). SA is a phenolic compound known to control the array of growth, physiological, and developmental processes. It also works as a direct or an indirect signaling response against different stresses and therefore improves photosynthetic functions, nutrient uptake and assimilation, proline metabolism, plant water relations; modifies antioxidant defense systems; and eventually nullifies ROS-induced consequences (Iqbal et al. 2015; Tari et al. 2015; Rasheed et al. 2020; Syyed et al. 2021). There exists a close relationship between SA and S where SA can regulate various aspects of plant responses under both stressful and optimal environments through signaling crosstalk with S-assimilation (Khan et al. 2015).

As a signaling molecule, ethylene acts as a major modulator of plant stress responses mainly by influencing the ROS production and the system involved in ROS metabolism (Cao et al. 2007; Kazan 2015; Tao et al. 2015; Khan et al. 2016; Jahan et al. 2021a, b; Sehar et al. 2021). In several studies, ethylene supplementation-mediated improvements in plant growth and development under abiotic stresses were reported to involve ethylene-induced strengthening of antioxidant defense system (Asgher et al. 2014; Khan et al. 2016; Zhang et al. 2016). Major ethylene signaling components such as ETHYLENE INSENSITIVE2-3 (EIN2 and EIN3) and several members of the AP2/ERF transcription factor gene family have complex regulatory roles in plants during abiotic stress adaptation (Kazan 2015). In a recent report, ethylene induced the expression of psbA and psbB and the production of GSH in wheat and protected the pigment system II activity and photosynthesis under salt stress (Sehar et al. 2021). Ethylene was also involved in optimization of proline metabolism and antioxidant system and eventually improved salinity tolerance in mustard (Jahan et al. 2021a). It is also important to mention here that S-assimilation is associated with ethylene (Masood et al. 2016); however, SA acts as an inhibitor of ethylene (Ahmed et al. 2020). SA-supplemented plants exhibited reduced ethylene generation in heat-exposed plants by decreasing the 1-amino-cyclopropane carboxylic acid (ACC) and ACC synthase (ACS) activity to an appropriate range (Khan et al. 2015). Ethylene-induced H2S was also found to negatively regulate ethylene biosynthesis by persulfidation of ACC oxidase in tomato under osmotic stress (Jia et al. 2018). Despite the mentioned facts, information on how ethylene is involved in SA and S-mediated plant salt tolerance is scanty in the literature.

As a widely cultivated species for oilseed, Indian mustard (Brassica juncea L.) stands second to soybean in world oilseed production (USDA 2018). It is cultivated mainly in the north-western agro-climatic region of India and suffers huge losses in its productivity mainly due to salinization of the cultivable land (Yousuf et al. 2016). Therefore, it is imperative to explore the salt tolerance mechanisms in B. juncea in order to sustain optimum growth and produce more in saline areas. Thus, the triad comprising SA, S, and ethylene is hypothesized herein to influence the response of B. juncea to salinity stress and helps this crop to protect its growth, metabolism and photosynthetic functions, and strengthen its antioxidant defense system against salinity stress impacts. Hence, the outcome of this research may contribute in so far elusive understanding on how ethylene gets involved in SA-mediated effect on salinity in the presence of S in B. juncea.

Materials and Methods

Reagents

Reagents and chemicals used in the present study were purchased from Sigma Aldrich (USA) or Himedia Pvt. Ltd (Mumbai, India) and used as received, unless otherwise stated.

Plant Culture and Treatments

Healthy seeds of Brassica juncea L. Czern & Coss. cultivar Pusa Vijay were surface-sterilized for 15 min with 0.01%
HgCl₂ followed by repeated washings through double distilled water. Sterilized seeds were sown in 23-cm diameter earthen pots filled with 5 kg of reconstituted soil including peat and compost (4:1, w/w) mixed with sand (3:1, w/w). Seeds were sown in each pot and were kept in natural day/night conditions with an average day/night temperatures of 20±3°C and 12±2°C, respectively, relative humidity of 60±5%, photosynthetically active radiation (PAR) was 750±25 μmol m⁻² s⁻¹ and a critical photoperiod of 10–12 h.

To assess the effect of 0.5 mM SA and 2.0 mM SO₄²⁻ alone or in coordination in alleviation of salinity, plants were supplied with 50 mM NaCl at 15 days after sowing (DAS), while S (2.0 mM SO₄²⁻) and SA (0.5 mM) were supplied at 20 DAS. Salicylic acid was dissolved in 100% ethanol and then added drop by drop to water (ethanol/water: 1/1000 v/v). Plants grown without NaCl or SA were sprayed with ethanol/water (1/1000 v/v) to maintain the control group. The application of SA was made as a foliar spray (30 mL) to the plants in each pot; and a surfactant teepol (0.5%) was mixed with the control and SA treatment solution. Hoagland nutrient solution (250 mL; Hewitt 1966) was added to the control plants every other day and 200 mL of double distilled water daily. Sulfur was provided in the form of MgSO₄ for obtaining 2.0 mM SO₄²⁻ concentrations, and Mg²⁺ was maintained at 2.0 mM in all treatments, including control, by the addition of appropriate MgCl₂.

The other experiment was performed to evaluate the involvement of ethylene in SA and S-mediated control of photosynthetic functions and growth using 200 μL L⁻¹ ethephon (an ethylene-releasing compound) or 100 μM 2,5-norbornadiene (NBD, ethylene action inhibitor) to SA and S treatment under salt stress. The concentrations of ethephon and NBD used in the present study were worked out earlier (Iqbal et al. 2017). Ethephon was chosen because it is known to alter ethylene evolution, and NBD was selected because it inhibits ethylene activity. Ethephon or NBD was sprayed on the upper surface of the foliage at 20 DAS together with 0.5% of a teepol surfactant. Per plant 25 mL of ethephon or NBD was applied with a hand sprayer. The treatments were set up in a completely randomized block design with four replicates (n=4) for each treatment and the parameters were studied after 30 DAS. Leaves of the same age were taken for determinations.

**Determinations**

**Photosynthetic and Growth Characteristics**

On a sunny day, net photosynthesis, stomatal conductance, and intercellular CO₂ concentration were measured in completely expanded topmost second leaves of plants in each treatment at around 11.00 to 12.00 at day light saturating intensity using Infra-Red Gas Analyzer (CID-340, Photosynthesis system, Bio-Science, Camas, WA, USA). At the time of measurement, the atmospheric conditions were as follows: photosynthetically active radiation, ~680 μmolm⁻²s⁻¹; air temperature, ~22°C; and relative humidity, ~70%.

Chlorophyll content in the leaves of plants taken from every treatment was determined using a SPAD chlorophyll meter (502 DL PLUS, Spectrum Technologies, Plainfield, IL, USA). The maximal PSII photochemical efficiency (Fv/Fm) of the fully expanded second leaf from the top of the plant was determined using a chlorophyll fluorometer (Junior-PAM, Heinz Walz, GmbH, Effeltrich, Germany). The details are given in Supplementary file S1.

Leaf area was measured by leaf area meter (LA 211; Systronics, Hyderabad, India). Dry weight was estimated after drying the samples in an oven at 80°C until the water evaporated and a consistent weight was achieved.

**Oxidative Stress Parameters**

The method of Okuda et al. (1991) was used to determine H₂O₂ content. Lipid peroxidation was determined by estimating thiobarbituric acid reactive substances (TBARS) following Dhindsa et al. (1981). Supplementary File S1 contains the details of the procedure.

**Assay of Antioxidant Enzymes**

Fresh leaves (0.2 g) were homogenized in a chilled mortar and pestle with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVP) in potassium-phosphate buffer (100 mM; pH 7.0). The homogenate was centrifuged at 15,000 × g for 20 min at 4°C. After centrifugation, the supernatant was collected and activity of superoxide dismutase (SOD; EC 1.15.1.1) and glutathione reductase (GR; EC 1.6.4.2) was measured. For the measurement of ascorbate peroxidase (APX; EC 1.11.1.11), the extraction buffer was supplemented with 2 mM AsA.

Giannopolitis and Ries (1977) and Beyer and Fridovich (1987) methods were used for the assay of SOD activity, where the suppression of photochemical reduction of nitro blue tetrazolium (NBT) was studied. Activity of APX was assayed using description of Nakano and Asada (1981) by measuring the decrease in ascorbate absorbance at 290 nm owing to enzymatic breakdown. Foyer and Halliwell (1976) method was followed for GSH-dependent oxidation of NADPH at 340 nm to evaluate the activity of GR. Supplementary File S1 contains the details of the procedure.
Ethylene Evolution

Ethylene evolution in leaves was assessed using gas chromatograph as described by Fatma et al. (2021). Briefly, cut leaf material (0.5 g) was placed into 30 mL tubes with moist paper. Tubes were stoppered with secure rubber caps in order to minimize evaporation from the tissue, and were placed in light for 2 h under the same condition used for plant growth. With a hypodermic syringe, 1-mL gas sample was withdrawn from the tubes and assayed on a gas chromatograph (Nucon 5700, Nucon New Delhi, India). This gas chromatograph was equipped with a 1.8-m Porapack™ N (80–100 mesh) column (Sigma-Aldrich, St. Louis, MO, USA), a flame ionization detector and data station. Nitrogen was used as carrier gas. The flow rates of nitrogen, hydrogen, and oxygen were 30, 30, and 300 mL min⁻¹, respectively. The detector was set at 150 °C. Ethylene was identified based on the retention time and quantified by comparison with peaks from standard ethylene concentration.

Determinations of ATP-S Activity and the Content of S, Cys, GSH and Redox State

The activity of ATP-S was measured using the Lappartient and Touraine method (1996). The turbidimetric method was used to determine tissue-S content, whereas determination of content of Cys and GSH was done by the methods of Gaïtonde (1967) and Griffith (1980), respectively, as described by Fatma et al. (2014). Supplementary File S1 contains the details of the procedures followed. The ratio of GSH/GSSG was used to calculate the redox status.

Assay of NR activity and N Content

Activity of nitrate reductase (EC 1.7.99.4) was assayed following the method of Kuo et al. (1982) as described by Iqbal et al. (2012). Leaf samples for the assay of NR activity were collected in the noon when maximum intensity of light was present that is required for the maximum activation of this enzyme. Nitrogen content of leaves was computed by Lindner (1944) technique, and altered by Novozamsky et al. (1983). File S1 contains the details of these procedures.

Histochemical Staining Method for Visualizing the Presence of Superoxide and H₂O₂

To visualize the presence of superoxide and H₂O₂ in the test leaf samples, histochemical staining techniques using nitro blue tetrazolium (NBT) and 3, 3-diaminobenzidine (DAB) were employed (Wang et al. 2011). Three leaves from each treatment were immersed in NBT (1.0 mg mL⁻¹) solution made in phosphate buffer (10 mM; pH 7.8) at room temperature for six hours under light. Samples stained with NBT or DAB revealed blue or brown dots. The pigmented samples were soaked in concentrated ethanol and then run through a 70% ethanol filter. Snapshots of the leaf samples that had been cleaned were taken with a NIKON digital camera (COOLPIX110).

Statistical Analysis

SPSS 17.0 for Windows was used to perform statistical analysis of variance (ANOVA), and the results were given as treatment mean SE (n = 4). The F-value was computed after an ANOVA was generated according to the experiment design. For the significant data, the least significant difference (LSD) was computed at p ≤ 0.05. The results are not statistically different by LSD test at p ≤ 0.05, if bars showing the same letter.

Results

Influence of Combined SA and S in Reversal of Salt Stress Effect on Photosynthetic Functions and Growth

Photosynthetic characteristics decreased under salt stress; however, the application of 0.5 mM SA or 2.0 mM SO₄²⁻ increased the photosynthetic characteristics. Under no stress, the plants treated with 0.5 mM SA or 2.0 mM SO₄²⁻ exhibited almost equal increase of ∼30.3%, 37.2%, 34.8%, and 30.7% in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control. When exposed to 50 mM NaCl, application of 0.5 mM SA resulted in 15.1%, 14.7%, 20.7%, and 12.7% increase in net photosynthesis, intercellular CO₂ concentration, stomatal conductance, and chlorophyll content, respectively, compared with the control.
compared with control. Similarly, in 0.5 mM SA-supplied plants but grown without salt the increase in leaf area and plant dry mass was by 32.6, 45.1% in comparison with the control. When exposed to 50 mM NaCl, the application of 0.5 mM SA or 2.0 mM SO$_4^{2-}$ increased leaf area and plant dry mass compared to both control and 50 mM NaCl treatment. But the maximum increase of 49.1% in leaf area and 51.2% in plant dry mass was found with the collective supply of 0.5 mM SA plus 2.0 mM SO$_4^{2-}$ to the salt grown plants compared with control (Table 1). This increase in photosynthetic and growth characteristics was maximum along with the combined application of SA and SO$_4^{2-}$ compared with either SA plus salt or SO$_4^{2-}$ plus salt suggesting that their combination is providing signal that is not conveyed individually. In our study, we speculated that this is because of optimum ethylene evolution and ethylene-mediated action with their combined treatment under salt stress.

### Table 1

| Treatments       | Leaf area (cm$^2$ plant$^{-1}$) | Plant dry mass (g plant$^{-1}$) | Chlorophyll content (SPAD value) | Maximal PSII Photochemical Efficiency | Net Photosynthesis (μmol CO$_2$ m$^{-2}$ s$^{-1}$) | Intercellular CO$_2$ Concentration (μmol H$_2$O m$^{-2}$ s$^{-1}$) | Stomatal Conductance (mmol H$_2$O m$^{-2}$ s$^{-1}$) |
|------------------|---------------------------------|---------------------------------|----------------------------------|----------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Control          | 140.7 ± 4.0 g                   | 1.64 ± 0.02 g                   | 36.8 ± 1.2 g                     | 0.74 ± 0.02 g                          | 14.5 ± 1.24 g                                   | 360 ± 07 g                                      | 396 ± 09 g                                      |
| NaCl             | 999.9 ± 3.4 h                   | 0.97 ± 0.03 h                   | 30.4 ± 1.1 h                     | 0.66 ± 0.01 h                          | 10.7 ± 1.20 h                                   | 266 ± 05 h                                      | 302 ± 08 h                                      |
| SA               | 186.7 ± 4.6c                    | 2.38 ± 0.04c                    | 48.1 ± 1.4c                      | 0.85 ± 0.03c                           | 18.9 ± 1.39c                                    | 494 ± 11c                                       | 534 ± 13c                                       |
| SO$_4^{2-}$       | 183.3 ± 4.7 cd                  | 2.36 ± 0.04 cd                  | 47.3 ± 1.3 cd                    | 0.84 ± 0.03 cd                         | 18.7 ± 1.37 cd                                   | 487 ± 11 cd                                     | 528 ± 13 cd                                     |
| SA + SO$_4^{2-}$  | 224.4 ± 5.8a                    | 2.59 ± 0.05a                    | 53.6 ± 1.6a                      | 0.92 ± 0.06a                           | 22.8 ± 1.44a                                    | 588 ± 15a                                       | 618 ± 15a                                       |
| NaCl + SA        | 168.2 ± 4.2f                    | 1.97 ± 0.06f                    | 41.5 ± 1.2f                      | 0.77 ± 0.02f                           | 16.7 ± 1.29f                                    | 413 ± 09f                                       | 478 ± 10f                                       |
| NaCl + SO$_4^{2-}$| 178.9 ± 4.4e                    | 2.19 ± 0.06e                    | 44.8 ± 1.3e                      | 0.82 ± 0.03e                           | 18.1 ± 1.33e                                    | 448 ± 10e                                       | 512 ± 11e                                       |
| SA + SO$_4^{2-}$ + NaCl | 209.8 ± 5.4b                | 2.48 ± 0.07b                    | 51.2 ± 1.6b                      | 0.88 ± 0.05b                           | 21.2 ± 1.41b                                    | 556 ± 13b                                       | 578 ± 14b                                       |

Plants were treated with 0.5 mM SA and/or 2 mM SO$_4^{2-}$ in presence or absence of 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at (p < 0.05). SA salicylic acid

### Table 2

| Treatments       | H$_2$O$_2$ (nmol g$^{-1}$ leaf FW) | TBARS (U mg$^{-1}$ protein min$^{-1}$) | SOD activity | APX activity | GR activity |
|------------------|-----------------------------------|----------------------------------------|--------------|--------------|-------------|
| Control          | 21.5 ± 0.76b                      | 3.35 ± 0.25b                           |              |              |             |
| NaCl             | 32.8 ± 0.90a                      | 5.60 ± 0.28a                           |              |              |             |
| SA               | 12.5 ± 0.23ef                     | 1.88 ± 0.08ef                          | 11.0 ± 0.38c | 2.19 ± 0.13c | 0.359 ± 0.011cd |
| SO$_4^{2-}$       | 12.8 ± 0.21e                      | 1.96 ± 0.09e                           | 10.9 ± 0.37cd | 2.17 ± 0.12cd | 0.353 ± 0.010cd |
| SA + SO$_4^{2-}$  | 08.6 ± 0.16h                      | 1.28 ± 0.06h                           | 13.4 ± 0.54a | 2.63 ± 0.23a | 0.391 ± 0.006b |
| NaCl + SA        | 15.8 ± 0.42c                      | 2.59 ± 0.23c                           | 09.1 ± 0.30f | 1.82 ± 0.06f | 0.305 ± 0.006f |
| NaCl + SO$_4^{2-}$| 13.5 ± 0.30d                      | 2.21 ± 0.20d                           | 09.8 ± 0.32e | 1.96 ± 0.09e | 0.328 ± 0.009e |
| SA + SO$_4^{2-}$ + NaCl | 10.7 ± 0.19 g                | 1.63 ± 0.08 g                           | 12.2 ± 0.52b | 2.46 ± 0.20b | 0.371 ± 0.008a |

Plants were treated with 0.5 mM SA and/or 2 mM SO$_4^{2-}$ in presence or absence of 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at (p < 0.05). FW fresh weight, SA salicylic acid

**Influence of SA with S in Reducing Oxidative Stress by Accelerating the Activity of Antioxidant Enzymes**

Application of 0.5 mM SA or 2.0 mM SO$_4^{2-}$ resulted in decrease in H$_2$O$_2$ and TBARS content in non-stressed plants and plants grown with salt. Under no stress, SA decreased content of H$_2$O$_2$ TBARS content equally by about 43.8% compared with the control. However, when comparison was made with salt grown plant, the decrease was found to be 26.5% in H$_2$O$_2$ content and 22.6% in TBARS content with 0.5 mM SA. The supplementation of 2.0 mM SO$_4^{2-}$ to salt grown plants decreased H$_2$O$_2$ by 37.2% and TBARS by 34.0% in comparison with control. The maximum decrease in H$_2$O$_2$ and TBARS content was obtained with the application of SA with SO$_4^{2-}$ together under 50 mM NaCl which decreased H$_2$O$_2$ and TBARS content by 50.2% compared with control (Table 2).

Salt, S, or SA independently increased activity of antioxidant enzymes under no stress. Application of 0.5 mM SA to 50 mM NaCl-treated plants increased the activity of SOD,
APX, and GR increased by 68.5, 59.6, and 41.8%, respectively, when compared to the control. In plants grown with salt and SO$_4^{2-}$, the activity of SOD, APX, and GR increased by 81.4, 71.9, and 52.5%, respectively, compared to the control treatment. Under no stress and salt stress conditions, supplementing with 0.5 mM SA and 2.0 mM SO$_4^{2-}$ combined led in the greatest increase in SOD, APX, and GR activity when compared to the control (Table 2).

**Influence of SA with S on Nitrogen Assimilation**

Salt treatment reduced N content and NR activity compared with the control. The individual supplementation of 0.5 mM SA or 2.0 mM SO$_4^{2-}$ increased N content and NR activity equally by about 39.0% compared with the control under no stress. Under salt stress, application of 0.5 mM SA increased N content by 13.7% and NR activity by 21.3%, while 2.0 mM SO$_4^{2-}$ increased the content of N by 22.2% and the activity of NR by 21.3% compared with respective control. The maximum increase of N content by 64.8 or 50.8% and NR by 63.5 or 53.1% with combined treatment of SA plus SO$_4^{2-}$ was observed in no stress or salt stress, respectively, compared to the control (Fig. 1).

**Influence of SA in S-Mediated Reversal of Salt Stress through Increasing S-Assimilation**

Supplementation of 0.5 mM SA improved ATP-S activity, content of S, and cysteine by 73.6%, 53.8%, and 64.2%, respectively, under no stress and by 37.9%, 23.5%, and 42.5% under salt stress compared with the respective control. Similarly, the individual application of 2.0 mM SO$_4^{2-}$ also increased ATP-S activity, content of S, and cysteine under no stress and salt stress compared to the respective control. The combined application of 0.5 mM SA plus 2.0 mM SO$_4^{2-}$ to salt grown plants resulted in maximum increases of 86%, 64.7%, and 77.9% in ATP-S activity, contents of S, and cysteine, respectively, compared with control treatment under salt stress. The supply of 0.5 mM SA or 2.0 mM SO$_4^{2-}$ under no stress or salt stress treatment increased GSH content and redox state almost equally by 48.8 or 47.1% and 65.7 or 64.8% compared with the respective control. Moreover, the application of 0.5 mM SA and 2.0 mM SO$_4^{2-}$ together enhanced the content of GSH and the redox state maximally compared to control or salt treatment (Table 3).

**Effect of SA with S on the Presence of Superoxide and H$_2$O$_2$ Visualized through Histochemical Staining Method**

To ascertain the cellular status of oxidative stress markers O$_2^{•−}$ and H$_2$O$_2$, these two traits were visualized in leaves employing histochemical staining methods through staining of leaves with DAB and NBT stains. The observation of leaves made after 6 h revealed brown spots and blue spots with higher intensities showing higher levels of H$_2$O$_2$ and O$_2^{•−}$ in plants exposed to 50 mM NaCl (Fig. 2B; in both panel 1 and 2). The application of 0.5 mM SA or 2.0 mM SO$_4^{2-}$ showed the lesser intensity with less brown and blue spots in comparison with control or salt treatment under no stress and salt stress conditions. Significantly the maximal lowering in the H$_2$O$_2$ and O$_2^{•−}$, indicated by lowest intensities of brown or blue spots were noted in the leaves of 0.5 mM SA plus 2.0 mM SO$_4^{2-}$-treated plants under 50 mM NaCl (Fig. 2H; in both panel 1 and 2).

**Effect of SA with S on the Chloroplast Ultrastructure under Salt stress**

Chloroplast ultrastructure was presented as TEM micrographs (Fig. 3). Under no stress, chloroplasts had a normal shape with well-organized thylakoid systems (Fig. 3A). The samples from 50 mM NaCl showed disorganized thylakoids (Fig. 3B). Plants receiving individual application of SA or SO$_4^{2−}$ showed developed thylakoid stacks (Fig. 3C, D). However, chloroplast of NaCl-treated plants receiving SA plus SO$_4^{2−}$ exhibited a regular form with well-organized thylakoid systems and a significantly higher number of thylakoid stacks (Fig. 3E).

**Effect of SA and S on Ethylene Evolution**

In order to assess if the SA induced reversal of salt stress in presence of SO$_4^{2−}$ involved ethylene, the plants were grown with ethephon plus salt stress and also with NBD applied to SA- and SO$_4^{2−}$-treated plants under salt stress. Ethylene evolution decreased with SA, SO$_4^{2−}$, or their combination in salt stress. Maximum evolution of ethylene was observed under salt stress, which was 3.47 times higher compared to control. When SA or SO$_4^{2−}$ was supplemented to salt-stressed plants, ethylene evolution was decreased. In the presence of SA without salt stress, the decrease was by 26.31% and with SO$_4^{2−}$ it was 21.0% compared with the respective control. In the presence of SO$_4^{2−}$, SA under salt stress caused a decrease of 42.4% in ethylene evolution in comparison with salt-treated plants, while SA plus salt caused decrease of 56.1% and SO$_4^{2−}$ plus salt caused 48.5% decrease in ethylene evolution in comparison with salt-treated plants (Fig. 4).

**Involvement of Ethylene in SA-Induced and S-mediated Reversal of Salt stress for Photosynthesis through Increased S-assimilation capacity, Activity of Antioxidant Enzymes, and Ethylene Evolution**

To assess if the SA induced reversal of salt stress in the presence of SO$_4^{2−}$ involved ethylene, the plants were subjected to ethephon under salt stress or with NBD to SA and
Fig. 1 Content of nitrogen A and activity of nitrate reductase B of mustard (Brassica juncea L.) leaves at 30 days after sowing. Plants were treated with 0.5 mM SA and/or 2 mM SO$_4^{2-}$ in presence or absence of 50 mM NaCl. Data are presented as means ± SE ($n=4$). Data followed by the same letter are not significantly different by LSD test at ($p<0.05$). DW dry weight, FW fresh weight, SA salicylic acid.
Table 3  Activity of ATP-sulfurylase (ATP-S) and content of cysteine (Cys), sulfur (S), reduced glutathione (GSH) and redox state of mustard (Brassica juncea L.) leaves at 30 days after sowing

| Treatments                  | ATP-S Activity (µmol g⁻¹ protein s⁻¹) | S content (mg g⁻¹ DW) | Cys content (nmol g⁻¹ Leaf DW) | GSH content (nmol g⁻¹ Leaf DW) | Redox state (GSH/GSSG) |
|-----------------------------|---------------------------------------|-----------------------|-------------------------------|-------------------------------|-------------------------|
| Control                     | 1.29 ± 0.018 h                        | 3.40 ± 0.21f          | 05.90 ± 0.31 g                | 42.20 ± 3.09 h                | 20.70 ± 1.10f           |
| NaCl                        | 1.47 ± 0.019 g                        | 2.80 ± 0.11 g         | 07.10 ± 0.45f                 | 50.50 ± 2.86 g                | 17.60 ± 0.90 g          |
| SA                          | 2.24 ± 0.029c                         | 5.23 ± 0.21c          | 09.69 ± 0.55c                 | 62.80 ± 3.18c                 | 34.30 ± 1.03c           |
| SO₄²⁻                      | 2.19 ± 0.029 cd                       | 5.20 ± 0.21c          | 09.56 ± 0.55c                 | 62.10 ± 3.10 cd               | 34.13 ± 1.05c           |
| SA + SO₄²⁻                  | 2.51 ± 0.043a                         | 6.00 ± 0.24a          | 11.16 ± 0.64a                 | 77.10 ± 3.60a                 | 39.70 ± 1.12a           |
| NaCl + SA                   | 1.78 ± 0.017f                         | 4.20 ± 0.20e          | 08.41 ± 0.44e                 | 54.40 ± 2.61f                 | 30.20 ± 1.22e           |
| NaCl + SO₄²⁻                | 2.04 ± 0.019e                         | 4.90 ± 0.21d          | 09.10 ± 0.30d                 | 59.50 ± 3.12e                 | 32.60 ± 1.03d           |
| SA + SO₄²⁻ + NaCl           | 2.40 ± 0.042b                         | 5.60 ± 0.23b          | 10.50 ± 0.61b                 | 72.20 ± 3.40b                 | 37.80 ± 1.18b           |

Plants were treated with 0.5 mM SA and/or 2.0 mM SO₄²⁻ in presence or absence of 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at (p < 0.05). DW dry weight, SA salicylic acid

Fig. 2  Histochemical staining method of NBT (panel 1; A–H) and DAB (panel 2; A–H) for the visualization of H₂O₂ and superoxide ion of mustard (Brassica juncea L.) leaves at 30 days after sowing. Leaves were represented as under control (A), 50 mM NaCl (B), 0.5 mM SA (C), 2.0 mM SO₄²⁻ (D), SA plus 2.0 mM SO₄²⁻ (E) and in the combination of 50 mM NaCl, leaves represented as with 0.5 mM SA (F), 2.0 mM SO₄²⁻ (G), and SA plus 2.0 mM SO₄²⁻ (H). DAB, 3, 3-diaminobenzidine, NBT nitro blue tetrazolium, SA salicylic acid

Fig. 3  Ultrastructure of chloroplasts in mustard (Brassica juncea L.) leaves using transmission electron microscopy at a magnification of 6000 × under control A 50 mM NaCl B, 0.5 mM SA C, 2.0 mM SO₄²⁻ D, and 0.5 mM SA plus 2.0 mM SO₄²⁻ with 50 mM NaCl E at 30 d after sowing. Bars (−); (A–E) = 500 nm. Thythylakoid membranes, SA salicylic acid
SO$_4^{2-}$ treatment under salt stress. Plants receiving SA plus SO$_4^{2-}$ in presence of salt showed inhibited photosynthetic characteristics, stomatal behavior and growth when NBD was supplemented to the treatment. It was found that the photosynthetic characteristic including net photosynthesis, stomatal conductance and plant dry mass was reduced by 28.4, 17.5, and 26.2%, respectively, when ethylene action inhibitor NBD was applied to SA plus SO$_4^{2-}$ in presence of salt. However, the results of photosynthetic characteristics with exogenous ethephon to salt-treated plants was significantly equal to the plants receiving salt with SA and SO$_4^{2-}$, which was found to cause 38.1% increase in net photosynthesis, 53.7% in stomatal conductance and by 52.5% in plant dry mass compared with control. Moreover, application of ethephon to salt-stressed plants caused 38.1% increase in net photosynthesis, 53.7% in stomatal conductance and by 52.5% in plant dry mass compared with control. The application of ethephon to salt-stressed plants were equally effective in reducing the H$_2$O$_2$ content by 53.7 or 54.6%, respectively, in comparison with control. Contrarily, the H$_2$O$_2$ content was increased by 16.4% in SA plus SO$_4^{2-}$ plants treated with NBD compared to control (Table 4).

Analysis of stomatal behavior by SEM also showed reduction in presence of NBD and the results were reversed with the application of ethephon or SA plus SO$_4^{2-}$ to salt-treated plants (Fig. 5A–J). The image of the stomatal width aperture, stomatal length, and stomatal frequency were apparently visible under different treatments when SEM was done. The stomatal frequency was reduced by 18.9% compared to the control. However, supplementation of NaCl treated with SA plus SO$_4^{2-}$ improved stomatal frequency by 27.2% compared to the control. The application of ethephon to NaCl-treated plants resulted in increase of 23.5%, whereas 6.8% reduction was observed in stomatal frequency with NBD compared to the control (Fig. 6A). Leaf samples under control conditions had normal stomata with the characteristic guard cells having stomatal width aperture and stomatal length of 6 µm and 7.2 µm (Fig. 6B, C), whereas the impact of salt stress on stomatal closure was clearly seen as the diameter of the stomatal width aperture and stomatal length
were of 1 µm and 5.5 µm, respectively. The stomatal width aperture and stomatal length were improved with SA plus \( \text{SO}_4^{2-} \) in presence of NaCl by 13 µm and 14.2 µm, respectively. Application of ethephon counteracted the effects of NaCl for stomatal width aperture and stomatal length (Fig. 5G, H; Fig. 6B, C). However, stomatal width aperture and stomatal length reduced in presence of NBD by closing the stomatal width aperture and stomatal length by 3 µm and 6.6 µm, respectively (Fig. 5I, J; Fig. B, C).

In this study, individual application of both SA and S in the form of \( \text{SO}_4^{2-} \) were found effective in enhancing S-assimilation, photosynthesis, and growth in salt stress. A common point in their alleviation strategy was ethylene because SA was inhibiting ethylene biosynthesis and S was reducing the oxidative stress by directly increasing GSH and reducing stress ethylene formation by directing the Cys to GSH and not to the ethylene pathway via Met. However, it was an interesting issue to discuss that what could be the effect of their combination and how will they influence ethylene, as this is still not discussed in any study. We observed that the combination was best in stress alleviation, and this could be explained with ethylene synthesis and signaling. In combination of SA and S, SA reduced the formation of stress ethylene, whereas S increased S-assimilation and generation of ethylene by modulating its emission through the Met pathway (S-assimilation leads to Met, and SAM which is precursor for ethylene via ACC). The observation was verified by using both ethephon and ethylene action inhibitor NBD, which modulated both its synthesis and signaling. In the first experiment, addition of SA or \( \text{SO}_4^{2-} \) to salt-stressed plants reduced ethylene evolution compared to salt stress. Compared to \( \text{SO}_4^{2-} \), SA caused greater reduction in ethylene but when they were applied in combination we observed a greater increase in ethylene evolution that was sufficient to signal plants for increased antioxidative enzymes, S-assimilation, photosynthesis, and growth in salt stress.

Thus, in the second experiment we took the best dose of SA and \( \text{SO}_4^{2-} \) combined treatment and compared it with salt and ethephon treatment. We observed significantly equal increase in S-assimilation, ethylene evolution, photosynthesis, and growth in both the treatments suggesting ethylene to be the key molecule. The supplementation of ethephon to salt-treated plants increased antioxidant activities significantly equal to the plants receiving salt with SA and \( \text{SO}_4^{2-} \) (Table 4). The results showed that the effects of SA in reversal of salt stress through S mediation also involved ethylene. The results on ethylene formation with exogenous ethephon to salt-treated plants was significantly equal to SA plus \( \text{SO}_4^{2-} \) in salt stress. The results were further substantiated using ethylene action inhibitor to confirm that it is ethylene signaling that affected the alleviation pathway. In the presence of NBD, salt-stressed plants receiving SA plus \( \text{SO}_4^{2-} \)-exhibited decrease in the selected parameters for S-assimilation such as content of Cys, S, and GSH by 12, 13.5, and 11.9%, respectively. Moreover, antioxidant enzymes activity was also minimal under NBD treatment with SA plus \( \text{SO}_4^{2-} \) under 50 mM NaCl (Table 5). Plants receiving salt with SA and \( \text{SO}_4^{2-} \) showed optimum ethylene evolution by 24.10%, respectively, compared to control which was reversed with NBD (Fig. 7). Thus, there exists a crosstalk between SA and ethylene in reversal of salt stress in presence of S because S-assimilation leads to formation of ethylene through S-adenosyl methionine and SA application induced S-assimilation.

### Discussion

Salicylic acid potentially regulates the major physiological and molecular mechanisms adopted by plants for the alleviation of the harmful effects of salt stress. So, the efficiency of SA in the alleviation of salt stress through S-supplementation was determined in the present study. The roles and underlying mechanisms of SA in oxidative

| Treatments                  | \( \text{H}_2\text{O}_2 \) content (nmol g\(^{-1}\) leaf FW) | Net Photosynthesis (μmol CO\(_2\) m\(^{-2}\) s\(^{-1}\)) | Stomatal conductance (mmol H\(_2\)O m\(^{-2}\) s\(^{-1}\)) | Plant dry mass (g plant\(^{-1}\)) |
|-----------------------------|---------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|----------------------------------|
| Control                     | 22.5 ± 0.72c                                            | 16.5 ± 0.7b                                          | 388 ± 15b                                            | 1.60 ± 0.07b                     |
| NaCl                        | 34.9 ± 0.81a                                           | 09.8 ± 0.4d                                          | 301 ± 11d                                            | 0.89 ± 0.06d                     |
| NaCl + SA + \( \text{SO}_4^{2-} \) | 10.2 ± 0.70d                                          | 22.8 ± 0.8a                                          | 590 ± 19a                                            | 2.42 ± 0.08a                     |
| NaCl + Eth                  | 10.4 ± 0.68d                                          | 23.1 ± 0.9a                                          | 496 ± 16a                                            | 2.44 ± 0.09a                     |
| NaCl + SA + \( \text{SO}_4^{2-} \) + NBD | 26.2 ± 0.77b                                         | 11.8 ± 0.5c                                          | 320 ± 12c                                            | 1.18 ± 0.07c                     |

Plants were treated with 0.5 mM SA plus 2 mM \( \text{SO}_4^{2-} \) and/or 200 μL L\(^{-1}\) ethephon (Eth) in presence of 50 mM NaCl. The treatment 100 μM norbornadiene (NBD) was applied with combination of 0.5 mM SA plus 2 mM \( \text{SO}_4^{2-} \) to plants raised with 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at (p < 0.05). FW fresh weight.
Fig. 5 Stomatal behavior of mustard (*Brassica juncea* L.) leaves (A, B) under control, (C, D) 50 mM NaCl, (E, F) 0.5 mM SA plus 2.0 mM SO$_4^{2-}$ in 50 mM NaCl, (G, H) 200 µL L$^{-1}$ ethephon plus 50 mM NaCl, and (I, J) 0.5 mM SA plus 2.0 mM SO$_4^{2-}$ with 50 mM NaCl and 100 µM NBD at 30 days after sowing. The opening and closing of stomata were observed under scanning electron microscope at a magnification of 500x (A, C, E, G, I) and 2500x (B, D, F, H, J). Bars (A, C, E, G, I) = 50 µm; bars (B, D, F, H, J) = 5 µm. *NBD* norbornadiene, *SA* salicylic acid
Fig. 6 Stomatal frequency A, stomatal aperture length B and stomatal width aperture C in mustard (Brassica juncea L.) leaves at 30 days after sowing. Plants were treated with 0.5 mM SA plus 2.0 mM \( \text{SO}_4^{2-} \) and/or 200 µL L\(^{-1} \) ethephon in presence of 50 mM NaCl. The treatment with 100 µM NBD was applied with combination of 0.5 mM SA plus 2.0 mM \( \text{SO}_4^{2-} \) with 50 mM NaCl. Data are presented as means ± SE \((n = 4)\). Data followed by the same letter are not significantly different by LSD test at \((p < 0.05)\). NBD norbornadiene, SA salicylic acid.
Table 5 Activity of ascorbate peroxidase (APX) and glutathione reductase (GR) and content of cysteine, sulfur, and reduced glutathione (GSH) of mustard (Brassica juncea L.) at 30 days after sowing

| Treatments                  | APX (U mg⁻¹ protein min⁻¹) | GR (nmol g⁻¹ leaf DW) | Cysteine (mg g⁻¹ leaf FW) | Sulfur (mg g⁻¹ leaf FW) | GSH (mg g⁻¹ leaf FW) |
|-----------------------------|----------------------------|-----------------------|---------------------------|-------------------------|----------------------|
| Control                     | 1.19 ± 0.03c               | 0.211 ± 0.005c        | 05.80 ± 0.5c              | 3.70 ± 0.4b             | 41.8 ± 3.0c          |
| NaCl                        | 1.36 ± 0.04b               | 0.244 ± 0.003b        | 06.80 ± 0.6b              | 2.60 ± 0.2d             | 50.4 ± 3.4b          |
| NaCl + SA + SO₄²⁻           | 2.43 ± 0.05a               | 0.360 ± 0.007a        | 10.80 ± 0.7a              | 5.66 ± 0.6a             | 74.3 ± 4.2a          |
| NaCl + Eth                   | 2.47 ± 0.06a               | 0.365 ± 0.007a        | 10.90 ± 0.7a              | 5.70 ± 0.6a             | 74.6 ± 4.3a          |
| NaCl + SA + SO₄²⁻ + NBD     | 1.04 ± 0.02d               | 0.180 ± 0.003d        | 05.10 ± 0.4d              | 3.20 ± 0.3c             | 36.8 ± 2.7d          |

Plants were treated with 0.5 mM SA plus 2 mM SO₄²⁻ and/or 200 µL L⁻¹ ethephon (Eth) in presence of 50 mM NaCl. The treatment with 100 µM norbornadiene (NBD) was applied with combination of 0.5 mM SA plus 2 mM SO₄²⁻ to plants raised with 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at (p < 0.05). DW dry weight, FW fresh weight

Fig. 7 Ethylene evolution in mustard (Brassica juncea L.) leaves at 30 days after sowing. Plants were treated with 0.5 mM SA plus 2.0 mM SO₄²⁻ and/or 200 µL L⁻¹ ethephon (Eth) in presence of 50 mM NaCl. The treatment with 100 µM norbornadiene (NBD) was applied with combination of 0.5 mM SA plus 2.0 mM SO₄²⁻ with 50 mM NaCl. Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at (p < 0.05). FW fresh weight, NBD norbornadiene, SA salicylic acid
stress, S- and N-assimilation, antioxidant metabolism, and photosynthetic characteristics are discussed. The individual SA or S application decreased the negative effects of salt stress, but maximum reduction resulted with combined application of SA plus S in both no stress and salt stress conditions. However, the efficiency of SA plus S in the alleviation of salt stress was due to the result of optimal ethylene formation. For this, the results were verified with the application of exogenous ethylene and its action inhibitor in salt stress. The present study showed that SA might be inhibiting stress ethylene, while with S, it may be promoting S-assimilation to enhance optimal ethylene formation and ethylene sensitivity to eventually affect the salt tolerance process.

**Influence of SA and S in the Alleviation of Salt stress**

The phytohormone SA is known to improve photosynthetic functions and growth in abiotic stressed plants (Nazar et al. 2011; Miura and Tada 2014; Gururani et al. 2015; Rasheed et al. 2020). In the present study, 50 mM NaCl-exposed plants supplied with 0.5 mM SA and 2.0 mM SO$_4^{2-}$ had significantly improved maximum chlorophyll content, PS II efficiency, and gas exchange parameters when compared to individual applications of 0.5 mM SA or 2.0 mM SO$_4^{2-}$. The involvement of SA in the synthesis of photosynthetic pigments, increase in PS II efficiency, rate of photosynthesis in abiotic stressed plants has been extensively studied (Khan et al. 2015). Enhancements in the rate of net photosynthesis and PS II efficiency were reported in salinity-exposed *Vigna radiata* supplied with SA alone (0.5 mM) (Nazar et al. 2011). Yoshida and Noguchi (2009) also found SA-mediated enhancements in S uptake and level of GSH in ozone-exposed *Arabidopsis thaliana*. The supplied SA-mediated protection of the photosynthetic machinery in salinity stressed *B. juncea* and *Vigna radiata* was also argued to involve increased ATP-S activity and serine acetyl transferase, and Cys and GSH content and decreased salinity-acquired oxidative stress (Nazar et al. 2015). On the other hand, individual supply of excess-S enhanced GSH production and improved photosynthetic efficiency and growth in salinity-stressed plants in *B. juncea* (Fatma et al. 2021). As observed herein with 0.5 mM SA and 2 mM SO$_4^{2-}$, an increased level of S-containing compounds including Cys and GSH were previously shown associated with higher photosynthesis at varying levels (Wirtz and Droux 2005). Additionally, increased net photosynthesis, up-regulated NR activity, improved chlorophyll content, carboxylation efficiency, normal thylakoid membranes, and light-mediated reactions were reported in cowpea plans grown with low or optimal levels of SA (Moharekar et al. 2003). Regarding the effect of SA and SO$_4^{2-}$ effect on growth, particularly under stress conditions, approaches for improving the plant photosynthetic functions can greatly help in improving plant growth and related parameters. In the present study, the addition of 0.5 mM SA to 50 mM NaCl, together with 2.0 mM SO$_4^{2-}$ maximally restored the salinity-induced loss in the studied herein growth parameters namely plant dry mass and leaf area. The significance of SA in plant growth and development has also received a lot of attention (Rivas-San Vicente and Plasencia 2011; Khan et al. 2015). The supplied SA and SO$_4^{2-}$-mediated improvements in plant dry mass and leaf area can be attributed to the supplied SA and SO$_4^{2-}$-mediated significant reductions in oxidative stress that are mainly involved in salinity-accrued decreases in photosynthesis, and plant growth and development (Munns and Tester 2008). The application of SA with SO$_4^{2-}$ to NaCl-exposed plants increased the uptake of S and N through induced activity of their assimilatory enzymes ATP-S and NR in order to produce high S-containing compounds to be utilized in ROS metabolism and thereby salinity tolerance in the present study. Application of SA and SO$_4^{2-}$-mediated increases in leaf-GSH and antioxidant activity can also be attributed in the photosynthetic functions; and hence, in increased plant dry mass and leaf area. On the other hand, the role of SA and SO$_4^{2-}$-induced antioxidant defense system strengthening, improved S-containing defense metabolites, elevated ROS (H$_2$O$_2$) metabolism, decreased TBARS, and improved photosynthetic functions in SA and SO$_4^{2-}$-mediated improvements in plant growth in terms of leaf area and plant dry mass under salt stress has been widely argued (Khan et al. 2013; Fatma et al. 2016; Hussain et al. 2019, 2021). SA-induced improvement in growth was also considered a result of the supplied SA (0.5 mM)-mediated rise in net photosynthetic rate in salinity-exposed *Torreyra grandis* (Li et al. 2014). Overall, the combined supply of SA and SO$_4^{2-}$ minimized oxidative stress and thereby improved photosynthetic functions and S-containing compounds and eventually improved growth under 50 mM NaCl stress in *B. juncea*.

Plants adopt varied physio-biochemical mechanisms to cope with the consequences caused due to elevated accumulation of oxidative stress. The foliar supply of 0.5 mM SA with 2.0 mM SO$_4^{2-}$ decreased O$_2^•−$, H$_2$O$_2$, and TBARS content in leaves. The observed herein, activity of antioxidant enzymes including APX, GR, and SOD and also the cellular level of non-enzymatic antioxidants including GSH are induced in order to scavenge O$_2^•−$ and H$_2$O$_2$ and have a tight control over lipid peroxidation, tested in this study as the leaf TBARS content. Notably, the 0.5 mM SA application added a significant momentum in the SO$_4^{2-}$-mediated metabolism of O$_2^•−$ and H$_2$O$_2$ and minimized the TBARS through strengthening antioxidant defense system against
salinity stress. Interplay between SA and redox signals in the plant stress defense response has also been reported previously (Khan et al. 2014, 2015). Notably, apart from its signaling function, SA also has an antioxidant role in concert with reduced GSH in plant response to stress (Herrera-Vásquez et al. 2015). Additionally, as observed in the present study where 50 mM NaCl-caused elevation in ROS (O$_2^•−$ and H$_2$O$_2$) levels, the generation of ROS including O$_2^•−$ and H$_2$O$_2$ in cellular organelles such as chloroplasts and peroxisomes triggers the biosynthesis of SA, which has been argued essential for main outputs of the defense responses including the transcriptional reprogramming, cell death, and stomatal closure (Herrera-Vásquez et al. 2015).

In this study, a higher dismutation of O$_2^•−$ resulted in a higher production of H$_2$O$_2$ in plants. Thus, to avoid H$_2$O$_2$-mediated consequences, efficient scavenging of H$_2$O$_2$ in chloroplast needs the parallel initiation of its metabolizing enzymes and other associated components in AsA-GSH pathway. The SA-mediated induction in the activity of enzymes involved in O$_2^•−$ dismutation, H$_2$O$_2$ metabolism, and GSH egeneration been widely reported (Khan et al. 2015). Earlier, the exogenously supplied SA (0.5 mM) increased APX and GR enzymes activities, improved GSH content, and decreased leaf ROS and TBARS levels in salinity-exposed plants (Nazar et al. 2011, 2015). In another report, exogenously applied 0.5 mM SA-mediated up-regulation of the transcriptome level of antioxidant genes of key H$_2$O$_2$-metabolizing enzymes was suggested to protect Triticum aestivum against salinity stress in another study (Li et al. 2013). It is worthy to mention here that there occurs a close relation not only between the plant-GSH but also between GSH/GSSG redox state and with the plant SA-status. To this end, over-accumulation of SA increased GSH levels and also that of the reducing power (GSH/GSSG ratio) (Mateo et al. 2006). Higher GR activity can restore the high ratio of GSH to GSSG, occurring under optimal growth conditions can be restored by means of higher GR activity (Szalai et al. 2009). Earlier, the supply of 0.5 mM SA to 50 mM NaCl-exposed B. juncea brought significant enhancements in H$_2$O$_2$-scavenging APX activity and GSH-regenerating (GR) enzymes (Nazar et al. 2015). Furthermore, increased GSH content was argued to maintain the proper functioning of enzymes of AsA-GSH pathway, and results in the increased activity of GR and APX with the application of 0.5 mM SA under salt stress (Nazar et al. 2011, 2015). Thus, it can be said that in 50 mM NaCl-exposed Pusa Vijay cultivar, exogenously supplied SA-mediated improved plant health involved its role in control of NaCl-acquired oxidative stress via modulating O$_2^•−$-dismutation, H$_2$O$_2$-metabolizing, and GSH-regenerating enzymes; and the pool of GSH and its redox state.

The effects of SA and S in the alleviation of salt stress were attributed to lowering stress ethylene to an appropriate level, and ethylene favorably controlled GSH production via control of AsA-GSH cycle enzyme activity and protected photosynthetic functions (Khan et al. 2016; Sehar et al. 2021). Under salt stress, the greater reduced state (GSH/GSSG) generated as a result of ethylene-induced GSH synthesis safeguarded and enhanced photosynthetic performances and plant development (Sehar et al. 2021).

**Involvement of Ethylene in SA and S-mediated Reversal of Salt stress**

Ever since SA acts as an inhibitor of ethylene and S-assimilation is associated with ethylene synthesis (Khan et al. 2014; Nazar et al. 2015; Fatma et al. 2016). Hence, the effort was made herein to determine the role of ethylene in the coordinated role of SA and SO$_4^{2−}$-mediated salt tolerance. The information of the earlier experiment that SA plus SO$_4^{2−}$ maximally alleviated salt stress was used to find the role of ethylene in such effect. Therefore, for the confirmation of the involvement of ethylene in SA plus SO$_4^{2−}$-mediated reversal of salt stress effect, next experiment was performed, where ethephon was exogenously applied to salt-stressed plants and NBD to SA and S treatment under salt stress. Ethephon supplementation increases ethylene formation that helps in salinity tolerance (Jahan et al. 2021a) and NBD is involved in inhibition of ethylene action. The use of both these ethylene modulators showed that ethylene synthesis and signaling are important regulators in salt tolerance effect by combined SA and SO$_4^{2−}$. Interestingly, the effects of ethephon and salt treatment were found to be significantly equal to the plants receiving salt with SA and SO$_4^{2−}$. However, when SA plus SO$_4^{2−}$ receiving plants under salt stress were treated with NBD, inhibition in photosynthetic characteristics, stomatal behavior and growth occurred was observed. Such plants also showed reduced capacity of S-assimilation, activity of antioxidant enzymes and GSH content emphasizing that ethylene was essential to modulate the antioxidative enzymes and S-assimilation in salt stress and SA plus SO$_4^{2−}$-treated plants.

Involvement of ethylene in salt tolerance has been investigated earlier (Riyazuddin et al. 2020; Sehar et al. 2021). Under salt stress, ethylene has been shown to maintain Na$^+$/K$^+$ homeostasis and increased antioxidants to scavenge ROS. It also influences nutrient uptake and enhances nitrate and sulfate assimilation (Riyazuddin et al. 2020). In Arabidopsis, both ethylene production and its signaling genes are implicated in salt tolerance (Yang and Guo 2018). In B. juncea cultivars differing in photosynthetic capacity, ethylene increased the assimilation of S and N, and improved photosynthesis (Iqbal et al. 2012). Moreover, by binding to the ethylene receptors, NBD inhibited ethylene sensitivity and activity in such plants, resulting in no increase in N- and
S-assimilation or photosynthesis. NBD is one of the major chemical inhibitors of ethylene action (Iqbal et al. 2017).

This study tended to enlighten further the interaction between ethylene, SA, and SO$_4^{2−}$ in salt tolerance. Under stressful conditions, there is a strong relationship between SA and ACC, with SA inhibits ethylene production by restricting the conversion of ACC to ethylene and protecting plants from stress-related effects (Leslie and Romani 1986; Khan et al. 2014; Nazar et al. 2015). S-adenosyl methionine (SAM, ethylene production-precursor) modulates ethylene biosynthesis, and thereby forms a strong relationship between S and ethylene (Masood et al. 2012; Fatma et al. 2016). Thus, interplay between SO$_4^{2−}$, SA, and ethylene was assumed in this experiment in the control of 50 mM NaCl-accrued consequences. With NBD treatment to plants grown with SA and S under salt stress, the ameliorative effect of SA and S was not perceived and reduction occurred in the cysteine and GSH content, together with reduced photosynthesis and growth suggesting ethylene action was necessary was the SA and S effect. Moreover, the application of ethephon under salt stress showed significantly similar result as SA plus SO$_4^{2−}$ under salt stress again proving that ethylene evolved under these treatments (both showed significantly equal ethylene evolution) was responsible for stress alleviation. Ethephon increased the content of GSH, stomatal behavior and photosynthetic characteristics in salt stress plants, which was significantly similar to the effect of SA and SO$_4^{2−}$ combined in salt stress. Masood et al. (2012) have shown that both ethephon and S under cadmium stress showed similar effect on photosynthesis and growth suggesting ethylene involvement in S-mediated effect. In this research the similar results of combined SA and S under salt stress to ethephon plus salt stress showed that the effects of SA and SO$_4^{2−}$ are mediated by ethylene under salt stress. This is because both ethephon or the SA and SO$_4^{2−}$ treatment under salt stress optimized ethylene evolution by decreasing stress ethylene and this optimal ethylene signals for maximum increase in photosynthesis and growth performance.

Give above, it can be emphasized that inhibition of stress ethylene and optimization of ethylene under salt stress with SA plus SO$_4^{2−}$ treatment resulted in maximum benefit in photosynthesis and growth, which could be explained by the effects of SA and S on the formation of ethylene. SA inhibited ethylene production by inhibiting its synthesis (ethylene evolution decreased with SA under salt stress) and S increased GSH synthesis and optimized ethylene formation under salt stress. The ethylene production was its most suitable level on application of SA and S together that induced plants’ responses to S-assimilation, antioxidants and growth. Moreover, it is important to note that it is ethylene action that is mediating the SA and S induced effect on salt alleviation through optimal range of ethylene formed with SA plus SO$_4^{2−}$ and its action regulated the synthesis of GSH and redox state. Thus, this study has shown that ethylene intervenes the effect of SA in the presence of SO$_4^{2−}$ to increase S-assimilation and antioxidants activity, and imparts tolerance to salt in mustard plants. This is the first report on the photosynthetic responses of plants induced by the involvement of ethylene in reversal of salt stress by SA in the presence of S in B. juncea.

**Conclusion**

The supply of SA and SO$_4^{2−}$ improved photosynthetic functions and growth mainly as a result of improved levels of S-containing compounds (Cys and GSH), and eventually improved growth under salt stress. The optimization of ethylene with SA plus SO$_4^{2−}$ resulted in reversal of salt stress effect. Notably, the use of ethephon and NBD involved in modification of ethylene synthesis and action, helped in verifying the involvement of ethylene in SA-induced S-assimilation, antioxidant system, photosynthetic response and tolerance to salt stress in the presence of SO$_4^{2−}$. NBD-treatment decreased S-assimilation capacity and regulation of antioxidants, and thereby reversed the SA plus SO$_4^{2−}$-mediated changes in S-assimilation and photosynthesis. Significantly, similar results obtained with exogenous supplement of salt stressed B. juncea with ethephon alone or SA plus SO$_4^{2−}$, where the inhibition of stress ethylene and formation of optimized ethylene resulted in ethylene sensitivity to enhance photosynthesis of plants.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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