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

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Abstract

The involvement of ethylene in reversal of salt stress inhibited photosynthetic activity and growth by salicylic acid (SA) together with sulfur (S) was studied in mustard (*Brassica juncea* L.) plants. Application of SA (0.5 mM) plus SO_4^{2-} (2.0 mM) improved photosynthetic activity through markedly increased S-assimilation, antioxidant enzymes activity and optimized ethylene and glutathione (GSH) production for reduced reactive oxygen species (ROS) in plants under 50 mM NaCl stress. As 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 SO_4^{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 SO_4^{2-} treatments. The ethephon application to salt treated plants suppressed stress ethylene and optimized ethylene formation and increased ethylene sensitivity to enhance photosynthesis of plants by affecting antioxidative capacity of plants. Application of NBD to plants receiving SA plus SO_4^{2-} in presence of salt showed inhibited photosynthetic characteristics, stomatal behavior and growth. These plants exhibited minimal capacity of S-assimilation and antioxidant enzymes activity and GSH content. This explained that ethylene was involved in the reversal of salt stress by SA plus SO_4^{2-}. Thus, the study showed that ethylene intervenes the effect of SA in the presence of SO_4^{2-} to upregulate the antioxidants that lead to increased S-assimilation, and imparted tolerance to salt in mustard plants.

Introduction

One of the most damaging abiotic stresses has been soil salinity, which has resulted in significant losses in cultivated land area, crop yield, and quality (Yamaguchi and Blumwald 2005; Shahbaz and Ashraf 2013: Fatma et al. 2021; Jahan et al. 2021; Sehar et al. 2021; Syeed et al. 2021). It has been estimated that the rising salinization of world cultivable lands at an annual rate of 10% per year would result into salinization of more than half of all arable lands by 2050 (Jamil et al. 2011). Plants exposed to salinity have shown reduced growth and development due to oxidative stress caused by the production of reactive oxygen species (ROS) (Isayenkov and Maathuis 2019). On the other, stressed plants tend to build up their antioxidant defense system to control the cellular ROS-level and minimize ROS-accrued consequences including lipid and protein oxidation. Major antioxidant enzymes (such as ascorbate peroxidase, APX; catalase, CAT) and non-enzymatic antioxidants (such as ascorbate, AsA; reduced glutathione, GSH; and *α*-tocopherol, flavonoids and proline), alone and/or cumulatively scavenge varied ROS (Sharma and Dietz 2009; Ashraf 2009; Mittal et al. 2012).

Markedly, major phytohormones, mineral nutrients, and/or their interaction outcomes have been widely reported to significantly modulate and improve the plant's defense system against stress impacts (Khan et al. 2015; Jahan et al. 2020). Sulfur (S) is one of the most important nutrient elements for plants, and has been the subject in plant and agricultural study. 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, thioredoxin system and GSH, a major antioxidant metabolite and non-protein thiol (Fatma et al. 2013; Anjum et al. 2015). Applied S improved 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, restricting ROS formation, and thus, minimizing oxidative stress and its consequences (Fatma et al. 2021).

Salicylic acid (SA), a natural phenolic compound has been identified to control the array of growth, physiological and developmental processes. Salicylic acid also works as signaling response directly or indirectly against different stresses improving photosynthetic functions, nutrient-uptake and assimilation, proline metabolism, plant water relations, modifying antioxidant defense systems and nullifying ROS and their consequences (Iqbal et al. 2015; Tari et al. 2015; Rasheed et al. 2020). Through signaling cross-talks with S-assimilation, there is a close relationship between SA and S, where SA can govern numerous elements of plant responses in both stressful and optimal conditions (Khan et al. 2015, Hussain et al. 2021). However, it is also remarkably stated that SA acts as an inhibitor of ethylene (Ahmed et al. 2020) and S-assimilation is associated with ethylene (Masood et al. 2016). Ethylene, as a signaling molecule, acts as a major modulator of plant stress responses by influencing the ROS production (Cao et al. 2013; Tao et al. 2015; Khan et al. 2016; Jahan et al. 2021; Sehar et al. 2021). It has previously been observed that ethylene supplementation increases the activity of enzymatic and non-enzymatic antioxidants, which act as a first line of defense against abiotic stress (Asgher et al. 2014; Khan et al. 2016; Zhang et al. 2016). Recently, we have shown that ethylene protects pigment system II activity and photosynthesis in salt stress by inducing the expression of *psb*A and *psb*B and the production of GSH in wheat (Sehar et al. 2021), and by optimization of proline metabolism and antioxidant system in mustard (Jahan et al. 2021) According to the report of Khan et al. (2015), SA-supplemented plant reduced ethylene generation in heat-exposed plants by decreasing the 1-aminocyclopropane carboxylic acid (ACC) and ACC synthase (ACS) activity to an appropriate range. But the information on how ethylene is involved in SA and S-mediated salt tolerance is scanty in the literature.

As a widely cultivated species for oilseed, Indian mustard (*Brassica juncea* L.) stands second in world oilseed production (USDA 2018). It is cultivated mainly in the North-Western agro-climatic region of India and suffers huge losses in productivity mainly due to the salinization of the cultivable land (Yousuf et al. 2016). Therefore, in order to sustain optimum growth and produce more in saline environments, it is critical to investigate the salt tolerance mechanisms in *B. juncea*. Thus, the triad comprising SA, S and ethylene is hypothesized herein to influence the response of *B. juncea* to salinity and helps this crop to protect growth, metabolism and photosynthetic functions, ROS-metabolism and strengthening antioxidant defense system against salinity stress-impacts. However, information on how ethylene gets involved in SA-mediated effect on salinity in the presence of S to in oilseeds such as *B. juncea* has remained elusive.

Materials And Methods

Plant Culture and Treatments

Experiments were performed at the Department of Botany, Aligarh Muslim University, Aligarh, India. Healthy seeds of *Brassica juncea* L. Czern & Coss. cultivar Pusa Vijay were undergone surface sterilization for 15 min with 0.01% $HgCl_2$ followed by washings for five times through double distilled water. Sterilized seeds were sown in a diameter of 23-cm 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 ± 3ºC and 12 ± 2ºC, 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 SO_4^{2-} 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 SO_4^{2-}) and SA (0.5 mM) were supplied at 20 DAS. Salicylic acid was introduced to water after being dissolved in 100% ethanol and then added drop by drop (ethanol/water: 1/1000 *v/v*). Plants cultivated 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 test plant on each pot; and a surfactant teepol (0.5%) was mixed with the control and SA treatment solution. Hoagland solution (250 mL) was added to the control plants (Hewitt 1966) every other day and 200 mL of double distilled water daily. Sulfur was provided in the form of $MgSO_4$ for obtaining 2.0 mM SO_4^{2-} concentrations, and Mg^{2+} was maintained at 2.0 mM in all treatments, including control, by the addition of appropriate $MgCl_2$.

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 concentration of ethephon and NBD has been 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 CO_2 concentration were evaluated in completely expanded topmost second leaves of plants in every treatment around 11.00 to 12.00 at day light saturating intensity using Infra-Red Gas Analyzer (CID-340, Photosynthesis system, Bio-Science, USA). At the time of measurement, the atmospheric conditions were as follows: photosynthetically active radiation, ~680 μmolm^{-2} s^{-1}; air temperature, ~22ºC and relative humidity, ~70%.

Chlorophyll content in the leaves of plants taken from every treatment was determined using a SPAD chlorophyll meter (SPAD 502 DL PLUS, Spectrum Technologies).

Using a chlorophyll fluorometer, the maximal PSII photochemical efficiency (Fv/Fm) of the fully expanded second leaf from the top of the plant was calculated (Junior-PAM, Heinz Walz, Germany). The details are given in Supplementary file S1.

Leaf area meter (LA 211; Systronics, Hyderabad, India) was used to determine the leaf area. 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 Biomarkers

Okuda et al. (1991) approach was used to determine H_2O_2 content (1991). 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 leaf tissues (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 minutes at 4°C. After centrifugation, the supernatant was collected and activity of superoxide dismutase (SOD) and glutathione reductase (GR) was measured. For the measurement of ascorbate peroxidase (APX), the extraction buffer was supplemented with 2 mM AsA.

Giannopolitis and Ries (1977) and Beyer and Fridovich (1987) methods were used for SOD (EC 1.15.1.1) by studying the suppression of photochemical reduction of nitro blue tetrazolium (NBT). Activity of APX (EC 1.11.1.11) was measured 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 (EC 1.6.4.2). Supplementary File S1 contains the details of the procedure.

Ethylene Evolution

Ethylene evolution in leaves was assessed using gas chromatograph by the process defined previously by Fatma et al. (2021).

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 determination of content of S, Cys and GSH was done by methods of turbimetric, Gaitonde (1967) and

Griffith (1980), respectively described by Fatma et al. (2014). Supplementary File S1 contains the details of these procedures. 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 evaluated by Kuo et al. (1982 as described by Iqbal et al. (2012). 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 H_2O_2

To visualize the presence of superoxide and H_2O_2 in the test leaf samples, histochemical staining techniques using nitro blue tetrazolium (NBT) and 3, 3-diaminobenzidine (DAB) were utilized (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).

Scanning and Transmission Electron Microscopy

The technique of Daud et al. (2009) and Sandalio et al. (2001) with small modifications was used to prepare leaf samples for scanning and transmission electron microscopy, respectively. File S1 contains the details of these procedures.

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 $p \leq 0.05$. The results are not statistically different by LSD test at $p \leq 0.05$, if bars showing the same letter.

Results

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

Photosynthetic characteristics decreased under salt stress in plants, but the application of 0.5 mM SA or 2.0 mM SO_4^{2-} increased the photosynthetic characteristics. Under no stress, the plants treated with 0.5 mM SA or 2.0 mM SO_4^{2-} exhibited almost equal increase of ~30.3, 37.2, 34.8 and 30.7 in net photosynthesis, intercellular CO_2 concentration, stomatal conductance and chlorophyll content, respectively compared with the control. In plants 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 CO_2 concentration, stomatal conductance and chlorophyll content, respectively compared with salt treatment but lesser than the treatment of 2.0 mM SO_4^{2-} under stress. The combined supplementation of 0.5 mM SA and 2.0 mM SO_4^{2-} maximally increased net photosynthesis, intercellular CO_2 concentration, stomatal conductance and chlorophyll content under no stress or salt stress compared to 50 mM NaCl treatment (Table 1). PSII activity response was also similar to these treatments. Maximum increase of 18.9 % in PSII activity was noted with combined application of 0.5 mM SA plus 2.0 mM SO_4^{2-} to the salt grown plants 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.

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_2O_2 and TBARS content in non-stressed plants and plants grown with salt. Under no stress, SA decreased content of H_2O_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_2O_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_2O_2 by 37.2% and TBARS by 34.0% in comparison with control. The maximum decrease in H_2O_2 and TBARS content was obtained with the application of SA with SO_4^{2-} together under 50 mM NaCl which decreased H_2O_2 and TBARS content by 50.2% compared with control (Table 2).

Salt, S or SA independently increased activity of antioxidant enzymes. As 0.5 mM SA was applied to 50 mM NaCl-treated plants, the activity of SOD, APX, and GR increased by 68.5, 59.6, and 41.8%, respectively, when compared to the control. The activity of SOD, APX and GR increased by 81.4, 71.9, and 52.5%, respectively in plants grown with salt and SO_4^{2-} 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 37.9, 23.5 and 42.5% under salt stress compared with the respective control. 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 86.0, 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 to Visualize the Presence of Superoxide and H_2O_2 through Histochemical Staining Method

To ascertain the cellular status of oxidative stress markers $O_2^{\bullet-}$ and H_2O_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_2O_2 and $O_2^{\bullet-}$ 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_2O_2 and $O_2^{\bullet-}$, 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). Micrographs showed that under no stress, chloroplasts had a normal shape with well-organized thylakoid systems (Fig. 3A), whereas disorganized thylakoids were seen in the treatment of 50 mM NaCl (Fig. 3B). The chloroplast

ultrastructure of the plants receiving SA plus SO_4^{2-} to NaCl-treated plants was significantly altered. As seen in Fig. 3C, chloroplast had a regular form with well-organized thylakoid systems and a significantly higher number of thylakoid stacks.

Effect of SA and S on Ethylene Evolution

Ethylene evolution decreased with SA, SO_4^{2-} or their combination in salt stress. We observed maximum evolution of ethylene under salt stress which was 3.47 times compared to control. When SA or SO_4^{2-} was supplemented to salt stressed plants, we observed decrease in ethylene evolution. 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 compare 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 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 as 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-}. Treatment 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. Moreover, application of ethephon to salt treated or application of SA plus SO_4^{2-} to salt treated plants were equally effective in reducing the H_2O_2 content by 53.7 or 54.6% respectively, in comparison with control. Contrarily, the H_2O_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 aperture 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 as shown in the Fig. 5. The image of the stomatal aperture was apparently visible under different treatments when SEM was done. Leaf samples under control conditions had normal stomata with the characteristic guard cells having stomatal aperture diameter of 6 µM (Fig. 5 A-B), whereas the impact of salt stress on stomatal closure was clearly seen as the diameter of the stomatal aperture was of 1 µM (Fig. 5 C-D). The stomatal aperture improved with SA plus SO_4^{2-} in presence of NaCl with opened stomatal aperture by 13 µM (Fig.

5 E-F). The application of ethephon counteracted the effects of NaCl for stomatal aperture (Fig. 5 G-H). However, stomatal aperture reduced in presence of NBD by closing the stomatal aperture size by 3 µM (Fig. 5 I-J),

We observed in our study that individually both SA and S in the form of SO_4^{2-} are 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 stress ethylene formation while 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 to modulate both its synthesis and signaling. In the first experiment addition of SA or SO_4^{2-} to salt stressed plants reduced ethylene evolution compared to salt stress. Compared to 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 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 treatment 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 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 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 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 SO_4^{2-} under 50 mM NaCl (Table 5). Plants receiving salt with SA and SO_4^{2-} showed optimum ethylene evolution by 24.10% respectively compared to control which was reversed with NBD (Fig. 6). Thus, there exists a cross-talk 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 physiological and molecular mechanism of plants and affects S assimilation in 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 stress, S and N assimilation, antioxidant metabolism and photosynthetic characteristic 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 S promoted S-assimilation to enhance optimal ethylene formation and ethylene sensitivity to affect the salt tolerance process.

Influence of SA and S in the Alleviation of Salt stress

The role of SA in improving photosynthetic functions and growth in abiotic stressed plants has been widely reported (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 applied 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. 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 SA (0.5 mM) supplied *Vigna radiata* under salinity exposure (Nazar et al. 2011). Fatma et al. (2021) have shown that excess-S enhanced GSH production and improved photosynthetic efficiency and growth in salinity stressed plants in *B. juncea.* Yoshida and Noguchi (2009) also stated 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-accrued oxidative stress (Nazar et al. 2015). An increased level of S-containing molecules such as Cys and GSH were previously shown associated with higher photosynthesis at varying levels, as seen here with 0.5 mM SA and 2.0 mM SO_4^{2-} (Wirtz and Droux 2005). Additionally, cowpea plans grown with low or optimal levels of SA showed increased net photosynthesis, up-regulated NR activity, improved chlorophyll content, carboxylation efficiency, normal thylakoid membranes, and light mediated reactions (Moharekar et al. 2003). Regarding SA and SO_4^{2-} effect on growth, particularly in stress conditions, approaches for improving the plant photosynthetic functions can greatly help in improving plant growth and related parameters. The addition of 0.5 mM SA to 50 mM NaCl, together with 2.0 mM SO_4^{2-} restored the salinity-induced loss in plant dry mass and leaf area in the present study. 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 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_2O_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). Li et al. (2014) reported SA-induced growth was mainly because of the supplied SA (0.5 mM)-mediated rise in net photosynthetic rate in salinity-exposed *Torreya grandis*. 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. In leaves of the Pusa Vijay cultivar of *B. juncea*, foliar supply of 0.5 mM SA with 2.0 mM SO_4^{2-} decreased $O^{2\bullet}$, H_2O_2 and TBARS content. The observed herein, the activity of antioxidants enzymes including APX, GR and SOD and also the cellular level of non-enzymatic antioxidants including GSH are induced in order to scavenge $O_2^{\bullet-}$ and H_2O_2 and have a tight control over lipid peroxidation (tested herein as the leaf TBARS content). Notably, the 0.5 mM SA application added a significant input in the SO_4^{2-}-mediated metabolism of $O_2^{\bullet-}$ and H_2O_2 and minimized the TBARS through strengthening antioxidant defense system against salinity stress. It has also been previously demonstrated that SA and redox signals interact in the plant stress defense response (Khan et al. 2014, 2015). Apart from its signaling function, SA also has an antioxidant role in the stress response when combined with reduced GSH (Herrera-Vásquez et al. 2015). In this study, a higher dismutation of $O_2^{\bullet-}$ resulted in a higher production of H_2O_2 in plants. Thus, to avoid H_2O_2-mediated consequences, efficient scavenging of H_2O_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 $O_2^{\bullet-}$-dismutation, H_2O_2-metabolism and GSH-regeneration 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). Exogenously applied 0.5 mM SA-mediated up-regulation of the transcriptome level of antioxidant genes of key H_2O_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 decreasing control (GSH/GSSG ratio) (Mateo et al. 2006). Higher GR activity can restore the high ratio of GSH to GSSG that occurs under optimal growth conditions (Szalai et al. 2009). Earlier, the supply of 0.5 mM SA to 50 mM

NaCl-exposed *B. juncea* brought significant enhancements in H_2O_2-scavenging APX activity and GSH-regenerating (GR) enzymes (Nazar et al. 2015). Furthermore, increased GSH content maintained the appropriate functioning of AsA-GSH pathway enzymes, resulting in higher GR and APX activity when 0.5 mM SA was applied under salt stress (Nazar et al. 2011, 2015). Thus, it can be said that exogenously supplied SA-mediated plant health involved the control of NaCl-accrued oxidative stress via modulating $O_2^{\bullet-}$-dismutation, H_2O_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 ascorbate-glutathione 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), as a result, the efforts of the present study were to determine the role of ethylene in the coordinated role of SA and SO_4^{2-}mediated salt tolerance. Ethephon supplementation increases ethylene formation that helps in salinity tolerance (Jahan et al. 2021) 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.

Ethylene's involvement in salt tolerance has been investigated (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 sulphate assimilation (Riyazuddin et al. 2020). In *Arabidopsis*, both ethylene production and its signaling genes are implicated in salt tolerance (Yang and Guo 2018, Fricke 2004). Iqbal et al. (2012) reported the role of ethylene in increasing S and N-assimilation and thus photosynthesis in *B. juncea* cultivars differing in photosynthetic capacity. 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).

We tried to investigate 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) impacts ethylene biosynthesis, forming 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. 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. Thus, 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. This could be explained by the SA and S effect on ethylene formation. 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, the 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 mustard.

Conclusion

Conclusively, the optimization of ethylene with SA plus SO_4^{2-} resulted in reversal of salt stress effect. 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-} was verified by using chemicals that modify ethylene synthesis and action, ethephon and NBD. Application of SA plus SO_4^{2-} on S-assimilation and photosynthesis were reversed with NBD due to the decrease of S-assimilation capacity and regulation of

antioxidants. However, plants receiving exogenous ethephon to salt or SA plus SO_4^{2-} under salt stress showed significantly similar results and showed that the inhibition of stress ethylene and formation of optimized ethylene resulted in ethylene sensitivity to enhance photosynthesis of plants. The supply of SA and SO_4^{2-} improved photosynthetic functions and S-containing compounds (Cys and GSH), and eventually improved growth under salt stress. Considering the involvement of ethylene by SA in the presence of S, it would be very imperative to explore more biochemical and molecular insights into the metabolic interrelationships among the major components of SA-biosynthesis enzyme (benzoicacid 2-hydroxylase; cinnamate-4-hydroxylase (C4H); phenylalanine ammonia lyase); S-assimilation (mainly the activity of ATP-S and serine acetyl transferase); enzymes involved in GSH synthesis (such as γ-ECS and GSHS); and ethylene actions (SAM, ACC and ACS) in abiotic stressed crop plants.

Declarations

Conflict of Interest: There is no conflict of interest.

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Figures

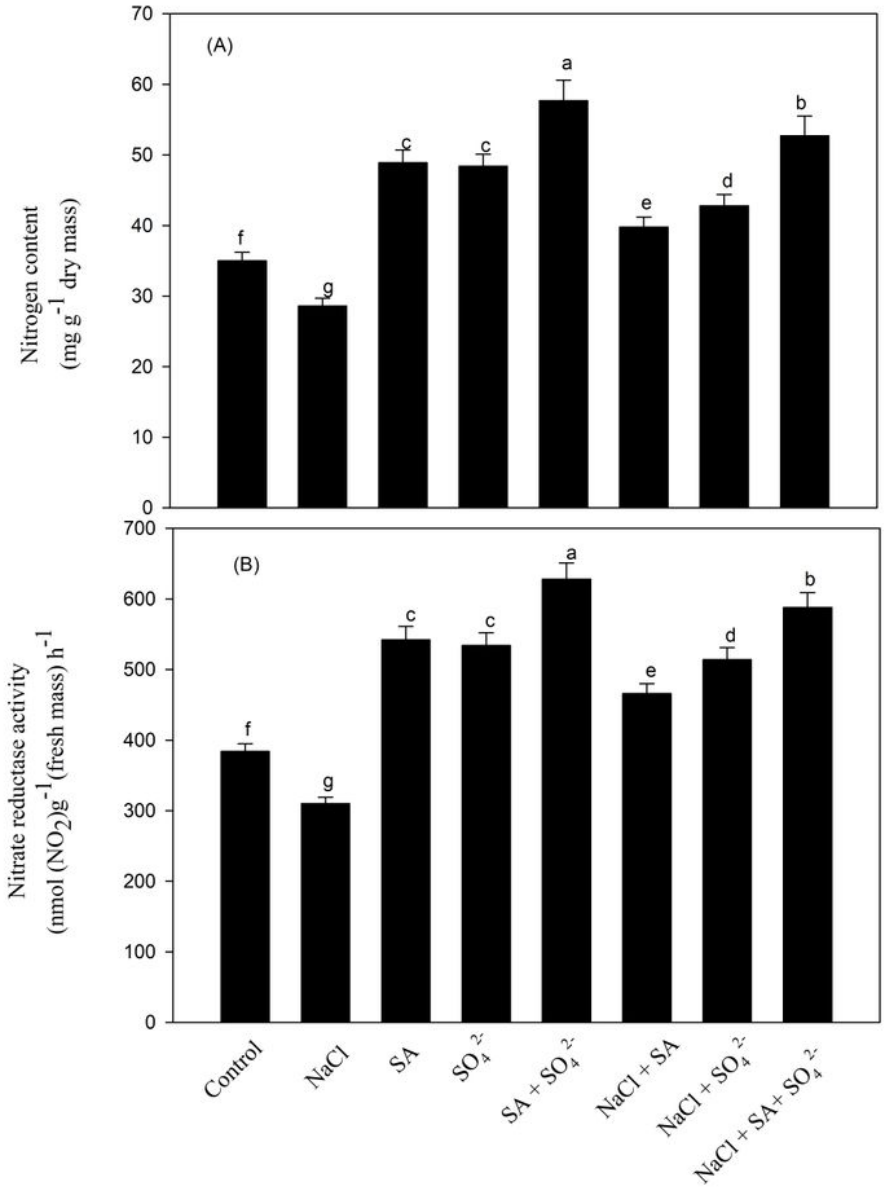

Figure 1

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 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 ($p < 0.05$). SA, salicylic acid.

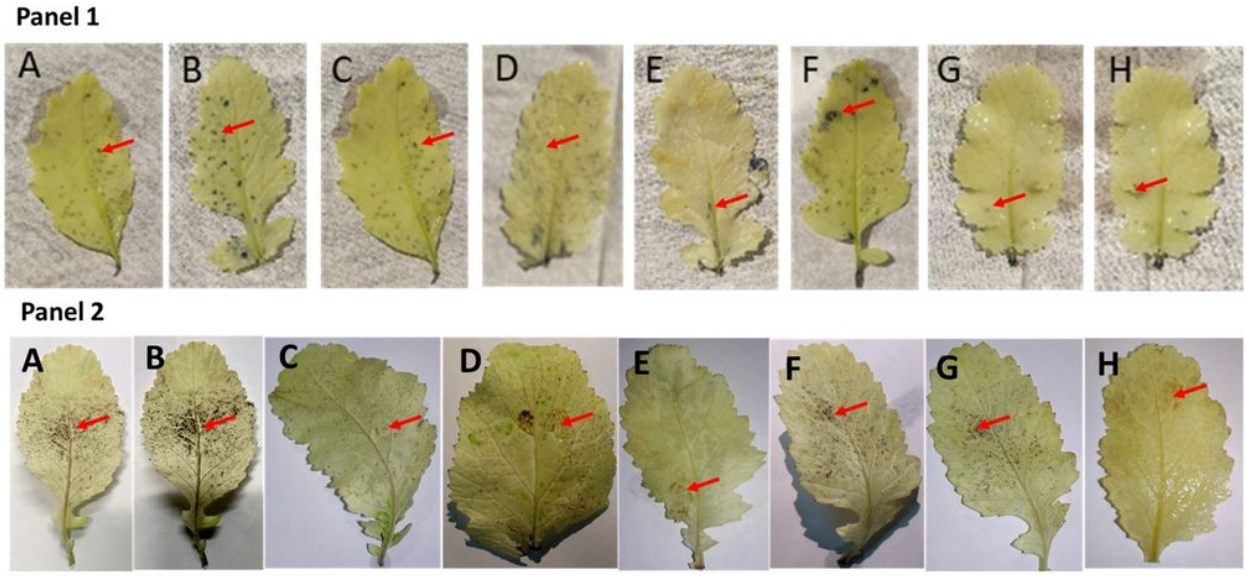

Figure 2

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). Data are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at ($p < 0.05$). DAB, 3, 3-diaminobenzidine; NBT, nitro blue tetrazolium; SA, salicylic acid.

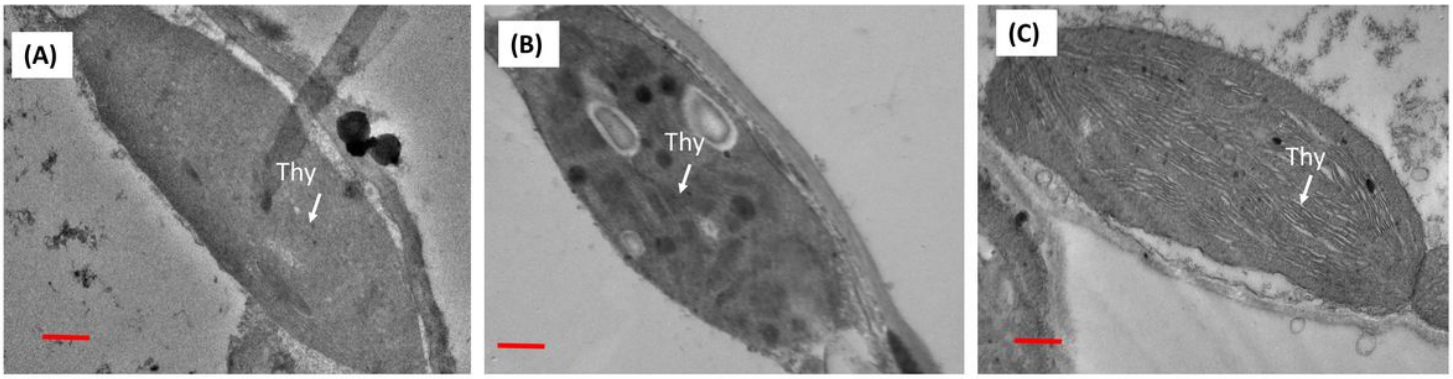

Figure 3

Ultrastructure of chloroplasts in mustard (Brassica juncea L.) leaves using transmission electron microscopy at a magnification of 6000x under control (A) 50 mM NaCl (B) and 0.5 mM SA plus 2.0 mM SO42- with 50 mM NaCl (C) at 30 d after sowing. Bars (A–C) = 500 nm. Thy = thylakoid membranes. SA, salicylic acid.

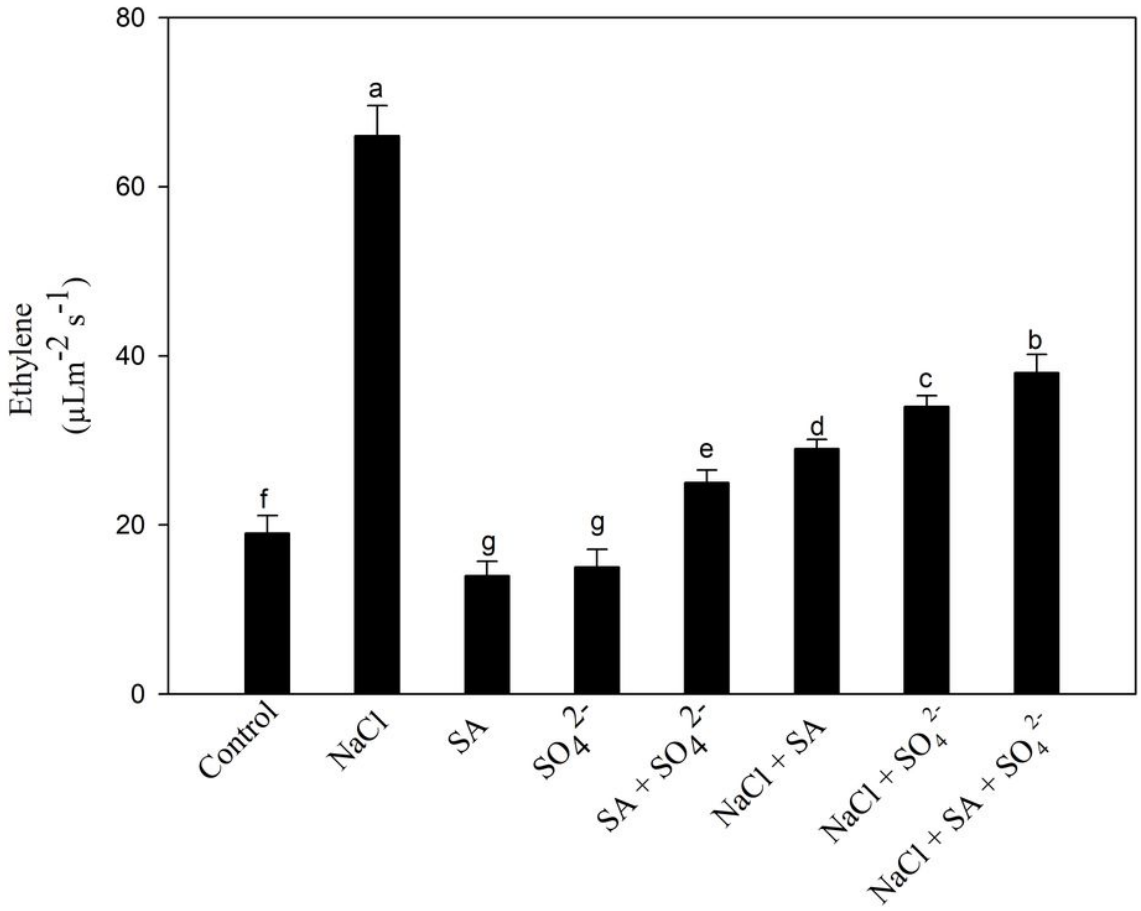

Figure 4

Ethylene evolution in mustard (Brassica juncea L.) leaves at 30 days after sowing. 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 ($p < 0.05$). SA, salicylic acid.

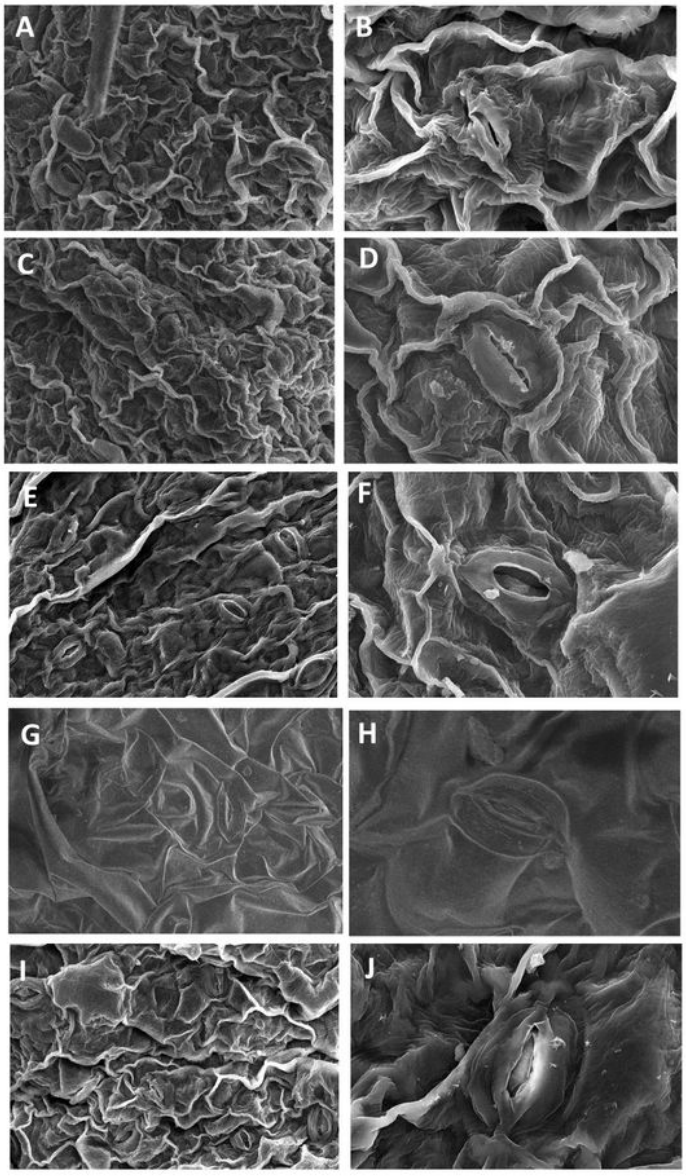

Figure 5

Stomatal behavior of mustard leaves (A, B) under control, (C, D) 50 mM NaCl, (E, F) 0.5 mM SA plus 2.0 mM SO42- 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 SO42- 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.

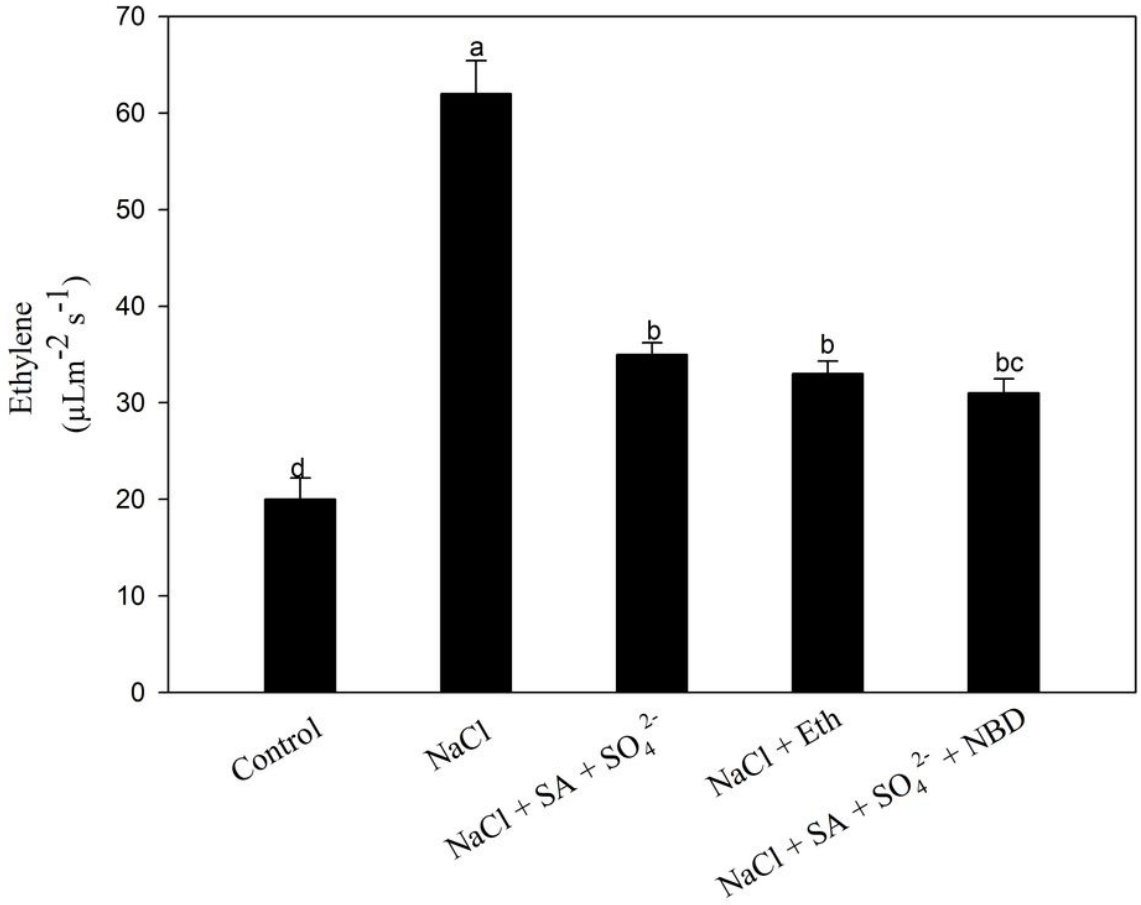

Figure 6

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 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 SO42- with 50 mM NaCl. Data are presented as means ± SE (n = 4). 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.