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Article

Ethylene Supplementation Combined with Split Application of Nitrogen and Sulfur Protects Salt-Inhibited Photosynthesis through Optimization of Proline Metabolism and Antioxidant System in Mustard (*Brassica juncea* L.)

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Abstract: In the present study, the potential of ethylene as ethephon (an ethylene source) was investigated individually or with a combination of the split dosage of nitrogen (N) and sulfur (S) soil treatments for the removal of damaging effects of salt stress (100 mM NaCl) in mustard (*Brassica juncea* L.). Plants were grown with 50 mg N plus 50 mg S kg^{-1} soil at sowing time and an equivalent dosage at 20 days after sowing ($[N50 + S50]_{0d}$ + $[N50 + S50]_{20d}$). Ethephon at 200 µL L^{-1} was applied to combined split dosage of N and S with or without NaCl. Plants subjected to NaCl showed a deceased in growth and photosynthetic characteristics as well as N and S assimilation, though, proline metabolism and antioxidants increased. The application of ethephon to plants grown with split N and S dosages significantly enhanced the photosynthetic efficiency by increasing the assimilation of N and S, improving the content of proline and induction of the antioxidant system with or without NaCl. The regulation of ethylene and/or split form N and S application may be the potential tools for overcoming salt stress effects in this species and in related Brassicaceae.

Keywords: antioxidants; *Brassica juncea;* ethylene; nitrogen assimilation; proline metabolism; sulfur assimilation.

1. Introduction

A remarkable increase in the population at the global level, combined with speedy industrialization in emergent countries, caused issues for global food and energy needs. According to the United Nations and Food and Agricultural Organization, the world population will extent up to 9.7 billion in 2050, and will face extreme challenges at various fronts, among which attaining food security is a high-priority issue [1]. Climate change can be considered as the foremost and exacerbating topic for production of crop as it is associated with harsh environmental states, as drought, salt, low and high temperature, flooding, UV radiation, and toxicity of heavy metal occurring consecutively and concurrently. These are the major factors limiting enhanced agricultural yield in nearly all areas of the world [2]. This circumstance is predicted to worsen with the increase in

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climate in the upcoming years and is an enormous challenge for the food security at the global level [3].

Among various abiotic stress factors, salt stress is one of the important problems worldwide that limits the reliable crop production and the security of food globally [4]. Salt stress reduces about 27.5 billion USD and affects an area of approximately 936 Mha yearly nationwide [5,6]. It induces different physio-biochemical abnormalities, toxic ions uptake, as sodium (Na^+) and chloride (Cl^-), unbalances essential nutrients including dual hyperosmotic effects, disturbs the homeostasis of water that declines plant growth rate and yield productivity [7,8]. Additionally, salt stress damages cellular membranes through the reactive oxygen species (ROS) accumulation. The resulting declination of molecular O_2 to H_2O yields the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) which are toxic and reactive [9,10]. The ROS arrangement actuated by salt stress may prompt oxidation of proteins and layer lipids, or may cause DNA damage in different cellular compartments [11]. Therefore, tissues harmed by oxidative stress augmented carbonylated proteins and malondialdehyde (MDA) content [12].

To lessen the damaging effects of salt stress, plants developed several processes which involved antioxidant defense systems, compartmentation of ions, and amended osmolytes accumulation [7,8]. The assimilation of antioxidant enzymes works as an observable defense properties in the stressful condition that interacts with ROS detoxification [7,10,13]. The antioxidant defense system also helps in the resistance of numerous stress and transport of amino acid across the membranes, alongside satisfying different works involved in redox sensing and signaling, then it affords a protection from salt stress. At the present time, for strengthening tolerance mechanisms against salt stress, various systems are being tried and embraced, including traditional and biotechnological and regular methodologies [14]. The most recent couple of years have focused on mineral nutrients supplements and plant development controllers for improving the development and yield execution of plants under salt stress environments [14,15].

Being static, plants as immovable organisms sense the stressors in their environment and respond to them by committed stress response pathways. Nitrogen (N) and sulfur (S) are involved in a crucial role in several abiotic and biotic stresses tolerance through the production of osmolytes, plant hormones and non-enzymatic and enzymatic antioxidants [7,16]. Nitrogen and S are intricate in the biosynthesis of essential organic complexes, comprising protein, amino acids, nucleic acids and several other cellular components [17-19]. In addition, S-cointaining metabolites, including a large range of crucial metabolites as thiols, reduced glutathione (GSH), amino acids; cysteine (Cys) and methionine (Met), have played an important part in the tolerance of salt [7,20]. Besides, the assimilatory pathways of N and S have been considered practically merged, and all around composed, as the accessibility of one component controls the other [7,21,22].

The use of phytohormones is one of the best pragmatic approaches for coping with salt stress and has also shown in various studies with success in alleviating the toxicity of salt stress [7,8,23]. The gaseous phytohormone ethylene (ET) is a stress-responsive hormone and functions as an important contributor to plant development and growth under abiotic stress conditions [13,24,25]. Ethylene has a fundamental role in salt stress

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responses through the Na^+ and K^+ ions uptake and accumulation and the control of osmotic stress [26]. Iqbal et al. [27] have reported that ET stimulated the stomatal response, permitting further entry of CO_2 for carboxylation, as well as enhanced photosynthesis. Moreover, Lin et al. [28] showed that ET decreases ROS accumulation induced by high salinity degrees and ultimately enhances plant tolerance to excess salt. Ethylene increases the assimilation of N and controls proline synthesis in plants in both optimal or stress environments [27,29,30]. Asgher et al. [31] have explained the role of ET in the restoration of chromium (Cr)-mediated reduction in photosynthesis through increased S and N-assimilation in *Brassica juncea*. A recent study by Fatma et al. [26] showed that the supplementation of ET and S regulates ABA content, antioxidant system and enables the responses of stomata, chloroplast ultrastructure, and photosynthetic characteristics in *B. juncea* experiencing salt stress.

Given that studies on N or S assimilation and salt stress tolerance revealed that these phenomena are critically linked, it is essential to identify and to analyze how much N or S assimilation is induced by phytohormones or vice versa to reduce the injurious action of salt stress in plants. The understanding of the role ethylene in regulating the assimilation of N or S can give more insight on how these elements can induce salt tolerance. In addition, *B. juncea* is a high N- and S-requiring species, very responsive to phytohormones [32], and it is a promising plant for phytoremediation purposes [33]. The soil availability of N and S may not be as beneficial to plants and alleviating salt stress as N and S availablility at changed growth time duration of plants. The N and S available at diverse plant growth phases may also have chance to be utilized fully by higher N and S assimilation induced by ethylene benefitting maximally for salt stress tolerance. On this basis, the present work provides a comprehensive investigation about the regulation of salt tolerance by split N and S application through biochemical and physiological processes, highlighting the prominent function of ethylene.

2. Materials and Methods

2.1. Plant Material, Growth Environments and Treatments

Experiment was set up on mustard (*Brassica juncea* L. Czern & Coss. var. Pusa Tarak) at the Botany Department, Aligarh Muslim University, Aligarh, India. The seeds were sterilized using $HgCl_2$ and washed by distilled water and then grown in the pots of 23-cm diameter that was full by 5 kg soil with compost and peat in the ratio of 1: 4 (w/w), and filled with sand at ratio 3:1 (w/w). Environmental conditions for growing plants in pots were: natural day/night conditions, 640 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). The day/night temperatures were at 24/18 °C (± 3 °C) and the relative humidity was at 68 ± 5%. In each pot, four plants were kept. In the experiment, ammonium sulphate was the source of N and S. The soil used for growing plants had available 100 mg each of N and S kg^{-1} soil which served as control. Supplementation of N or S was done in split dosage as 50 mg N kg^{-1} soil (N50) plus 50 mg S kg^{-1} (S50) provided at sowing time (0 days) and the similar dose at 20 days after sowing (DAS) [$(N50 + S50)_{0d}$ + $(N50 + S50)_{20d}$]. Salt stress application was carried out at alternative days at the concentration of 100 mM NaCl for 15 DAS. An amount of 100 mL each of NaCl and distilled water was given alternately for 15

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days. For evaluating the role of ethylene in salt stress alleviation through regulation of the assimilation of N and S, proline metabolism and antioxidant system, 200 µL L^{-1} ethephon (Eth; 2-chloroethyl phosphonic acid) as ethylene source was sprayed on the foliage of control and split N and S receiving plants at 20 DAS. A surface active agent teepol (0.5%; v/v) was mixed in the treatment of control and ethephon. The treatment were set in a totally randomized square plan. The number of duplicates for every treatment was four (n = 4). At 40 DAS, determination of different plant sampled were done.

2.2. Oxidative Stress

2.2.1. H_2O_2 Content and Lipid Peroxidation

Oxidative stress level as content of H_2O_2 and TBARS was done by the technique of Okuda et al. [34] and Dhindsa et al. [35].

2.3. Histochemical Staining

The generation of $O_2^{\bullet-}$ level was analyzed by histochemical staining process with nitro-blue tetrazolium chloride (NBT), and the technique of Wang et al. [36] was used to stain the leaves.

2.4. Nitrogen Assimilation

2.4.1. Activity of Nitrate Reductase and Nitrogen Content

The technique of Kuo et al. [37] was used for the leaf nitrate reductase activity and N content in leaves was computed by the Kjeldahl digestion process as defined by Lindner [38].

2.5. Sulfur Assimilation

2.5.1. ATP-sulphurilase Activity and Sulfur Content

Activity of ATP-sulphurylase was examined by the process of Lappartient and Touraine [39]. Turbidimetric procedure of Chesnin and Yien [40] was used for S content.

2.5.2. Cysteine Content

Leaves Cys was resoluted by the method of Gaitonde [41].

2.5.3. Glutathione Content and Redox State

Reduced glutathione (GSH) was evaluated by Anderson [42]. The GSH to GSSG ratio was computed for redox state.

2.6. Ativities of Antioxidant Enzymes

The antioxidant enzymes as superoxide dismutase (SOD) catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) was amplified by the process of Beyer and Fridovich [43] and Giannopolitis and Ries [44], Aebi [45], Nakano and Asada [46] and Foyer and Halliwell [47], respectively.

2.7 Proline Metabolism

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2.7.1. Estimation of Proline Content

Proline content in leaf was estimated by accepting the ninhydrin process of Bates et al. [48].

2.7.2. Determination Of Glutamyl Kinase and Proline Oxidase Activities

The activity of glutamyl kinase and proline oxidase was examined after the methods of Hayzer and Leisinger [49] and Huang and Cavalieri [50], respectively.

2.8 Ethylene Metabolism

2.8.1. ACS Activity

1-aminocyclopropane-1-carboxylic acid synthase activity was done by the process of Avni et al. [51] and of Woeste et al. [52].

2.8.2. Ethylene

Ethylene evolution was as done previously by Fatma et al. [25].

2.9 Photosynthetic and Growth Characteristics

Photosynthetic characteristics were examined in fully extended plant leaves by using the Infrared Gas Analyzer (CID-340, Photosynthesis system, Bio-Science, USA) at light saturating intensity on a mid of the day (PAR; 720 μmol m^{-2} s^{-1}) and at 390 ± 15 μmol mol^{-1} atmospheric CO_2.

The details of the information about the maximal PS II photochemical efficiency chlorophyll content, leaf area and plant dry weight have been given in Fatma et al. [25].

2.10. Electron Microscopy

2.10.1. Scanning Electron Microscopy

The technique of scanning electron microscopy (SEM) in leaves was worked out by the process of Daud et al. [53].

2.11. Statistical Analysis

Statistics was done on data using analysis of variance (ANOVA) by SPSS (ver. 17.0 Inc., USA) for Windows and presented as means ± standard error (SE). The treatments had four sets (n = 4). The least significant difference (LSD) was calculated for the significant data at $p < 0.05$. Bars showing the same letter were not significantly different by LSD test at $p < 0.05$.

3. Results

3.1. Oxidative Stress

Exposure of plants to NaCl showed larger H_2O_2 and TBARS content, compared to the respective control values. Exogenous ethephon to plants grown under no stress

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reduced TBARS and H_2O_2 by 21.9 and 35.0%, compared with control plants. The decrease in TBARS and H_2O_2 content was prominent in plants obtaining split dosage of N and S at $(N50 + S50)_{0d}$ + $(N50 + S50)_{20d}$ with ethephon in the absence and presence of salt, compared to the respective control plants which had available N100 + S100 (Figure 1).

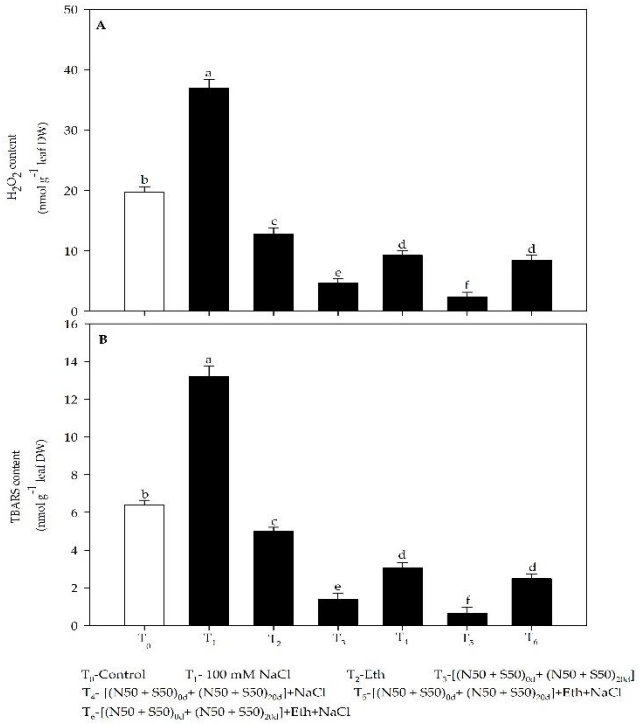

Figure 1. Content of (A) H_2O_2 and (B) TBARS in mustard at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at p < 0.05. DAS: days after sowing; DW; dry weight; N: nitrogen; S: sulfur; H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid reactive substances.

3.2. ROS accumulation

Salt stress brought about ROS overproduction in plants. The enhanced content of ROS led to oxidative damage in the mustard plants, causing to increase lipid peroxidation and the damage of cell membrane. The generation of $O_2^{\bullet-}$ level in leaves was computed by using NBT as a histochemical process. The level of generation of $O_2^{\bullet-}$ was viewed as a result of blue staining after 6 h of treatment of leaves with NBT. The staining spots were more conspicuous in salt-treated leaf discs, compared to control leaves. Ethephon to the salt-treated plants decreased the $O_2^{\bullet-}$ spots, compared to salt-alone treatment. Adding ethephon with split dosage of N and S to plants showed no toxic effects in the absence of salt, and limited staining spots were viewed in plants treated with ethephon with split dosage of N and S under salt treatment, compared to the NaCl treated plants value (Figure 2).

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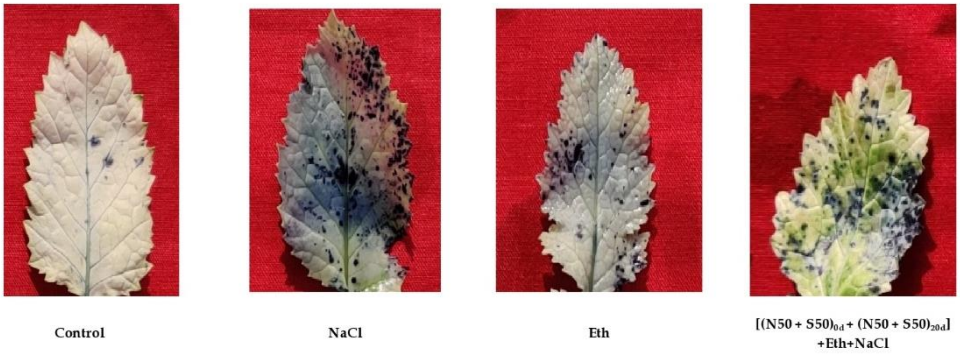

Figure 2. *In situ* determination of level of generation of superoxide ion ($O_2^{\bullet-}$) through nitro blue tetrazolium staining in mustard afterward the dehydration of leaves at 40 DAS. Plants were raised with 0, 100 mM NaCl alone or in combination with 200 µL L^{-1} ethephon (Eth) with split dosage of N and S [$(N50 + S50)_{0d}$ +$(N50 + S50)_{20d}$].

3.3. Antioxidant Enzymes and Salt Tolerance

Salt treated plants increased the antioxidant enzymes activities, compared to the control values. Ethephon individually increased CAT, APX, GR and SOD activity by 30.0, 93.9, 44.3 and 85.4%, respectively, compared to the control. Plants obtaining split dosage of N and S increased antioxidant enzymes activities in salt or without salt, compared to the respective control values. However, the antioxidant enzymes activities was extremely enhanced in ethephon combined with split dosage of N and S either with or without NaCl. Ethephon under salt stress with split dosage of N and S more conspicuously increased the SOD by 162.9%, CAT by 72.0%, APX by 210.6% and GR activity by 97.9% compared to the control (N100 + S100) plants (Table 1).

Table 1. The antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), reduced glutathione (GSH) content and redox state in mustard at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing.

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Treatment	SOD activity	CAT activity	APX activity	GR activity	GSH	Redox state GSH/GSSG
	(U mg^{-1} protein min^{-1})				(nmol g^{-1} leaf DW)	
Control	6.58 ± 0.16^{f}	118.01 ± 2.96^{f}	1.32 ± 0.03^{g}	0.192 ± 0.005^{f}	65.13 ± 1.64^{g}	17.91 ± 0.45^{f}
NaCl	8.80 ± 0.35^{e}	139.00 ± 5.56^{e}	1.85 ± 0.07^{f}	0.230 ± 0.009^{e}	79.27 ± 3.18^{f}	08.42 ± 0.33^{g}
Eth	12.20 ± 0.30^{d}	153.43 ± 3.86^{d}	2.56 ± 0.06^{e}	0.277 ± 0.007^{d}	87.25 ± 2.20^{e}	25.43 ± 0.63^{e}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$]	17.70 ± 0.27^{b}	207 02 ± 3.16^{b}	4.37 ± 0.06^{b}	0.397 ± 0.006^{b}	126.50 ± 1.92^{b}	36.76 ± 0.56^{b}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + NaCl	15.50 ± 0.32^{c}	178.03 ± 3.70^{c}	3.57 ± 0.07^{d}	0.329 ± 0.006^{c}	102.25 ± 2.12^{d}	30.55 ± 0.63^{d}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth	19.20 ± 0.29^{a}	232.07 ± 3.54^{a}	4.62 ± 0.07^{a}	0.428 ± 0.007^{a}	134.34 ± 2.04^{a}	38.76 ± 0.59^{a}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth + NaCl	17.03 ± 0.36^{b}	203.07 ± 4.22^{b}	4.10 ± 0.08^{c}	0.380 ± 0.008^{b}	118.18 ± 2.45^{c}	34.27 ± 0.71^{c}

3.4. Nitrogen and Sulfur Assimilation

The importance of N and S or ethephon in S assimilation, ATP-S activity, content of S and Cys and GSH and redox state were analyzed. The treatment of 100 mM NaCl reduced S content and redox state but increased the ATP-S activity, content of Cys and GSH, compared to the respective control values. Ethephon application markedly improved the content of N by 16.4% and NR activity by 41.8%, compared with the respective control. Application of the split form of N and S [(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] enhanced N content and the activity of NR strikingly under stress or without stress . Furthermore, the toxicity of 100 mM NaCl on the content of N and the activity of NR were least in plants which got [(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + 200 µL L^{-1} ethephon, compared to the respective control plants (Table 2). Application of ethephon led to increase of ATP-S activity, Cys and GSH content by 94.9, 31.3 and 34.0%, respectively, compared to the control. Application of ethephon enhanced the S content and redox state by 26.3 and 41.9%, respectively, compared to control . Nevertheless, the combination of ethephon and split dosage of N and S [(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] with set to salt treated plants increased ATP-S activity, content of S, Cys, GSH and redox state more conspicuously than other treatments under both stress and no stress conditions, compared to the control (Table 2).

Table 2. Activity of nitrate reductase (NR) and ATP-sulfurylase (ATP-S), content of nitrogen (N) and sulfur (S), cysteine (Cys) in mustard plants at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing; DW: dry weight.

Treatments	NR activity (nmol NO_2 h^{-1})	ATP-S activity (µmol g^{-1} protein sec^{-1})	N content (mg g^{-1} DW)	S content (mg g^{-1} DW)	Cys content (nmol g^{-1} leaf DW)

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Control	414.35 ± 10.40^{e}	1.36 ± 0.03^{f}	35.91 ± 0.90^{f}	4.83 ± 0.12^{e}	6.41 ± 0.16^{f}
NaCl	325.27 ± 13.03^{f}	1.59 ± 0.06^{e}	23.85 ± 0.95^{g}	3.83 ± 0.15^{f}	7.44 ± 0.29^{e}
Eth	587.48 ± 14.84^{d}	2.65 ± 0.07^{d}	41.87 ± 1.05^{e}	6.10 ± 0.15^{d}	8.46 ± 0.21^{d}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$]	739.48 ± 11.36^{b}	3.62 ± 0.05^{b}	54.98 ± 0.84^{b}	8.57 ± 0.13^{b}	11.67 ± 0.17^{b}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + NaCl	652.84 ± 13.66^{c}	3.13 ± 0.06^{c}	48.24 ± 1.01^{d}	7.43 ± 0.15^{c}	9.88 ± 0.20^{c}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth	784.53 ± 11.91^{a}	3.83 ± 0.05^{a}	58.94 ± 0.89^{a}	9.28 ± 0.14^{a}	12.78 ± 0.19^{a}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth + NaCl	713.03 ± 14.81^{b}	3.57 ± 0.07^{b}	51.84 ± 1.07^{c}	8.42 ± 0.17^{b}	11.23 ± 0.23^{b}

3.5. Proline Metabolism under Salt Stress

Salt-stressed plants showed a significant response in terms of proline metabolism. Proline accumulation increased in plants in salt stress and with the supplementation of combined dosage of N and S compared to the control plants. Salt treatment increased proline content by 37.1%, compared to the respective control plants, whereas individual application of ethephon considerably increased proline accumulation by 45.6%, compared to the respective control plants. Plants receiving ethephon under salt stress increased proline accumulation considerably compared to the control. Moreover, ethephon together with the split dosage of N and S further increased the content of proline in absence or presence of salt compared to the NaCl treated plants or control (Table 3).

Application of ethephon individually enhanced glutamyl kinase (GK) activity by 41.9%, compared to control plants. Plants getting split dosage of N and S [(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] in the absence or presence of salt improved GK activity, compared to the respective control. Conversely, ethephon application under salt stress together with the split dosage of N and S more conspicuously increased the GK activity by 71.1%, compared to the plants under control states. The split dosage of N and S [(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] with ethephon showed a maximum reduction in proline oxidase (PROX) activity, compared to the respective control (Table 3).

Table 3. The activity of glutamyl kinase (GK), proline oxidase (PROX) and the content of proline in mustard plants at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and were treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing.

Treatments	**GK activity (U mg^{-1} protein min^{-1})**	**PROX activity (U g^{-1} protein min^{-1})**	**Proline content (mg g^{-1} FW)**
Control	0.65 ± 0.02^{g}	90.05 ± 2.04^{a}	05.63 ± 0.14^{g}

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NaCl	0.99 ± 0.04^{f}	61.05 ± 1.44^{b}	08.92 ± 0.35^{f}
Eth	1.12 ± 0.03^{e}	53.07 ± 1.08^{c}	10.32 ± 0.26^{e}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$]	1.59 ± 0.02^{d}	29.04 ± 0.41^{d}	17.96 ± 0.27^{d}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + NaCl	2.13 ± 0.04^{b}	06.21 ± 0.12^{f}	19.48 ± 0.40^{c}
[(N50 + S50)$_{0d}$+(N50 + S50)$_{20d}$] + Eth	1.71 ± 0.03^{c}	25.00 ± 0.40^{e}	21.88 ± 0.33^{b}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth + NaCl	2.25 ± 0.05^{a}	1.13 ± 0.01^{g}	23.49 ± 0.48^{a}

3.6. Ethylene Biosynthesis under Salt Tolerance

Salt grown plants exhibited improved ethylene production and ACS activity by 6.7 and 4.9-times, compared to the respective control values. However, ethephon application to plants developed under no salt led to decreased ethylene production and ACS activity by 85.8 and 82.4%, correspondingly, compared to the salt-grown plants. Plants grown with ethephon under salt stress with the split dosage of N and S increased ACS activity by 64.9% and ethylene production by 113.1%, compared to the respective control (Figure 3).

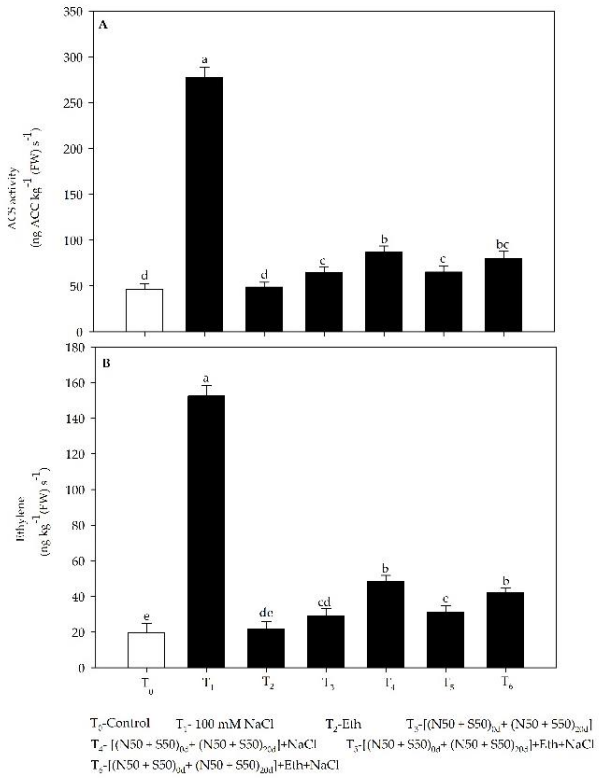

Figure 3. (A) Activity of 1-aminocyclopropane carboxylic acid synthase (ACS) activity and (B) ethylene production in mustard plants at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and were

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treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE (n = 4). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing; FW: fresh weight.

3.7. Photosynthetic and Growth Characteristics

The impact of split dosage of N and S, ethephon and ethephon + N and S on salt-stressed mustard plants was examined by analyzing photosynthetic efficiency.

Exogenous application of ethephon exhibited increased PSII efficiency (21.5%), intrinsic PS II efficiency (6.8%), actual PS II efficiency (6.4%), qP (7.7%) and ETR (9.3%), compared to the respective control plants. Conversely, NPQ was declined by ethephon by 11.8%, compared to the respective control. Split dosage of N and S enhanced the above individualities in presence or absence of salt, compared to the respective control plants. Besides this, ethephon with split dosage of N and S [$(N50 + S50)_{0d}$ + $(N50 + S50)_{20d}$] under salt stress noticeably declined the injurious effects of 100 mM NaCl on chlorophyll fluorescence and led to an increase in maximum PS II efficiency by 89.8%, intrinsic PS II efficiency by 64.6%, actual PS II efficiency by 106.5%, qP by 242.9% and ETR by 293.3%, and NPQ declined by 68.1%, compared to salt treatment obtaining plants (Figure 4).

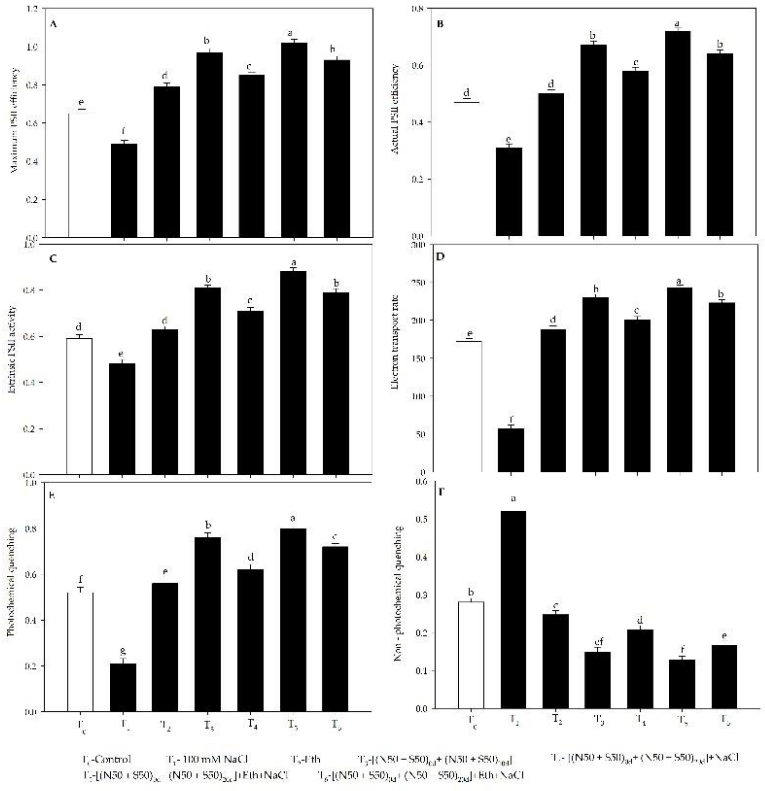

Figure 4. (A) Maximum PS II efficiency, (B) actual PS II efficiency, (C) intrinsic PS II efficiency, (D) electron transport rate, (F) photochemical quenching, and (G) non-photochemical quenching of mustard plants at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and plants were treated with 200 µL L^{-1} ethephon (Eth) in the

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absence or presence of 100 mM NaCl. Values are presented as means ± SE ($n = 4$). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing.

The plant getting split dosage of N and S or ethephon exhibited values of higher photosynthetic characteristics, compared to the respective control under no salt stress. Plants obtaining N and S plus ethephon under salt stress mitigated the toxicity of salt and improved photosynthesis, compared to the respective control values. Ethephon individually increased net photosynthesis by 52.8%, stomatal conductance by 25.5% and intercellular CO_2 concentration by 25.7% and chlorophyll content by 33.3%, compared with control plants. Application of [$(N50 + S50)_{0d} + (N50 + S50)_{20d}$] increased net photosynthesis by 116.2%, stomatal conductance by 65.5, and intercellular CO_2 concentration by 64.7% and chlorophyll content by 73.0%, compared to the respective control under no stress.

Exogenously applied ethephon increased photosynthetic efficiency of plants more effectively accompanied with the split dosage of N and S both under no stress and salt stress (Table 4). Exogenous ethephon with split dosage of N and S increased net photosynthesis by 268.4%, stomatal conductance by 106.6%, intercellular CO_2 concentration by 137.6% and chlorophyll content by 151.0%, compared to respective NaCl-treated plants (Table 4).

The growth characteristics were adversely affected by salt stress treatment. Ethephon led to enhanced leaf area by 34.1% and plant dry mass by 23.5% under no stress, compared to the respective control. Split dosage of N and S increased leaf area, compared to the control. Still, supplementation with split form of N and S to the NaCl treated plants markedly restored plant dry weight and leaf area, compared to NaCl-treated plants. Applying ethephon with split dosage of N and S $(N50 + S50)_{0d} + (N50 + S50)_{20d}$ to the salt, augmented leaf area by 244.0% and plant dry mass by 250.0%, compared to the NaCl-treated plants (Table 4).

Table 4. Net photosynthesis, chlorophyll content, stomatal conductance, intercellular CO_2 concentration, leaf area and plant dry mass in mustard at 40 DAS. Plants were raised with split dosage of N and S (50 + 50 mg kg^{-1} soil) at the sowing time (0 DAS) and at 20 DAS, and plants were treated with 200 µL L^{-1} ethephon (Eth) in the absence or presence of 100 mM NaCl. Values are presented as means ± SE ($n = 4$). Data followed by the same letter are not significantly different by LSD test at $p < 0.05$. DAS: days after sowing.

Treatments	Net photosynthesis (µmol CO_2 m^{-2} sec^{-1})	Chlorophyll content (SPAD value)	Stomatal conductance (mmol CO_2 m^{-2} sec^{-1})	Intercellular CO_2 concentration (µmol CO_2 mol^{-1})	Leaf area (cm^2 $plant^{-1}$)	Plant dry mass (g $plant^{-1}$)
Control	14.24 ± 0.36^{f}	28.22 ± 0.71^{e}	368.17 ± 9.26^{e}	272.11 ± 6.85^{f}	129.26 ± 3.24^{f}	2.34 ± 0.06^{f}
NaCl	07.93 ± 0.32^{g}	19.25 ± 0.77^{f}	289.43 ± 9.12^{f}	178.12 ± 7.12^{g}	62.53 ± 2.5^{g}	1.02 ± 0.04^{g}
Eth	21.71 ± 0.55^{e}	37.66 ± 0.95^{d}	462.12 ± 10.60^{d}	342.36 ± 8.61^{e}	173.64 ± 4.35^{e}	2.89 ± 0.07^{e}

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[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$]	30.71 ± 0.47^{b}	48.85 ± 0.75^{b}	609.54 ± 12.40^{b}	448.55 ± 6.84^{b}	227.05 ± 3.46^{b}	3.77 ± 0.06^{b}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + NaCl	25.50 ± 0.53^{d}	42.95 ± 0.89^{c}	537.71 ± 11.10^{c}	392.21 ± 8.16^{d}	198.82 ± 4.12^{d}	3.32 ± 0.07^{d}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth	33.46 ± 0.51^{a}	52.71 ± 0.81^{a}	643.56 ± 13.82^{a}	476.94 ± 7.27^{a}	239.51 ± 3.65^{a}	4.02 ± 0.06^{a}
[(N50 + S50)$_{0d}$ + (N50 + S50)$_{20d}$] + Eth + NaCl	29.13 ± 0.61^{c}	48.25 ± 1.00^{b}	597.22 ± 12.30^{b}	423.27 ± 8.81^{c}	215.68 ± 4.47^{c}	3.57 ± 0.07^{c}

3.8. Stomatal Behavior under Salt Stress

Stomatal response was studied in presence of ethephon supplementation with or without split dosage of N and S in presence or absence of salt. The width and length of stomata were 4.1 and 8.3 µm in the control plants. The analysis at SEM showed slight closing of the stomatal pore in salt stress, and the frequency of stomata decreased by 10.5%, compared to respective control plants. Exogenous ethephon with the split dosage of N and S application led to improved width and length of stomata by 1.7 and 7.5 µm, and the frequency of stomata by 42.1%, compared to control plants values. The treatment of ethephon with split dosage of N and S under stress or no stress showed a higher number of stomatal frequency, compared to control plants (Figure 5).

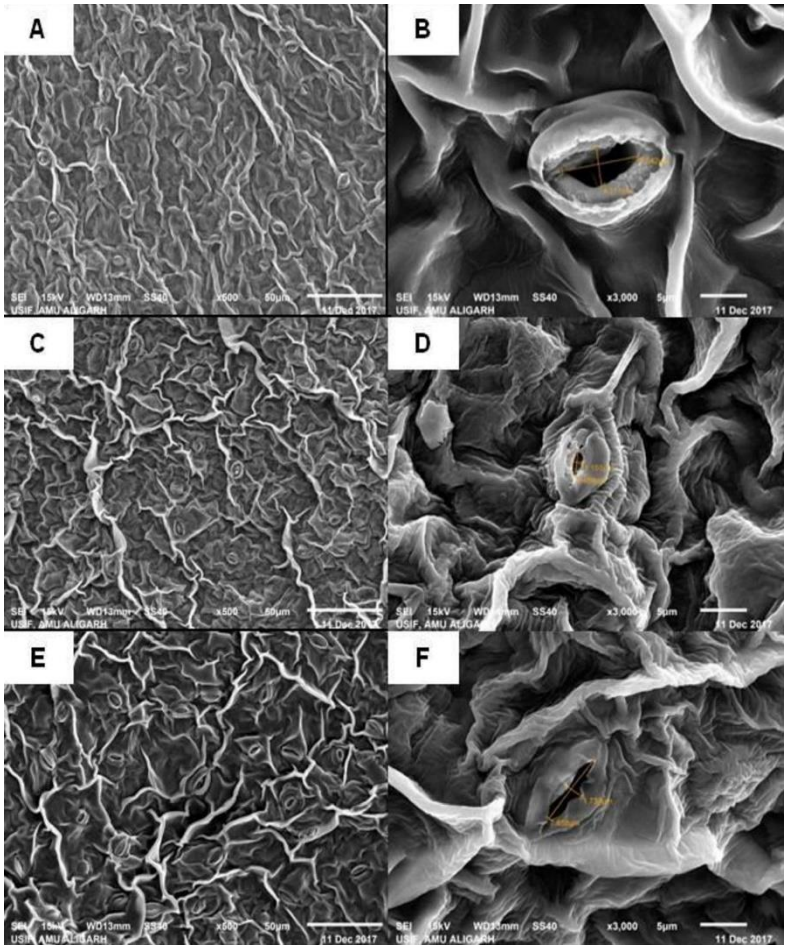

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Figure 5. Stomatal behavior of mustard was done under (A, B) control, (C, D) 100 mM NaCl, and (E, F) ETH with split dosage of N and S with 100 mM NaCl. The stomatal response of opening and closing was viewed in the SEM at 500× (A, C, E), 3000× (B, D, F) magnification in mustard plant developed under 100 mM NaCl at 40 DAS. Bars (A,C,E) = 50 μm; bars (B,D,F) = 5 μm;

4. Discussion

4.1. Ethephon application Plus N and S Mediated Oxidative Stress and Activity of Antioxidant System under Salt Stress

The uptake of Na^+ excessively strengthens its antagonistic connection with significant ions such as K^+ and Ca^{2+}. Excess assimilation of Na^+ ion in roots and its transport to shoots results in nutrients starvations by decreasing transportation, uptake and accumulations of mineral nutrients. For that reason, the keeping of intracellular balance of ions is one of the main step in the salt tolerance mechanisms in plants. Conversely, Sehar et al. [23], Khanna et al. [8], and Fatma et al. [25] stated the tolerance of plant was mediated by using exogenous plant hormone, nutrients, and antioxidants systems, via balancing homeostasis of ions. The present study gained novelty by using exogenous Eth on split dosage of N and S in salt-stressed plant that increased the antioxidant defense system and substantially decreased H_2O_2 and TBARS content.

It was interesting to note of that decreased oxidative stress in Eth treated with N and S was corresponded with huge up-regulation of the cell reinforcement framework in them. It was seen that cell reinforcement compounds, for example, APX, SOD, CAT, and GR were improved in salt stress, possibly to neutralize the oxidative damage brought about by salt stress. Plants conceivably enhance the antioxidant system to work on the harmful impact of abiotic stress. [13,24,54,55]. The enzymatic and non-enzymatic antioxidants work in cells to neutralize oxidative stress and to keep their concentration levels below toxic levels [56,57], but declined oxidative effects because of Eth or individual supplementation of N and S have been previously reported; though their interactive role is scanty for discussion. The present study focused on decreased rate of ROS accumulation in Eth treated with N and S may have directly added as a protecting key in cellular organelles and agree to their smooth working. This report allows with earlier studies in which application of Eth reduced oxidative stress and increased the antioxidant enzymes activity in *B. juncea* [54,58,59].

The present study showed that more promising results in enhancing the antioxidant system and lowering the oxidative stress were obtained when Eth and N and S were applied together. It is likely that Eth with split dosage of N and S treated plants lessened the oxidative stress more efficiently when plants received individual application of Eth, or N and S. It may be advisable that exogenous Eth with split dosages of N and S mitigated the ROS-arbitrated oxidative stress by activating the antioxidant enzymes.

4.2. Ethephon application plus N and S Reduced ROS Accumulation

The production of ROS accumulation excessively under salt stress caused the damage outcome in the plant cells [60]. The exogenous application of Eth combined with

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split dosage of N and S caused minimum staining because of the enhanced assimilation of N and S and synthesis of GSH. The noticeable effect of Eth plus N and S with least staining was because Eth plus N and S influenced ROS accumulation completely, compared to the individual effects. Ethylene plays a dynamic role as an amplifier for ROS production and develops tolerance to environmental stress by decreasing ROS accumulation in *B. juncea* [59]. Another reason is the GSH synthesis and increase in the antioxidant enzymes activity when co-applied Eth and N and S to salt-treated plants, which prompted a resilient capacity of ROS scavenging and diminished production rate of $O_2^{\bullet-}$.

4.3. Ethylene involvement in N- and S-Induced Increase in Redox State

Nitrogen metabolism is vital for the development of plants and likewise plays an imperative function in environmental fluctuations [8,61]. The change in the N metabolism can lead to different plant metabolism and limit the productivity of crop because of the alteration in their essential functions. The activity of NR and N content were severely exaggerated by salt stress because of the overproduction of ROS that caused membrane damage in the present study. Photosynthetic efficiency was directly related with decreased NR activity and any change in nitrate assimilation is accredited toward decreased admittance to CO_2, causing inactivation of NR enzyme [62]. However, exogenous application of Eth plus N and S showed a positive impact on N assimilation and the activity of NR in NaCl-treated plants. Iqbal et al. [63] have shown that Eth application functions as an important part in the metabolism of N and increases the activity of NR and the content of N in *B. juncea*.

Assimilation of S prompts to the GSH production and alleviates the injurious influence of salt stress. Exogenous application of Eth in salt stress significantly enhanced S and Cys content with GSH and activity of ATP-S, which improved redox status of cellular environment in the present study. A relative study executed in *B. juncea* has revealed that ET increases ATP-sulfurylase activity and S accumulation [64]. Reports on the effect of Eth with split N and S dosage on S-containing amino acids and reduced thiols (Cys, GSH) under salt stress are scanty in the literature. The treatment of Eth increased photosynthetic performances of salt-treated plants through improved thiol compounds and then led to the greater redox ratio in the presence of stress or absence of stress with split dosage of N and S. Cysteine is accountable for production of several important S containing compounds, such as methionine and GSH [65,66]. Besides this, the accumulation of GSH depends on Cys availability through S assimilation under salt stress condition [7,67]. Therefore, Cys biosynthesis in plants is critically important metabolic process that allows and serves as the branch point between S and N assimilation, as the carbon skeleton and amino group of Cys is derived from serine, a product of N assimilation [67]. Furthermore, S-assimilation pathway's first enzyme ATP-S regulates the synthesis of S-compounds. Thus, enhanced ATP-S activity can develop plants tolerance through increased thiol compounds under abiotic stress, which helped in removal of excess ROS [54,59]. Plants grown with salt stress showed higher ATP-S activity and exhibited greater tolerance to the salt stress. This study suggests that application of Eth is associated to S-assimilation via Cys, it is expected that S affects ET sensitivity, and ET is concerned in regulating GSH

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production and salt stress alleviation. In the previous study on *B. juncea* plants, Asgher et al. [31] have suggested that the application of Eth increased the content of reduced thiols such as Cys and GSH and improved photosynthesis and growth under Cr stress.

ROS overproduction can incite limited or harsh oxidation resulting in altered redox state, thus ROS metabolism and their constant stability is vital under stress states [68]. In addition, GSH is well known for the removal of excess ROS and protects plants from oxidative damage [69-71]. The homeostasis of GSH and GSSG keeps signaling of stress responsive proteins and regulates oxidative stress. Since GSH enhanced under Eth treated plants under salt stress that led to defense contrary to oxidative stress in *B. juncea* plants. The present study showed that Eth supplementation with split dosage of N and S led to the increase in the GSH content and keeping a suitable redox state that retains the cell free from salt toxicity.

In the present study, Eth was co-applied with N and S reversed salt stress effects by enhancing the ratio of GSH/GSSG and the GR activity in *B. juncea* plants which refurbished and amplified GSH content, so increasing the GSH/GSSG ratio. These results suggested that exogenous Eth plus N and S enhanced GSH biosynthesis and furthermore kept up redox homeostasis. The increment in thiols and redox state with Eth plus N and S aided the removal of toxic ions in salt stress.

4.4. Ethylene Improved Proline Metabolism on N and S Application

The connection between proline accumulation and dehydration resilience is the fundamental system to keep away from an unfavorable impact on many major physiological cycles, for example, leaf extension, the maintenance of cell osmotic potential, stomatal conductance, and photosynthesis. Thus, the raised proline content in our study, because of salt stress, may be a direct result of the expansion in proline biosynthesis, with a decline in its oxidation and kept up the water balance. Abiotic stress-actuated over amassing of proline was accounted for under different abiotic stressors in many past studies, which further offered assurance to protection to the stress instigated dehydration and oxidative stress [31,72] In the present study, Eth application improved the content of proline in salt stress. Ethephon application modulated the metabolizing enzymes and caused higher proline content with less oxidative damage brought by salt stress. The increased accumulation of proline afterward Eth application caused from GK activity induction and POX activity inhibition. The activity of P5CS and GK has a vital role in regulating the proline level and abiotic stress in plants [73]. Iqbal et al. [74] reported that the increment in proline metabolism affect ethylene levels in plants under salt stress. The present study suggested that Eth application with split dosage of N and S improved proline metabolism added to tolerance to the salt stress too increased photosynthetic efficiency. Ethylene induced proline accumulation and protected the photosynthetic system from salt-stress mediated oxidative stress by sustaining water relations and by diminishing oxidative stress in salt-stressed plants.

In addition, proline accumulation maintains osmotic adjustments of the salts-treated plants to provide optimal conditions for cellular reactions. Studies have shown that proline

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accumulates in heat stress in *Triticum aestivum* [75] drought stress in *B. juncea* [76], and Ni stress in *B. juncea* [24], with improved osmotic adjustments.

4.5. Influence of Ethylene with Supplementation of N and S on Ethylene Biosynthesis

Any environmental fluctuation causes rapid ET emission, which is called as stress ethylene. The present study revealed that exogenous application of Eth with N and S diminishes the salt stress toxicity then improved photosynthesis and growth mainly by its effects on ET synthesis. The link between ET and adverse effects on plants is well known and it has now become clear that many steps in ET biosynthesis and action can be altered by stress. It is well known that ET biosynthesis occurs through simple metabolic pathways [77-79] and ACC oxidase and ACC synthase are the two important enzymes involved in ET production. The study of ET biosynthetic pathway has shown that enzymes and the genes encoding those enzymes are responsible for the rapid production of ET under stress condition [77,78,80,81].

Salt-stressed plants exhibited enhanced ET production, compared to respective control plants, and thus stress ET formed was accountable for salt mitigated injurious effects on plant function. It has been advised that increased ET production under abiotic stress induces oxidative stress and photosynthetic processes were affected by them [30, 82]. The combined application of Eth together with N and S significantly more interacted with ET production under salt stress and limited the stress ET production to the most effective range favoring enhanced plant metabolism.

4.6. Ethylene Supplementation with N and S Improved Photosynthetic and Growth Performance under Salt Stress

Modification of photosynthetic efficiency in environmental stress is crucial for plant persistence [83] and plants accept this scheme by moderating some gas exchange attributes, containing stomatal conductance and intercellular CO_2 concentration and chlorophyll content [7,25]. To explain the salt stress effects and interaction with ET and N and S on photosynthesis, we investigate gas exchange together with Chl fluorescence characters in this study.

Exogenous application of Eth enhances photosynthetic capacity in *B. juncea* [25,59, 63] in abiotic stress. Exogenous application of Eth increases photosynthetic performance by enhancing stomatal conductance and also activity of Rubisco in *B. juncea* [59,64]. Application of Eth increased photosynthesis due to increased diffusion rate of CO_2 through intercellular spaces and increased photosynthetic pigments and stomatal aperture [60]. The application of Eth with split dosage of N and S was accomplished more in augmenting photosynthetic capacity under salt stress. The split dosage of N and S at the two developmental steps of plants, one at the time of sowing and other at 20 DAS made possible for better N and S assimilation and formation of reduced S compounds through enhanced antioxidant system. These processes were favored by ethylene due to Eth application. This led to protection of photosynthetic machinery from salt-induced oxidative stress. The positive mechanisms of ET alongwith N and S on photosynthesis are accredited in protecting chlorophyll, decreased ROS, and increased antioxidant enzymes

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activities in salt stress. Iqbal et al. [27] have stated that the N availability disposed ET evolution and affect the efficiency of stomatal conductance and photosynthesis.

The results also showed that salt stress decreased the chlorophyll content, which agree with previous reports by Hussain et al. [61] for *Vigna radiata* and Wang et al. [84] in *Medicago sativa* seedling. Application of Eth plus N and S increased chlorophyll content, led to active photosynthetic activity causing growth. Photosynthesis is directly related to crop production under several metabolic processes [83], and an ineffective photosynthetic capacity, which will lead to yield loss under stress [84]. The present study has shown that the salt induced decline in photosynthetic efficiency come up with lower reproductive growth.

The study of Chl fluorescence may highlight the impact on plants in stress conditions, with focusing on the potentiality of plants in environmental fluctuation [85,86]. The ratio F_v/F_m denotes the photosynthetic efficiency of whole PS II and the maximum quantum yield of PS II [87].The present study has shown that salt treatment reduce F_v/F_m, F_v'/F_m', qP, ΦPS II and ETR, the declination in photosynthesis was primarily inferable to photo-inhibition under the salt stress, however, increment in NPQ was obtained under salt-stress. Lu et al. [88] have reported that the decrease in Chl fluorescence under environmental stress destroys antenna pigments by the restricted block of electron transport from PS II to PS I. Exogenous Eth plus N and S reduced the decrease in photosynthetic efficiency as salt stress caused photo inhibition. The fraction of high lipid to chlorophyll reveals a little protein-packing density was reported in several studies [89,90]. The present study has shown that plants receiving Eth with N and S showed lower lipid peroxidation level and higher the content of chlorophyll than respective control or NaCl grown plants. The application of Eth enhanced F_v/F_m, F_v'/F_m', qP, ΦPSII and ETR, and decreased NPQ under Zn and Cr stress [13,31].

The expansion in development characteristics of salt-treated plants with Eth in addition to N and S is embraced to N and S-actuated ET-intervened changes in the photosynthesis. The most extreme easing of salt stress was seen with the treatment of Eth in addition to dosage of N and S clearly for more proficient thiols productions that bring about in greatest insurance of photosynthetic system then consequently leaf area and plant dry mass in salt-treated plants. Wang et al. [84] reported that ET mitigated the salt-toxicity in *Medicago sativa* by reducing oxidative stress. Moreover, the higher leaf area is related with ethephon-improved ET synthesis [28, 91]. Earlier, it has been reported that Eth application on *Arabidopsis*, *Nicotiana tabacum* increased leaf area, plant dry weight and pod number at low concentration however inhibited it at high concentration [92].The studies of Iqbal et al. [27] and Asgher et al. [31], Khan et al. [13] showed that ET cooperates with nutrients uptake and regulate plant responses under stress for improvement in plant dry weight and leaf area.

4.7. Ethylene with N and S Modulated the Stomatal Behavior under Salt Stress

Stomata are minute apertures in plant leaves that stimulate gas and water exchange among the plant and its related environs. Consequently, the closing and opening of the

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stomatal aperture is a major characteristic of keeping the transpiration rate and photosynthesis [93]. Salt treatment induced partial stomatal closure, because of the excessive accumulation of ions, the guard cells became flaccid but the stomata were found open in plants receiving Eth with N and S. The application of Eth plus N and S highly influenced the osmotic relations that resulted in stomatal opening. Furthermore, studies have shown ET to be involved in both stomatal opening and closure [94] depending on the situation. Additionally, abscisic acid (ABA), plant hormone played a central function in directive of stomatal closure through synthesis of second messengers, which involves ROS accumulation. Some studies report ET induced stomatal closure through NADPH oxidase-mediated ROS accumulation in the guard cells [95,96]. Further, another pathway involving EIN2 showed negative regulation of stomatal closure. Tanaka et al. [97] have showed that ABA influenced stomatal closure was inhibited by ET treatment. Zhang et al. [98] stated that flavonols accumulation in guard cells because of ET activity. These flavonols inhibit ROS production and stomatal closure. Ethylene and its precursor ACC stimulate H_2O_2 accumulation in guard cells and cause the closure of stomatal aperture in *Vicia faba* [99].

5. Conclusions

Supplemental N and S applied in a split dosage has a positive effect in mustard plants and alleviate the negative effects of salt stress. Salt stress remarkably decreased the photosynthetic efficiency of plants by increasing ROS generation. However, phytohormones with nutrients mitigated the salt stress induced damaging effects and improved plant growth by up-regulating antioxidant enzymes activities and the accumulation of osmolytes. Ethylene under salt stress played a noteworthy role in the N and S-mediated regulation of photosynthetic and growth characteristics. Furthermore, ET was involved in the protection and improvement of photosynthesis and plant growth under salt stress. In the light of the outcomes, we can infer that the antagonistic effects of salt on photosynthesis and growth were reversed significantly when Eth plus N and S were applied together, via a reduced salt-induced ROS production and an increased N and S-assimilation.

This comprehensive study delivered evidence that the split dosage of N and S more effectively alleviated the salt-induced damage in mustard plants grown in a saline environment. Therefore, combined split dosage of N and S application together with ET management can be advisable as a strategy for supporting salt tolerance in mustard plants. The results may be eventually extended in related Brassicaceae and other plant species.

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