

**Abscisic acid and nitrogen showed coordinated action on antioxidant system and osmotic adjustment to alleviate salinity inhibited photosynthetic potential in *Brassica juncea* L.**

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&These authors contributed equally in designing the experiments and in editing of the manuscript.

#### **Running Title:**

**ABA and N: Coordinately provides tolerance against salt stress in mustard plants.**

## Abstract

The present study assessed the effect of abscisic acid (ABA; 25  $\mu$ M) and/or nitrogen (N; 10 mM) in minimization of salinity (NaCl; 100mM)-impact on growth, photosynthetic efficiency, Rubisco activity, nitrogen and sulfur assimilation, oxidative stress ( $H_2O_2$ ), lipid peroxidation measured as thiobarbituric acid reactive substances, (TBARS), osmolyte (Proline) content, and the activity of antioxidant enzymes (superoxide dismutase, SOD glutathione reductase, GR; ascorbate peroxidase, APX) in cultivar RH0-749 of *Brassica juncea* L. NaCl stress caused significant elevations in  $H_2O_2$  and TBARS, and differentially modulated proline content, the activity of antioxidant enzymes, and impaired growth and photosynthetic functions. Exogenously applied 25  $\mu$ M ABA negatively affected plant growth and photosynthesis in *B. juncea* without NaCl. In contrast, exogenously applied 25  $\mu$ M ABA and 10 mM N, alone or in combination minimized oxidative stress, and maintained a fine-tuning between proline content and the activity of antioxidant enzymes, and thereby improved plant growth and photosynthetic functions in NaCl exposed *B. juncea*.

**Keywords:** Absciscic acid, *Brassica juncea*, Nitrogen, Salinity, Oxidative stress, Photosynthetic functions

## 1. Introduction

Soil salinity remains a growing problem for agriculture and has been found to restrain crop production over 800 million hectares of land globally [1,2]. Salt accumulation in cultivable land is mainly derived from seawater incursion into freshwater habitats and from irrigation with saline water [3]. Salt stress reduces the competence of plants to absorb soil water. Increased  $Na^+$  and  $Cl^-$  content within the plant system is harmful, resulting in oxidative stress and nutritional imbalance in plants [3,4]. These ions negatively affect plant growth and development, mostly because of the osmotic stress, decreasing photosynthetic efficiency, and debilitating metabolic processes [5,6]. Salt stress causes lipid peroxidation, disturbs the water and osmotic balance and nutrient uptake due to much higher production of reactive oxygen species (ROS) [2,7]. The over-production of ROS causes direct damage to proteins, lipids, nucleic acids, and photosynthetic functions [8]. Salinity decreases nitrogen uptake in plants and influences carbon and nitrogen metabolisms resulting in reduced growth and development [9,10].

To reduce the negative effects of salt stress, plants activate distinct mechanisms for ion and water homeostasis and cellular osmotic adjustment [11]. The prevalent strategy that

plants acquire is  $\text{Na}^+$  sequestration in the vacuoles and accumulation of compatible solutes in the cytosol [12,13]. Most osmolytes are N-containing metabolites and are important for osmotic adjustment [14]. By enhancing nutrient enrichment is another important strategy adopted by plants to reduce the detrimental effects of salt stress [15]. Among nutrients, N availability has a critical influence in salinity tolerance as it is the major component of enzymes, GSH, proline, and pigments [16]. Exogenously supplied N has been recorded to induce proline accumulation under salt stress, proline promotes the uptake of water, thus maintains osmotic balance, and protects the plants against over-production of ROS [17,18].

Phytohormones on other hand also enhances the activity of the antioxidant enzymes and induce the production of compatible solutes such as proline, thereby helps in the regulation of salinity stress in plants [19,20]. Among different phytohormones, ABA acts as a crucial signaling mediator in regulating physiological functions in response to various abiotic stresses such as salt, drought, low temperature [21-23]. In response to abiotic and biotic stress, ABA helps plants to survive by inducing a wide range of plant defenses, such as the expression of genes involved in antioxidant defense system [24,25]. ABA plays a crucial role in the stomatal movement of guard cells [26]. Increased level of ABA accumulation have been noticed in *Hordeum vulgare* [27], *Oryza sativa* [28], and *Zea mays* [29] under salt stress. Achard et al. [30] reported that ABA production is induced under salinity, and its signaling pathway is necessary for salt tolerance. In response to abiotic stresses, ABA regulates growth and development in plants [31,32].

It is obvious from the accessible literature that the exogenous supplementation of N or ABA alone enhances salinity tolerance in plants [33-35]. Nitrogen improves salinity tolerance by maintaining glutathione (GSH) production. However, the synergistic interaction of N with ABA in antioxidant defense system under salt stress in regulating the photosynthetic efficiency is still unexplored. In the present work, our aim was to study the interactive effects of ABA and N application in regulation of N and sulphur-assimilation, the antioxidant defense system in mustard plants under NaCl stressed condition.

## 2. Methodology

### 2.1 Experimental design and growth conditions

Seeds of mustard (*Brassica juncea* L. cv. RH0-749) obtained from IARI, New Delhi, was sterilized using  $\text{HgCl}_2$  solution (0.01%) and were washed repeatedly with double distilled water (DDW). Sterilized seeds were sown in clay pots filled with 5 kg of acid-washed sand

purified according to the method adopted by Hewitt [36]. The pots were kept in the greenhouse of the Department of Botany, Aligarh Muslim University, Aligarh, India, with an average day/night temperatures of  $23/14 \pm 3^{\circ}\text{C}$  and relative humidity of  $61 \pm 4\%$ . After germination, three plants/pots were maintained. For considering Absciscic acid (ABA) as an important regulator of NaCl stress alleviation, a preliminary experiment was conducted to assess the influence of 0, 5, 10, 25, and 50  $\mu\text{M}$  ABA treatment alone or in combination with 100 mM NaCl. ABA was dissolved in ethanol to prepare 100  $\mu\text{M}$  concentrations, which was then diluted in DDW to obtain the desired concentrations of the solution. ABA solution was sprayed on the foliage evenly at 15 days after germination using a hand sprayer. On the basis of results obtained from the preliminary experiment, 25  $\mu\text{M}$  ABA was considered as optimum concentration which was used for subsequent experiment. The concentration 10 mM N is considered the mustard's optimal concentration and thus was used to initiate N assimilation [37]. In main experiment, plants were treated with 0, 100 mM NaCl, 10 mM N, 25  $\mu\text{M}$  ABA with the following combination treatments N + ABA, N + NaCl, ABA + NaCl and N + ABA+ NaCl. Potassium nitrate was used as an N source for obtaining 10 mM N concentration, and  $\text{K}^{+}$  concentration was retained in entire treatments by the addition of potassium chloride. The control set of plants were supplemented with 250 ml of Hoagland nutrient solution only at alternate days and 250 mL of distilled water daily. Plants were given 100 mM NaCl or 10 mM N at 10 days after sowing (DAS), while 25  $\mu\text{M}$  ABA was sprayed on the leaf foliage evenly at 20 DAS using a sprayer pump. Sampling was done at 30 DAS. The design of the experiment was a randomized complete block design (RCBD), and for each treatment, the number of replicates were four.

## 2.2 Analyses of $\text{Na}^{+}$ and $\text{Cl}^{-}$ content

$\text{Na}^{+}$  and  $\text{Cl}^{-}$  content was measured in roots and leaves. 1 g of oven-dried plant tissue was dissolved in 4 ml concentrated  $\text{HNO}_3$  (68%) in a 100 ml glass beaker. The beaker containing the digested sample was heated on the water-bath until brown effervescence was observed. When the effervescence stopped, 38 mL of TAM solution (Tri acid mixture) was added dropwise till a clear solution was obtained. The solution containing plant samples was dried on the hot plate, and after that, dried samples were diluted with DDW to make a final volume of 100 ml. The  $\text{Na}^{+}$  content was measured using Flame Photometer (Systronics), and  $\text{Cl}^{-}$  content by titration against the 0.02 N silver nitrate using 5% potassium chromate solution as an indicator.

### 2.3 Analyses of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation (TBARS)

The H<sub>2</sub>O<sub>2</sub> content was measured in leaves by following the method of Okuda et al [38]. One gram leaves were grounded using 200 mM perchloric acid (ice-cold ) and centrifuged at 1200 g for 10 minutes, and the supernatant was neutralized by adding 4M potassium hydroxide. For measuring optical density (OD) at 590 nm, 2 ml of the eluate was mixed with 1ml solution containing 160  $\mu$ L of 3- methyl-2-benzothiazoline hydrazine, 40  $\mu$ L of peroxidase, and 800  $\mu$ L of 12.5 mM 3-(dimethylamino) benzoic acid

The contents of TBARS were measured in leaves by following the method of Dhindsa et al. [39]. One gram leaves were grounded in 0.25% thiobarbituric acid (TBA). 10% trichloroacetic acid (TCA) was used for preparing 0.25% TBA. The grounded mixture was heated for 30 min on a water bath and rapidly cooled in cold water, followed by centrifugation (10,000 g ) for 15 min. For measuring optical density (OD) at 532 nm, 2 ml of supernatant was mixed with 8 mL 20% TCA containing 0.5% TBA. The content of TBARS was calculated using an extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>.

### 2.4 Analyses of superoxide ion (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> by a histochemical staining method

In-situ determination of the level of superoxide ion (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> generation were visually detected by following the method of Kumar et al.[40] with slight modification. Freshly prepared Nitro blue tetrazolium (NBT) solution and Diaminobenzidine (DAB) solution were used for detecting (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub>, respectively. NBT solution was formed by dissolving 0.2 g of NBT in 100 ml of 50 mM sodium phosphate buffer (pH 7.5). DAB solution (pH 3.8) was formed by dissolving 100 mg of DAB in DDW in an amber-colored bottle. For NBT and DAB staining, leaf samples were soaked in NBT solution and DAB solutions, respectively, and incubated overnight at room temperature. The samples were then boiled for 20 min in absolute ethanol, and then photographs were taken.

### 2.5 Analyses of H<sub>2</sub>O<sub>2</sub> in roots by Confocal laser scanning microscopy

Root samples were immersed for 15 min in freshly prepared 12.5  $\mu$ M Dichlorofluorescein diacetate (H<sub>2</sub>DCFDA) solution. After repeated washing with DDW, temporary slides of stained samples were prepared, and fluorescence was monitored using a confocal laser scanning microscope (Model LSM 780) at excitation 400–490 nm and emission  $\geq$  520 nm.

## 2.6 Analyses of Photosynthetic parameters and rubisco activity

Net photosynthetic rate ( $P_N$ ), intercellular  $CO_2$  concentration ( $C_i$ ), and stomatal conductance ( $g_s$ ) was measured using an infrared gas analyzer (Model CID-340 ).

Chlorophyll content was measured on fully expanded young leaves using SPAD chlorophyll meter (Mode 502 DL PLUS ).

The maximum PSII efficiency ( $F_v/F_m$ ), photochemical fluorescence quenching ( $qP$ ), non-photochemical fluorescence quenching (NPQ), and Electron transport rate (ETR) was measured using a chlorophyll fluorometer (Model JUNIOR-PAM). ETR was calculated using formula:  $ETR = (PAR)(0.84)(0.5)YII$ . YII represents the photochemical quantum yield of photosystem II. The rubisco activity was measured in leaves by following the method of Usuda H [41].

## 2.7 Analyses of growth parameters

The plants were dried in the oven at  $80^\circ C$ , and dried material was weighed on an electrical balance. Leaf area (LA) was calculated with a leaf area meter (Model LA 211, Systronics, New Delhi, India)

## 2.8 Assay of antioxidant enzymes

Fresh leaves (0.2 g) were homogenized with extraction buffer using chilled mortar and pestle. Extraction buffer was prepared by dissolving 0.05% Triton X-100 and 1% polyvinylpyrrolidone in 100 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged (15,000 g) at  $4^\circ C$  for 20 min, and the supernatant was used for the assay of SOD and GR. For APX assay, 2 mM acrylonitrile styrene acrylate was added to the extraction buffer. The APX and SOD activity was calculated by following the method of Asada K [42] and Beyer et al [43] respectively. The activity of GR was measured according to the method of Foyer C H and Halliwell B [44].

## 2.9 Determination of nitrate reductase (NR) activity and N content

The NR activity was measured according to the method of Kuo et al. [45]. 1 g leaves was frozen in liquid nitrogen, grounded to powder, and then homogenized in 250 mM Tris-HCl buffer (pH 8.5), using chilled mortar and pestle. Buffer was prepared by dissolving 1 mM EDTA, 10 mM Cys, 1 mM DTT, 20  $\mu M$  FAD in 10% glycerol. The homogenate was centrifuged (10,000 g) at  $4^\circ C$  for 30 min. NR activity was measured as the rate of nitrite production at  $28^\circ C$  following the method of Nakagawa *et al.* [46]. In the reaction mixture,

NADH was used for initiating the reaction. Subsequently, after 20 min, the reaction was ended by adding 1 N HCl containing 1% sulphanilamide solution (1ml). After that, 0.02% aqueous NED (1 ml) was added. The reaction mixture (1.5 ml) containing enzyme extract, 10 mM KNO<sub>3</sub>, 0.065 M HEPES (pH 7.0), and 0.5 mM NADH in 0.04 mM phosphate buffer (pH 7.2) was used for measuring absorbance at 540 nm using a spectrophotometer after 10 min.

N content was measured by the Kjeldahl digestion method, as described by Lindner [47]. A 20 ml aliquot of the digested leaf sample was taken in a 100 ml volumetric flask. To this flask, 10% sodium silicate (2ml) and 4 ml of 2.5 N sodium hydroxide solutions were added to prevent turbidity and neutralize the excess of acid, respectively. The volume was made up to the 100 ml mark with DDW. In a 20 ml test tube, 10 ml aliquot was taken, and 1 ml Nessler's reagent was added. The final volume was maintained with DDW. The optical density was recorded on a spectrophotometer at 525 nm.

#### 2.10 Analysis of proline content

Proline content was calculated according to the method of Bates et al.[48]. 1 g fresh leaf tissues were homogenized in 10 mL of 3% sulphosalicylic acid. 4 ml each of acid ninhydrin and glacial acetic acid was added to the filtrate. The test tubes containing homogenate filtrate were heated on a water bath for one hour, followed by cooling the test tubes in ice-cold water. Afterward, the mixture was extracted with toluene, and the optical density was measured in a spectrophotometer at 520 nm using L-proline as a standard.

#### 2.11 Analysis of S, Cys, GSH content, and ATP-S activity.

For measuring S content, 0.2 g oven-dried leaves were grounded and dissolved in a solution containing 70% strength HNO<sub>3</sub> and 60% strength HClO<sub>4</sub> (85:15, v/v). S content was measured following the turbidimetric method. For turbidity development, 2.5 mL gum acacia solution (0.25%), 1.0 g BaCl<sub>2</sub>, was added to 5 mL aliquot, and the final volume was made 25 mL using DDW. Within 10 min after the turbidity development, the optical density was measured at 415 nm.

The Cys and GSH content was measured according to the method of Giatonde and Anderson [49,50] respectively. In fresh leaves; the ATP-S activity was measured according to the method of Lappartient and Touraine [51].

#### 2.12 Analyses of stomatal behavior

Fresh leaves were fixed with 2.5% glutaraldehyde solution for 4-5 h at room temperature.



Following repeated washing steps using phosphate buffer (15 min at each step), the samples were dehydrated through a graded series of ethanol solutions (60%, 70%, 80%, and 95%) for about 20 min at each step. After that, samples were placed in absolute ethanol. The small sections of dehydrated samples were coated with gold-palladium and observed under the scanning electron microscope (Model; Carl Zeiss EVO 40 ) at a magnification of 250 x and 3000 x. The stomata were visualized using SEM images.

### Statistical analysis

All data were subjected to statistical analysis using one-way analysis of variance (ANOVA), and Duncan's multiple range test was used to compare means of different treatments by IBM SPSS software (version 22.0). No of the replicates for each treatment were four, and data is presented as a mean  $\pm$  SE. The least significant difference (LSD) obtained at levels of  $P < 0.05$  was considered as significant. Different small case letters above bars indicate significant differences at  $P < 0.05$ .

### 3. Results

The effect of the treatment of N, ABA alone, or in combination under NaCl stress was studied. The concentration of ABA was optimized in the preliminary experiment, followed by studying the effectiveness of the role of 25  $\mu$ M ABA and 10 mM N alone or in combination in the presence or absence of 100 mM NaCl. During screening of ABA treatments, it was observed that in absence of NaCl, exogenously supplied ABA (greater than 5  $\mu$ M) significantly decreased photosynthetic and growth attributes but did not influence  $\text{Na}^+$  and  $\text{Cl}^-$  content,  $\text{H}_2\text{O}_2$ , and TBARS in comparison to control plants. However, in presence of NaCl treatment, photosynthetic and growth attributes were favorably influenced by exogenously supplied ABA in comparison to the NaCl stressed plants. Under salt stress, 25  $\mu$ M ABA was most efficient (than 5  $\mu$ M, 10  $\mu$ M and 50  $\mu$ M ABA) in increasing  $P_N$  (108.1%),  $g_s$  (55.6%),  $C_i$  (59%), chlorophyll content (56.6%), LA (50.2%), PDM (60.38%) and in reducing  $\text{H}_2\text{O}_2$  content (59.3%), TBARS (54%), and  $\text{Na}^+$  and  $\text{Cl}^-$  content (32.3% and 38.4% resp ) in comparison to the NaCl stressed plants (**Table 1**).

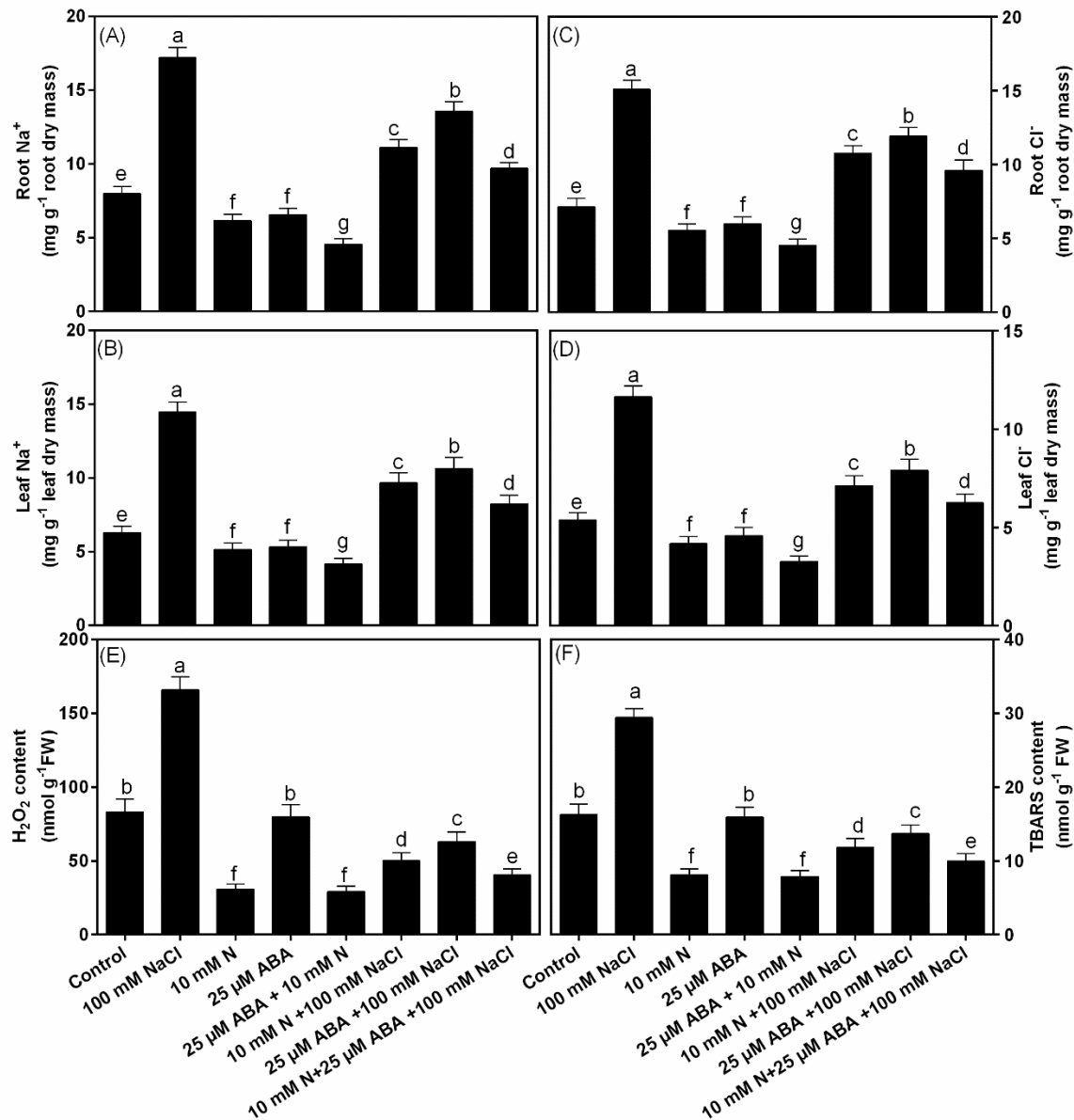
#### 3.1 Impact of N and ABA on $\text{Na}^+$ and $\text{Cl}^-$ accumulation and oxidative stress

The  $\text{Na}^+$  and  $\text{Cl}^-$  content in both leaves and roots of plants supplemented with 10 mM N and 25  $\mu$ M ABA alone or with the combination were analyzed for determining the potency of



N and ABA in inverting the accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  content under salt stress. The  $\text{Na}^+$  and  $\text{Cl}^-$  content increased with 100 mM NaCl, and the accumulation was higher in roots (17.1 mg  $\text{g}^{-1}$  root DW and 15.091 mg  $\text{g}^{-1}$  root DW resp) than in leaves (14.5 mg  $\text{g}^{-1}$  leaf DW and 11.63 mg  $\text{g}^{-1}$  leaf DW resp). Exogenous applied N exerted a positive effect in lowering  $\text{Na}^+$  and  $\text{Cl}^-$  ion accumulation in comparison with both control and salt-stressed plants. Treatment of plants with ABA individually was not effective in lowering the content of these ions under control conditions. The combined treatment of N + ABA further reduced these ion contents; root  $\text{Na}^+$  (43%), leaf  $\text{Na}^+$  (33%), root  $\text{Cl}^-$  (36%), and leaf  $\text{Cl}^-$  (39%) in comparison to control plants.

However, under salt stress, both N and ABA alone or in combination decrease the accumulation of these ions as compared with NaCl treated plants. Under stress condition, N reduced leaf and root  $\text{Na}^+$  accumulation (about 33% & 35.2% resp) and  $\text{Cl}^-$  accumulation (38% & 28% resp) while ABA reduced leaf and root  $\text{Na}^+$  accumulation (26.6% & 21.1% resp) and  $\text{Cl}^-$  accumulation (32.1% & 20.9% resp) in comparison with the NaCl treated plants. The combined treatment of N + ABA + NaCl maximally reduced these ion content as compared to the NaCl treated plant (root  $\text{Na}^+$  (43.7%), root  $\text{Cl}^-$  (36.4%), leaf  $\text{Na}^+$  (43.1%) and leaf  $\text{Cl}^-$  (46.2%) respectively. (**Fig. 1. A-D**)



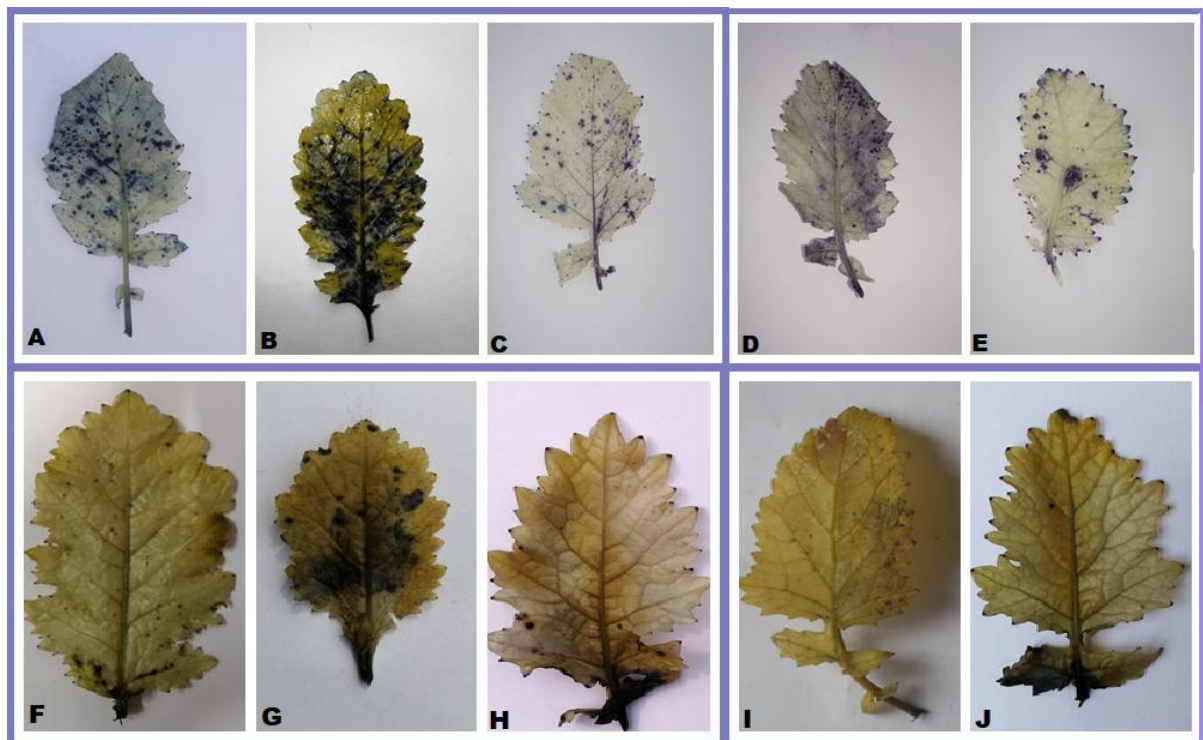
**Fig.1.** Leaf and root Na<sup>+</sup> and Cl<sup>-</sup> content (A-D), TBARS, and H<sub>2</sub>O<sub>2</sub> content (E-F) in RH0-749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100 mM NaCl, 10 mM N, and 25  $\mu$ M ABA or with combined treatment of N + ABA, N + NaCl, ABA + NaCl and N + ABA + NaCl. Data are presented as treatments mean  $\pm$  SE (n=4). The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

Content of H<sub>2</sub>O<sub>2</sub> and TBARS were quantified to evaluate the role of ABA and/or N in the mitigation of oxidative stress. Salt stress plants showed increased values for H<sub>2</sub>O<sub>2</sub> (99.5%) and TBARS (81%) in comparison with the control plants. Plants treated with N or ABA without NaCl showed that only N reduces H<sub>2</sub>O<sub>2</sub> and TBARS contents as compared to control,

and the result of ABA didn't differ with that of control plants. In the presence of NaCl, both N and ABA reduce the  $\text{H}_2\text{O}_2$  (69% & 59% resp) and TBARS (62% & 53% resp), while combined treatment of N + ABA reduced oxidative stress more conspicuously by reducing TBARS (66%) and  $\text{H}_2\text{O}_2$  (75.6%) content in comparison with the salt stress plants. (**Fig., 1. E-F**)

3.2 Generation of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  in leaves using a histochemical staining method and confocal laser scanning microscopy.

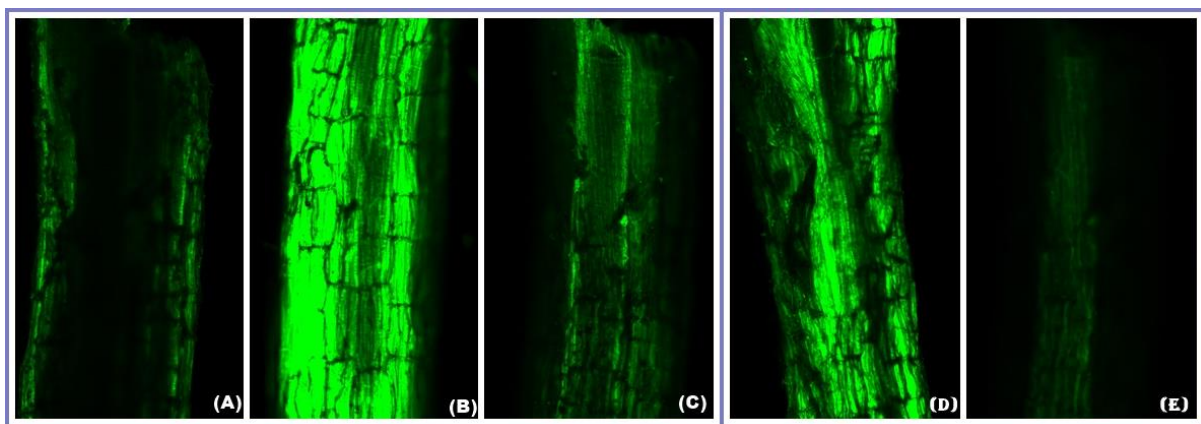
To visualize the oxidative stress in leaves, a histochemical staining method was employed to measure the level of generation of  $\text{O}_2^-$  (as shown by blue staining of leaves) and  $\text{H}_2\text{O}_2$  (as shown by blue staining of leaves) using NBT and DAB staining methods, respectively. The staining spots were more pronounced in NaCl treated leaf discs compared to the control, but restricted staining spots were observed in leaves of plants under salt stress treated with N or ABA alone in comparison with NaCl treated plant leaves. Moreover, N + ABA together more prominently reduced the staining spots in presence of NaCl stress (**Fig.2.A-J**).



**Fig.2.** In-situ determination of the level of generation of superoxide ions ( $\text{O}_2^-$ ) by nitro blue tetrazolium (NBT) staining of the leaves (A-E) and generation of  $\text{H}_2\text{O}_2$  by 3,3'-diaminobenzidine (DAB) staining of the leaves (F-G) in RH-0749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown with 0 (A,F), 100 (B,G), 200 (C,H), 300 (D,I), and 400 (E,J) mM NaCl.

mM NaCl (B,G), 10 mM N + NaCl (C,H), 25  $\mu$ M ABA + NaCl (D,I), and N + ABA+ NaCl (E, J). NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid

In our study, the analysis of dichlorofluorescein (DCF) fluorescence revealed the accumulation of  $H_2O_2$  in the roots. Roots of plants treated with 100 mM NaCl yielded higher intensity of green fluorescence (**Fig. 3. A-E**). However, roots of plants supplemented with N and ABA individually showed less intensity of green fluorescence, though the result was more conspicuous with N. Further, the combined application of N and ABA under salt stress most effectively reduced the  $H_2O_2$  content and thus showed lesser green fluorescence almost similar to observed in roots of control plants.



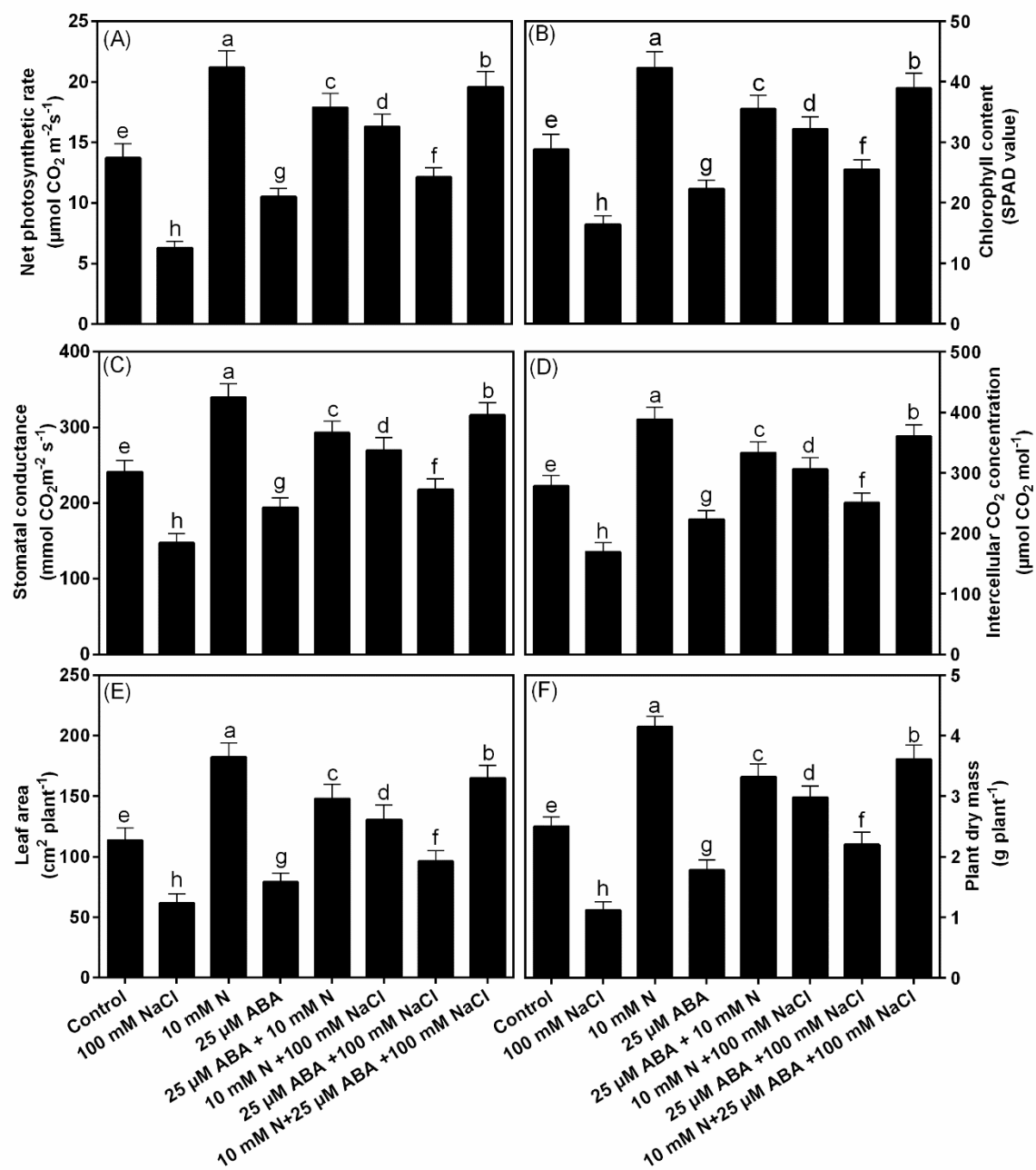
**Fig.3.** Confocal microscopic images of  $H_2O_2$  formation in roots using  $H_2DCFDA$  staining (A-E) at 30 days after sowing (DAS). Plants were grown with 0 (A), 100 mM NaCl (B), 10 mM N + NaCl (C), 25  $\mu$ M ABA + NaCl (D), and N + ABA+ NaCl (E). NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid

### 3.3 Impact of N and ABA on photosynthetic characteristics under NaCl stress

NaCl stress severely affected  $P_N$ ,  $g_s$ ,  $C_i$ , and chlorophyll content. The plants receiving N showed higher photosynthetic characteristics in comparison with the control plants, both under stress and non-stress conditions. Under non-stress conditions, ABA treated plants showed decreased values of photosynthetic parameters. In NaCl stressed plants, exogenous supplementation of ABA or N proved effective in increasing  $P_N$  by 109% and 181%,  $g_s$  by 63% and 101%,  $C_i$  by 52% and 98%, and Chl content by 55% and 96%, respectively, in comparison to the salt stressed plants. The combined supplementation of ABA and N showed a more prominent increase in  $P_N$  by 238%,  $g_s$  by 136%,  $C_i$  by 133%, and Chl content by 137%, in comparison to salt-stressed plants.

### 3.4 Impact of N and ABA on growth under NaCl stress

NaCl stress causes a decrease in LA and PDM, while N supplementation causes an increase in LA and PDM by 52% and 66%, respectively, under the non-stressed condition in comparison to control plants. Exogenously supplied ABA in the absence of NaCl also causes a reduction in LA (30%) and plant PDM (28.8%) in comparison to control plants. However, under NaCl stress condition, ABA or N increased LA by 48% and 115%, while the higher increase was more prominent with the combined application of ABA and N (176% and 222% respectively) in comparison to salt stressed plants. (**Fig 4. E-F**)



**Fig.4.** Net photosynthetic rate (A), chlorophyll content (B), stomatal conductance (C), intercellular CO<sub>2</sub> (D), leaf Area (E), and plant dry mass (F) in RH0-749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100 mM NaCl, 10 mM N, and 25  $\mu$ M ABA or with combined treatment of N+ABA, N + NaCl, ABA + NaCl and N + ABA + NaCl. The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

### 3.5 Impact of ABA and N on $F_v/F_m$ , QP, NPQ, and ETR under NaCl stress

Stressed plants exhibited reduced  $F_v/F_m$  (31%), QP (53%), ETR (47%), and increased NPQ (40.7%) in comparison to control plants. Supplementation of ABA both under control and stress condition decreased  $F_v/F_m$ , QP, and ETR, while increased NPQ as compared to control. Supplementation of N alone increased  $F_v/F_m$ , QP, and ETR, while decreased NPQ both under stress and non-stress conditions in comparison to control. Under stress, N increased  $F_v/F_m$  by 64%, ETR by 134%, QP by 145% respectively, and ABA increased  $F_v/F_m$  by 31%, ETR by 67%, QP by 58%, respectively in comparison to the NaCl treated plants; while supplementation of N or ABA reduced NPQ by 36% and 21% respectively as compared with NaCl treated plants. Further, when ABA + N was supplemented together under stress, it showed more prominent results than individual treatment of N and ABA in comparison to the NaCl treated plants (**Fig.5. A-D**).

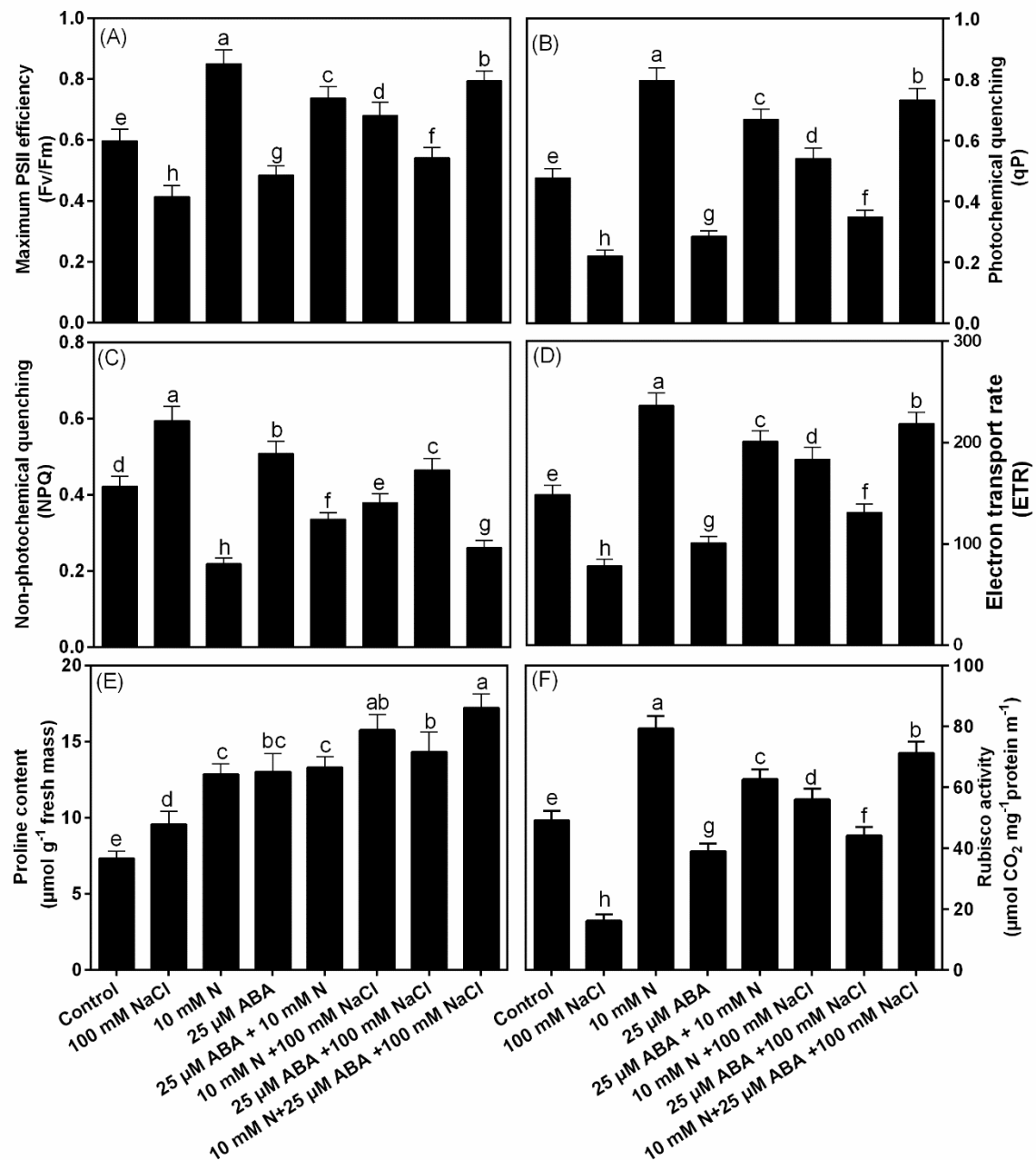
### 3.6 Impact of N and ABA on Proline content and Rubisco activity under NaCl stress

NaCl stressed plants showed increased proline content. Also exogenous supplementation of N and ABA individually showed increased proline content. However, the maximal increase was observed when ABA + N was supplemented together under both stressed, and non-stressed conditions (64% and 80% respectively) in comparison to NaCl treated plants (**Fig 5. E-F**).

NaCl stress severely affected the activity of rubisco in comparison to control plants. The plants supplemented with N showed higher rubisco activity both under control and stressed conditions. Under non-stress conditions, plants treated with ABA showed a reduction in rubisco activity compared with control plants. In stressed plants, supplementation of ABA or N individually was efficient in augmenting the activity of rubisco activity by 173% and 247%, respectively, in comparison to the NaCl treated plants. The supplementation of ABA and N together showed a maximal increase in the activity of rubisco by about 3-fold times in



comparison to the NaCl treated plants (**Fig 5. E-F**).

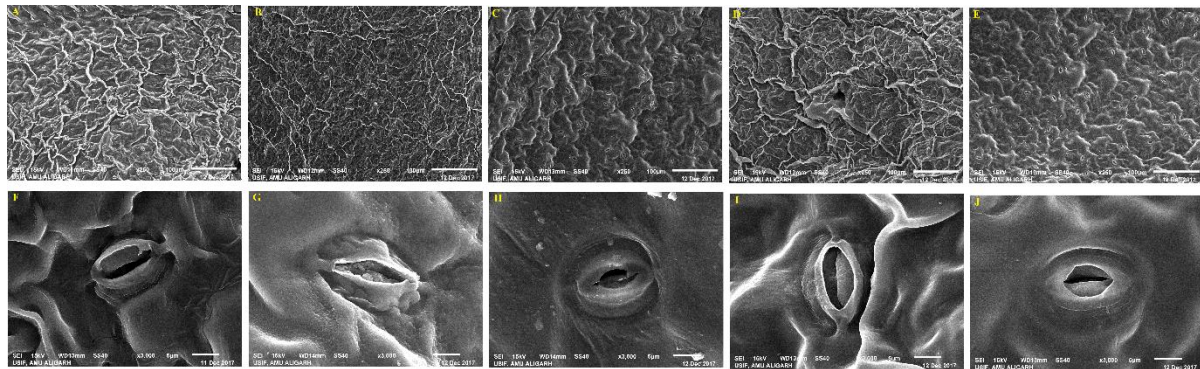


**Fig.5.** Maximum PSII efficiency (A), photochemical quenching (B), non-photochemical quenching (C), electron transport rate (D), proline content (E), and rubisco activity (F) in RH0-749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100 mM NaCl, 10 mM N, and 25 μM ABA or with combined treatment of N + ABA, N + NaCl, ABA + NaCl and N + ABA+ NaCl. Data are presented as treatments mean ± SE (n=4). The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.



### 3.7 Impact of N and ABA on stomatal behavior under NaCl stress

The stomatal behavior was observed with scanning electron microscopy. The leaf samples of NaCl treated plants showed closed stomata, and leaf samples of control plants showed open stomata. The treatment of ABA in the presence of NaCl causes stomatal closure. Plants treated with N alone or N+ABA in the presence of NaCl showed that stomata were partially opened, respectively. ( **Fig 6** )

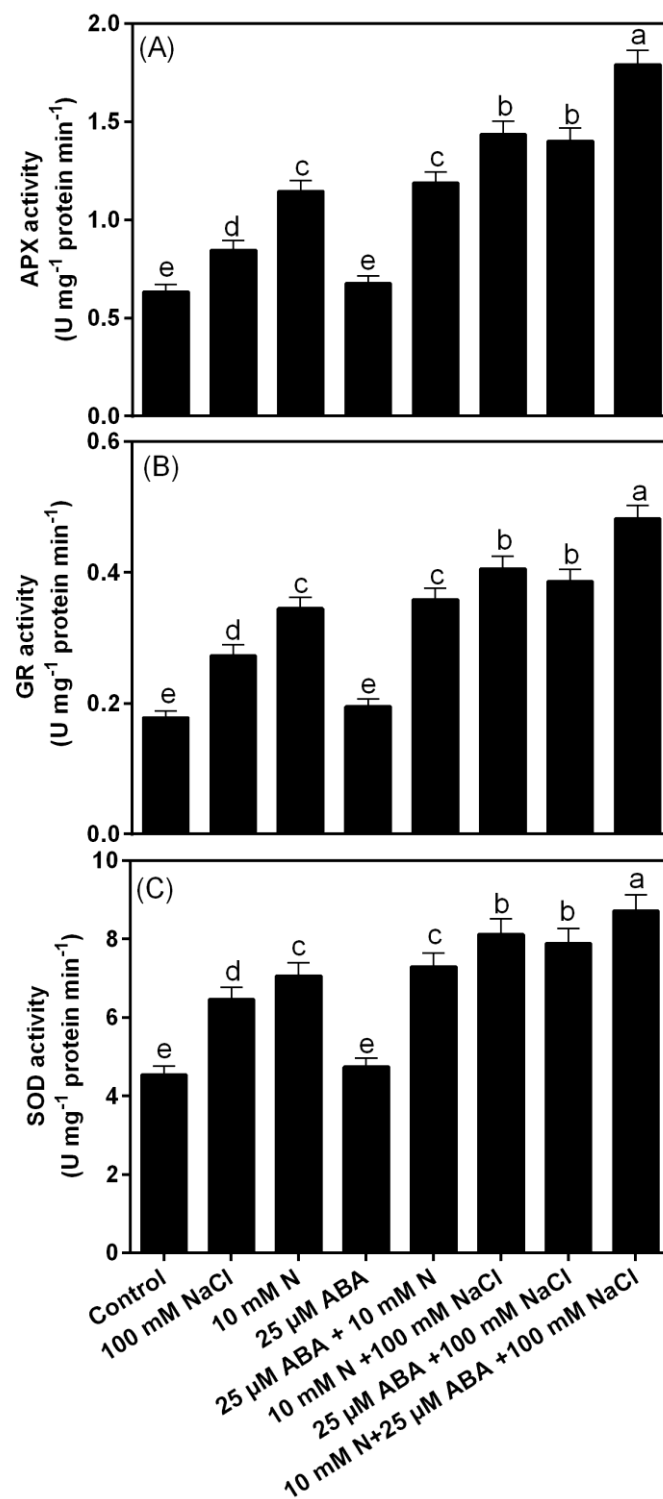


**Fig.6.** Leaf stomatal behaviour of mustard (*Brassica juncea* L.) under control (A,F), 100 mM NaCl (B,G), 10 mM N + NaCl (C,H), 25  $\mu$ M ABA + NaCl (D,I), and N + ABA + NaCl (E,J). The opening and closing of stomata were studied under the scanning electron microscopes at 250x (A-E) or 3000X (F-J) magnifications at 30 days after sowing (DAS). A single representative of each treatment is shown. NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

### 3.8 Impact of N and ABA on antioxidant metabolism under NaCl stress

The activity of APX, GR, and SOD, was measured for assessing their involvement in the antioxidant defense system of plants used against NaCl induced oxidative stress. Under NaCl stress, APX, GR, and SOD activity increased by 33%, 53%, and 42%, respectively in comparison to the control plants. Individual supplementation of N increased the activity of these enzymes both under stress and non-stress conditions. On the contrary individual supplementation of ABA under non-stress conditions does not show any significant change in the activity of these enzymes.

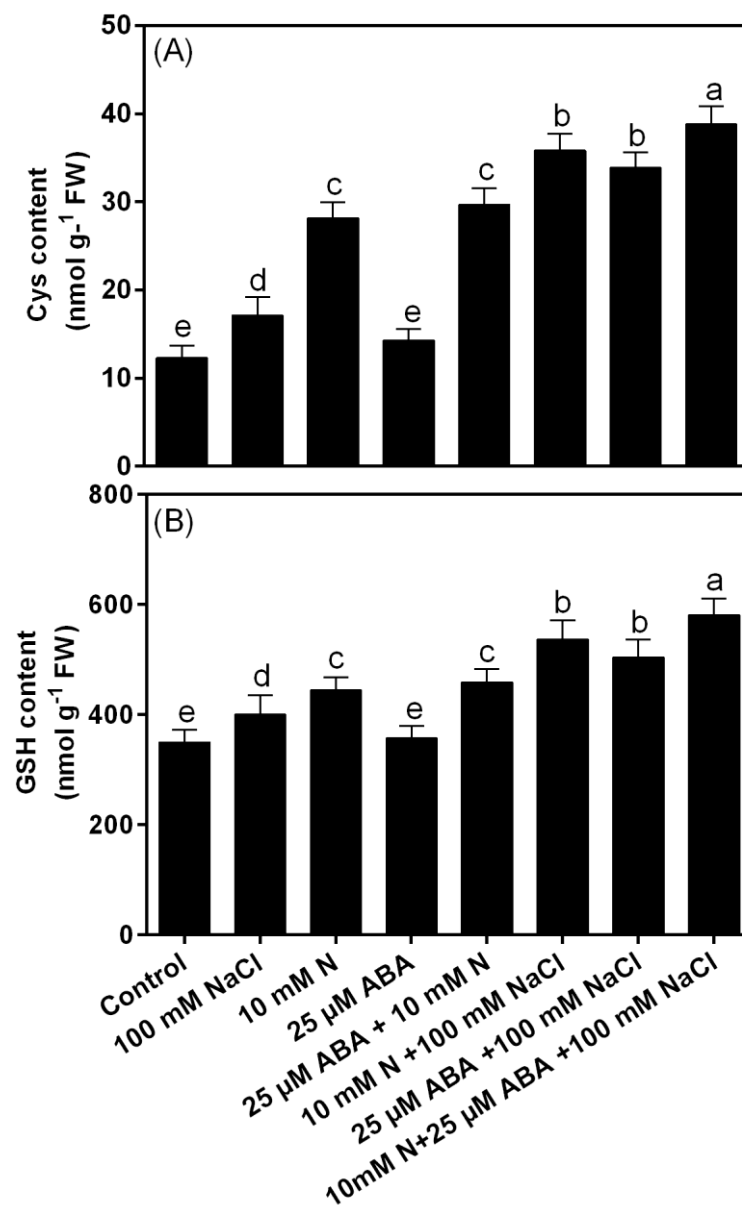
Under stress condition, plants supplemented with ABA exhibited an increase in the activity of APX by 120%, GR by 117%, and SOD by 74%, while plants receiving N exhibited an increase in APX by 120%, GR by 117%, and SOD by 78% in comparison to the control plants. Comparatively more pronounced increase was found when N + ABA were supplemented together than under NaCl stress condition. The combined application of N + ABA increases APX activity by 182%, activity by 175%, and SOD activity by 92% in comparison to the control plants under NaCl stress (**Fig.7. A-C**).



**Fig.7.** APX (A), GR (B), and SOD (C), activity in RH0-749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100 mM NaCl, 10 mM N, and 25  $\mu$ M ABA or with combined treatment of N+ABA, N+NaCl, ABA + NaCl and N + ABA+ NaCl. Data are presented as treatments mean  $\pm$  SE (n=4). The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

### 3.9 Impact of N and ABA on GSH and Cys content under NaCl stress

Under NaCl stress, GSH and Cys content increased by 14% and 39% respectively, in comparison to the control plants. Individual supplementation of N increased GSH and Cys content both under stress and non-stress conditions. However ABA alone under non-stress conditions was not beneficial in inducing GSH and Cys content. Under non-stress conditions, N increased GSH content by 27% and Cys content by 128% in comparison to the control. Under stress conditions, plants supplemented with ABA exhibited an increase in the GSH content by 26% and Cys content by 98%, respectively, while plants receiving N exhibited an increase in GSH content by 34% and Cys content by 109%, respectively, in comparison to the NaCl treated plants. Comparatively more pronounced increase was found under stress when N + ABA were supplemented together than their individual effects. The combined application of N + ABA in the presence of NaCl increases GSH by 45% and Cys by 126% in comparison to the NaCl treated plants. (**Fig 8**)

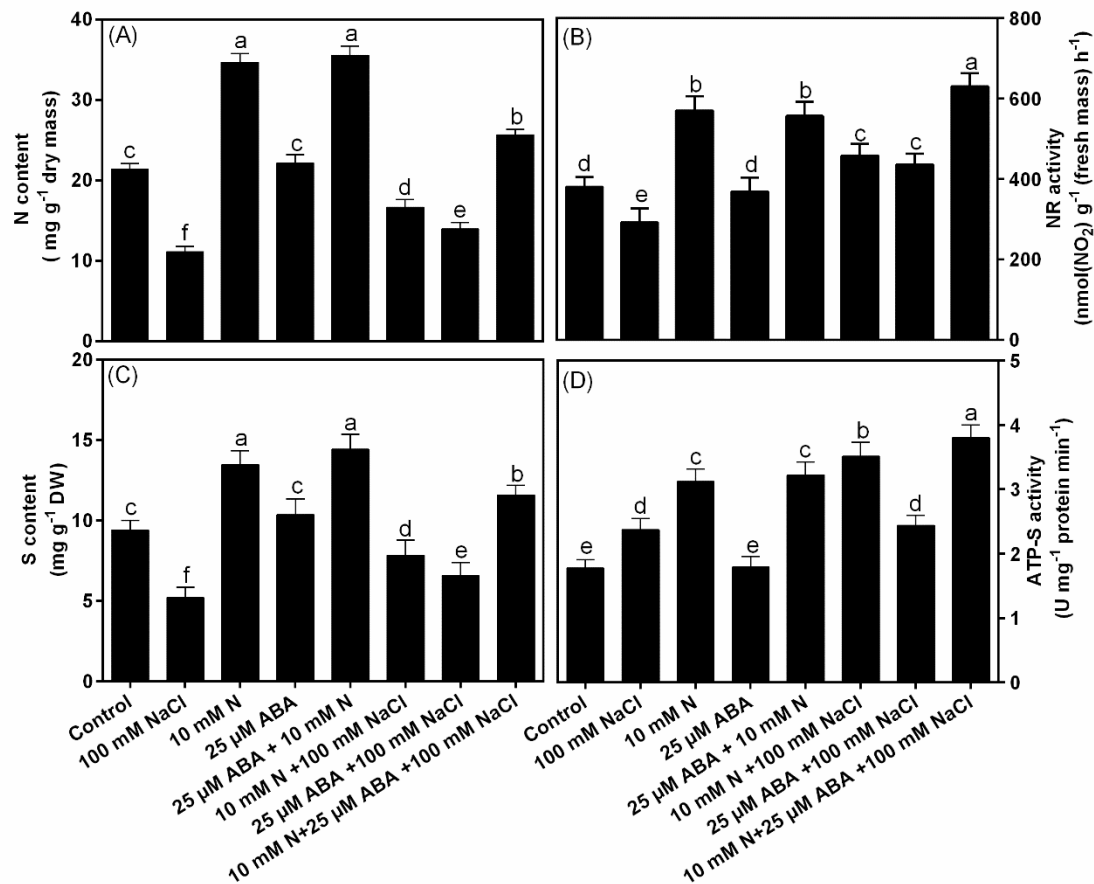


**Fig.8.** Cysteine and GSH content in RH0-749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100mM NaCl, 10mM N, and 25 μM ABA or with combined treatment of N+ABA, N+NaCl, ABA+NaCl and N + ABA+ NaCl. Data are presented as treatments mean  $\pm$  SE (n=4). The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

### 3.10 Impact of N and ABA on NR and N content under NaCl stress

NaCl stress caused the reduction in N content by 48% and NR activity by 23% in comparison to the control plants. Under non-stress condition, supplementation of N alone or

N+ABA increased N and NR content equally by about 62% and 46% respectively, while individual supplementation of ABA did not show any significant increase in N content and NR activity. However, in the presence of NaCl, plants supplemented with ABA or N showed an increase in the N content (25% or 49%) and NR activity (49% or 57%) respectively, while the combined treatment of ABA + N showed more prominent increase in N and NR content by 130% and 116% respectively in comparison to the NaCl treated plants. (Fig. 9. A-B)



**Fig.9.** The content of N (A), S(C), and the activity of NR (B) and ATP-S (D) in RH-0749 cultivar of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Plants were grown individually with 0, 100 mM NaCl, 10mM N, and 25 μM ABA or with combined treatment of N+ABA, N+NaCl, ABA+NaCl and N + ABA+ NaCl. Data are presented as treatments mean ± SE (n=4). The same letter above bars indicates that data did not differ significantly by the Duncan's multiple range test at  $P < 0.05$ . NaCl, Sodium chloride; N, Nitrogen; ABA, Absciscic acid.

### 3.11 Impact of ABA and N on S content and ATP-S activity under NaCl stress

Under NaCl stress, S content decreased by 44%, while ATP-S activity increased by 33%, in comparison to the control. Under non-stress conditions, supplementation of N increased S

content by 43% and ATP-S activity by 76%, while individual supplementation of ABA did not show any significant activity in comparison to the control. Plants receiving N + NaCl showed an increase in S content and ATP-S activity by 50% and 48%, respectively, while plants receiving ABA + NaCl increased S content and ATP-S activity by about 26% and 8%, respectively, in comparison to the NaCl treated plants. However, a more prominent increase was noted with combined treatment of ABA, and N, where the increase of 122% and 60% respectively was observed on comparison to the NaCl treated plants. (**Fig 9.C-D**)

#### 4. Discussion

Salt stress leads to a increased accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$ , which prevents uptake and homeostasis of essential nutrient elements and causes oxidative stress in plants [52,53]. In our study, the increased  $\text{Na}^+$  and  $\text{Cl}^-$  content in leaves and roots, treated with 100 mM NaCl (**Fig.1.A-D**), also causes oxidative stress due to the over-production of ROS. However, this increased oxidative stress enhances the production and activity of the various enzymatic and non-enzymatic antioxidants, which in turn protect plant tissues. In our study also the application of 25 $\mu\text{M}$  ABA or 10 mM N in NaCl stressed plant enhances the activity of APX, GR, and SOD, and considerably decreased the contents of TBARS and  $\text{H}_2\text{O}_2$  (**Fig. 7 A-C; Fig. 1 E-D**). These antioxidants also decline the over-production of ROS, as revealed by the reduced grade of synthesis of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  (**Fig. 2**), determined in leaves and roots (**Fig.3 A-E**). The effect was maximal when 25 $\mu\text{M}$  ABA and 10 mM N was supplied together to NaCl stressed plants. It has been reported that the increase in antioxidants activity has the potency to counterbalance excessive ROS generation and protect plants against NaCl toxicity [54]. Recently it has been shown that N supplementation stimulates the antioxidant defense system of the *Glycine max* [55]. The increase in the activity of the antioxidant enzymes with the application of ABA has been reported in cotton [56], tobacco [57], *Zea mays* L [58,59], sweet potato [60], *C. reinhardtii* [61] under different abiotic stresses. Response reactions induced by ABA to osmotic and salt stresses in higher plants have been well studied [62-64]. Exogenously supplied ABA causes mitigation of oxidative stress, thus resulting in the improvement of plant growth and survival [61]. Under NaCl stress conditions, significant SOD activity was observed in bean plants, proposing that SOD plays a vital role in ROS mitigation by converting  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  [65]. Our results also showed an increase in leaf SOD activities under NaCl stress. Similar results have been noticed in *Beta maritima* and *Beta vulgaris* plants under salt stress [66]. Since SOD transforms  $\text{O}_2^-$  into  $\text{H}_2\text{O}_2$ , and that also acts as ROS; therefore, high SOD activity alone cannot be regarded as accountable for mitigating

salt stress conditions. In our study, N and ABA alone or in combination also increased APX and GR activity in response to salt stress. (Fig.7). Since these are key enzymes of the ascorbate glutathione cycle [67] and therefore have the potential for acclimation to NaCl stress. APX mitigate  $O_2^-$  and  $H_2O_2$  non-enzymatically [42]. The role of glutathione and GR in the  $H_2O_2$  scavenging has been well recognized in the Halliwell-Asada pathway [68]. Several authors investigating salt-sensitive and salt-tolerant cultivars have proposed that salt tolerance is correlated with increased GR activity in salt-tolerant cultivars [69,70].

The reduction in growth and photosynthetic attributes (**Fig. 4**) under salt stress may be due to the overproduction of ROS, as obvious by increased TBARS and  $H_2O_2$  content (**Fig.1 E-D**). ROS interfere with the proper functioning of cell membrane lipids, proteins, and other important enzymes of metabolic pathways and thus, resulted in reduced growth and photosynthetic attributes in *Brassica juncea*. In our study ABA and N limited lipid peroxidation under salt stress as evidenced by a reduced content of TBARS and  $H_2O_2$ . (**Fig 1, E-D**). The supplementation of N showed protective effects on the membrane lipids and mitigated the NaCl induced lipid peroxidation. The present study shows more promising results in enhancing the antioxidant system and lowering the oxidative stress when N and ABA were applied together to NaCl stressed plants. It is likely that ABA grown plants reduced the oxidative stress more efficiently when plants received N through increased antioxidant metabolism. This was apparently because of the higher N-assimilation capacity of plants due to N and ABA treatment under NaCl stress. The present study, therefore, suggests a correlation between N and ABA in plants for alleviating NaCl stress as the greatest alleviation was found with the combined treatment of ABA and N.

In our study, exogenous supplied N increased leaf S and N content as well as S assimilation and N-assimilation as the activity of ATP sulfurylase, NR, and content of Cys was found increased in N supplied plants. (**Fig.8; Fig 9**). It has been reported that exogenous supplementation of N restores the ATP-S activity in nitrogen-deficient medium. [71]. Jamal *et al.* [72] also reported that exogenous supplementation of S enhanced the ATP-sulfurylase and NR activities in *Arachis hypogaea* when compared with plants grown without sulfur. S is linked to the N assimilation pathway and plays an important role in the functioning of NR, as it modulates the flow of  $NO_3-N$  into proteins [73]. The function of S in the regulation of NR is accordant with the earlier finding that L-Cys counteract repression of NR by several non S amino acids in the tobacco cells [74]. Thus sulfur has an important role in modulating NR activity and leaf N content, in addition to its role in modulating ATP-S and leaf S content. In the mustard plant, it has been reported that ATP-sulfurylase activity was low under S



deficiency, and supplementation of sufficient-S increased ATP-sulfurylase activity.[75]. In our study also exogenous supplementation of ABA and/or N enhances ATP-S activity and also increased S, Cys, and GSH under NaCl stress (**Fig. 9 C-D; Fig. 8**). These results, therefore, proposed that ATP-S plays a key role in maintaining Cys and GSH pool required for NaCl stress tolerance in mustard plants. This is in confirmation of the earlier findings that the exogenous supplemented GSH improved salt stress tolerance in *Glycine max* [76]. It has also been reported that GSH improves growth and development by detoxifying NaCl stress [77,78]. With an improved S assimilation pathway, the plant's potential to survive under oxidative stress conditions has been found [79,80]. Fatma et al.[81] have reported that increased ATP-S activity in *B. Juncea* indicated its higher sulfate accumulation capacity with increased PDM and photosynthetic attributes.

Several studies showed a decrease in PDM and LA under different concentrations of NaCl stress [82-84]. The reduction in LA under salt stress might be due to reduced growth as a result of the toxicity of  $\text{Na}^+$  and  $\text{Cl}^-$  in the shoot cells. In our study, exogenous supplementation of N prevented the reduction in LA and PDM (**Fig.4**). Nevertheless, the increased LA and PDM with N was attributed to an increased level of GSH synthesis and thus promoted growth [85]. In our study, NaCl stress severely affected  $\text{P}_\text{N}$ ,  $g_s$ ,  $\text{C}_i$ , and chlorophyll content in comparison with the control. The plants receiving N showed higher photosynthetic characteristics, both under stress and non-stress conditions. (**Fig.4**). This is in confirmation of the earlier findings that exogenously supplied N improve growth and development by increasing photosynthesis, chlorophyll content, proline production, nitrogen metabolism [86,87]. Our results are also in confirmation to the study of Akram and Ashraf [88], who reported that exogenously supplemented N improved growth of the *Helianthus* plant.

Exogenously supplied N triggers the synthesis of compatible solutes such as proline under salt stress conditions, and these compatible solutes have a vital role in an osmotic adjustment [89]. Our results also showed that exogenous supplementation of N and ABA increased proline content(**Fig.3**). However, the maximal increase was observed when ABA + N was supplemented together under both stressed and non-stressed conditions. Proline, besides a compatible solute, also has an important role in scavenging free radicals and protecting redox potential under NaCl stress [89,90]. Per et al. [91] reported that under stress conditions, proline and other compatible solutes are definitely regulated by phytohormones in addition to mineral nutrients. Hasanuzzaman et al. [92] reported that under salt stress exogenously supplemented proline mediate the upregulation of genes associated with the antioxidant

defense system, thus protecting rice seedlings from oxidative damage. Aleksza et al. [93] reported that proline biosynthesis is inflected by crosstalk amongst ABA signaling and phosphate homeostasis regulation through activation of the P5CS1 gene. It has been reported that both ABA and NaCl stress induces the activation of the P5CS1 gene in *Arabidopsis* [94], proving that proline accumulation is strongly dependent on salt stress and ABA, which is due to the activation of the P5CS1 gene.

Oxidative stress interrupts the structural and functional integrity of photosynthetic systems and reduces the efficiency of PSII and activity of Rubisco [95-96]. In our study, the NaCl stress reduced gas exchange parameters, Rubisco activity, and Chl content. It was, however, found that the supply of ABA or/and N improved these characteristics under salt stress. (**Fig. 4 A-D; Fig. 3**). The exogenous supply of N and ABA alone or in combined application favored N assimilation and GSH synthesis. This accumulatively shielded chloroplast and enzymes of the C3 cycle. The relationship between rubisco activity and S allocation in leaves has been shown [97]. It seems that the improved  $P_N$ ,  $g_s$ ,  $C_i$ , and chlorophyll content observed in our study was due to the recovery of photosynthetic efficiency resulted from ABA and N application. The efficiency of photochemical processes is provided by measuring Chl fluorescence in leaves. In the present study, Chl fluorescence parameters were decreased under salt stress, which contributed to the decrease in photosynthesis except non-photochemical quenching. NaCl stress reduced PSII efficiency (**Fig.5 A-D**). This observation is in good correspondence with the enhanced rate of lipid peroxides formation in leaves under NaCl stress.

Scanning electron microscopy study revealed the potential of ABA and N on stomatal responses of plants. In our study, comparatively closed stomata were found in NaCl treated plants than the control plants (**Fig. 6**). Treatment with N reduced the closing effect of NaCl stress on stomata. Combined treatment of ABA and N also reduced the effect of NaCl on stomatal width aperture. Under salt stress, the synthesis of ABA in leaves causes closure of stomata thus helps in protecting the plants against transpiration [98]. Generally, it has been found that ABA induces stomatal closure, but it has been found that an increase in intracellular GSH suppresses stomatal closure [99]. The combined treatment of ABA and N eminently influences GSH by modulating the production of GSH that resulted in the opening of stomata as compared with salt-treated plants. It is interesting to note that stomatal closure occurs by reducing intracellular GSH and that intracellular GSH regulates stomatal behavior [100]. ROS production in guard cells is required for stomatal [100,101]. This study, therefore, indicates the role of intracellular GSH in N and ABA-mediated regulation of NaCl-induced

ROS production and stomatal closure. S affects ABA biosynthesis through the availability of Cys [102]. Cys is also the substrate of ABA3, a Moco-sulfurylase that is crucial for AAO3 activity and for ABA synthesis in plants [103]. Therefore, AAO3 activity might link ABA biosynthesis via ABA3 to sulfur metabolism and thus to N metabolism. ABA is also known to impact S homeostasis by increasing the levels of GSH, an intermediary in S metabolism that has a critical role in maintaining the redox state of *Arabidopsis* [104].

## 5. Conclusion

In conclusion, the results indicate that NaCl stress severely affected photosynthetic attributes, plant growth and induced oxidative stress due to the over-production of ROS. Exogenously supplied N enhances the activity of antioxidant enzymes (SOD, APX, and GR), which in turn improve photosynthesis and growth parameters under normal as well as NaCl stress conditions. However, ABA supplementation improved these attributes only under stress conditions. In comparison to the individual influence of N and ABA, their combined application proved to be most effective in combating NaCl-induced toxic effects on photosynthesis and growth of plants. The positive influence of the combined application of N and ABA was through their effect on osmolytes, antioxidant enzymes, N and S-assimilation, and GSH production. The precise regulatory mechanism of ABA and/or N-induced NaCl stress tolerance requires to be examined.

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