

# Salicylic Acid Increases Photosynthesis of Drought Grown Mustard Plants Effectively with Sufficient-N via Regulation of Ethylene, ABA and Nitrogen-Use Efficiency

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## Research Article

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# Abstract

An essential approach to reduce drought in plants is to maximize the use of most limited resources. The increase in water-use efficiency (WUE) is important to maximally utilize the available water to increase photosynthesis and growth of plants under water-deficit stress. Both WUE and photosynthetic nitrogen use efficiency (PNUE), as the indices of resource-use efficiency were studied in mustard (*Brassica juncea* L.) plants grown under limited water conditions with low-N (100 mg N kg<sup>-1</sup> soil) and sufficient-N (200 mg N kg<sup>-1</sup> soil) N and sprayed with 0- and 0.5 mM salicylic acid (SA). Application of SA increased water potential, osmotic potential, WUE and incorporation of soil N into photosynthetic machinery by enhancing PNUE. It also increased photosynthesis of plants maximally by increasing stomatal conductance and intercellular CO<sub>2</sub> concentration under water-deficit stress. This increase was greater in the presence of sufficient-N where 0.5 mM SA maximally enhanced the N-metabolism, redox ratio that mitigated the oxidative stress. The application of SA on plants supplemented with N reduced ethylene and abscisic (ABA) synthesis. It could be inferred that SA enhanced N utilization at its optimal level to maintain redox ratio and inhibit ABA-mediated stomatal closure to enhance the resource utilization and photosynthesis. SA also enhanced glucose utilization which prevented photosynthetic repression by enhanced glucose under stress. Thus, SA application may impart a potential management tool for increasing photosynthetic NUE, WUE and photosynthesis under drought.

## Introduction

Drought is a severe environmental stressor that has a negative impact on plant growth and development. It can become chronic in climatic regions with limited water resources or in areas where rainfall is erratic (Tian and Lie 2006; Nawaz et al. 2014; Khan et al. 2020; Sehar et al. 2020; Seleiman et al. 2021). The rising severity of drought has been forecasted and thus, implied scientific inputs for sustainable agricultural productivity. Plants induce cellular mechanisms to minimize water loss upon drought condition to sustain growth and productivity. The primary mechanism is stomatal closure and reduced transpirational flux, but this would lead to reduced photosynthesis and overall crop yield loss due to less carbon availability in stroma of chloroplast (Mittler 2002). Another approach could be the increase of water use efficiency (WUE) to reduce water use under drought conditions. Plants adapt to the changing water limited conditions through utilizing the available resources for maximizing photosynthesis and growth (Weiss 2000). Nitrogen (N) is an essential nutrient element requisite for the synthesis of nucleic acids, proteins, enzymes, and pigment system and cell wall, and is therefore, required for normal growth and development of plants (Krapp 2015; Leghari et al. 2016; Souza and Tavares 2021). Iqbal et al. (2015) found that the availability of N to plants differently affects plants metabolism, while Nobel (1999) reported a linear relation between photosynthesis to leaf N; for some crops approximately 75% of leaf N was invested in photosynthesis. Moreover, a relationship between N and photosynthesis has also been reported to be influenced by water availability and plant growth regulators (Lawlor 2001).

Plant growth regulators (PGRs) improve physiological processes including photosynthesis at different nutrients availability (Lawlor 2001; Ahanger et al. 2014; Jahan et al. 2020). Additionally, salicylic acid

(SA) interacts with other signaling molecules and regulates various physiological and morphological responses in plants (Khan et al. 2015; Hussain et al. 2021). Per et al. (2017) reported the role of SA in regulating nutrients availability under abiotic stress. In mungbean, SA application was reported to enhance N and sulfur (S)-assimilation (Nazar et al. 2015), whereas SA raised the rate of calcium (Ca), magnesium (Mg), and potassium (K) in shoots and roots in arid circumstances and reduced the harmful effects of drought stress in wheat (El-Tayeb and Ahmed 2010). SA also interacts with N assimilation, affects proline metabolism and photosynthesis (Khan et al. 2015). Thus, increased SA synthesis under drought stress (Hussain et al. 2021; Kaya 2020; Khalvandi et al. 2021) might play a role in acclimation of plant through maximum utilization of the available water resources.

It is noteworthy that hormonal signals crosstalk under drought condition (Khan et al. 2020) may cooperate to govern drought stress responses, such as ethylene and ABA accumulation regulate many developmental processes and adaptive stress responses (Nazar et al. 2015; Sehar et al. 2020; Takahashi et al. 2020). Drought stress rapidly induces the expression of 9-cis-epoxycarotenoid dioxygenase 3 (NCED3), which is important in ABA synthesis (Behnam et al. 2013). Further, it has been reported that overexpression of *NCED3* enhanced water use efficiency (WUE) in several plant species while mutations in *nced3* reduced drought tolerance (Luchi et al. 2001; Tung et al. 2008). According to Ali et al. (2020), ABA improved drought tolerance in plants via regulating stomatal closure. Alam et al. (2013) reported that SA alleviated short-term drought stress in mustard seedlings via upregulating the antioxidant system.

Similarly, ethylene biosynthesis is stimulated under drought stress (Apelbaum and Yang 1981; Morgan and Drew 1997; Nazar et al. 2015); however it is stress ethylene that has been brought to an optimum level to initiate maximum utilization of available resource. Ethylene has been found to play a role in the auxin-induced opening of stomata in *Vicia faba* (Merritt et al. 2001) and in modulation of stomatal closure induced by ABA (Fatma et al. 2021). It has been shown that using ethephon (an ethylene-releasing compound) enhanced stomatal conductance, resulting in an increase in photosynthesis (Iqbal et al. 2012). Desikan et al. (2006) have found that H<sub>2</sub>O<sub>2</sub> generated by NADPH oxidase *AtrbohF* was responsible for ethephon induced stomatal closure in *Arabidopsis*. Thus, it may be said that under drought stress hormonal interaction might occur to regulate stomatal opening and photosynthesis. In the present study, the influence of SA and its coordination with ethylene and ABA has been studied on use efficiency of water and N and glucose accumulation to regulate stomatal conductance and protect photosynthetic functions of plants under drought. We hypothesized SA promotes photosynthesis of plants under drought by its effect on ethylene and ABA synthesis to regulate stomatal conductance and photosynthetic NUE. Besides, SA also increases the antioxidants to reduce oxidative stress and maintain redox state and helps utilization of glucose by plants to inhibit excess glucose-mediated photosynthetic repression. We, therefore, tested the influence of SA and N on stomatal conductance and measured photosynthetic N use efficiency (PNUE: net photosynthesis/N per unit leaf area) and intrinsic water-use efficiency (WUE: net photosynthesis/stomatal conductance), with associated changes in ethylene and ABA content of mustard under water-deficit stress.

# Materials And Methods

## Plant material and experimental conditions

Seeds of mustard (*Brassica juncea* L. Czern & Coss. cv. Pusa Jai Kisan) were sown in pots (23 cm diameter) filled with 5 kg soil which had ratio of sand, clay and peat as 70:20:10 on a dry weight basis. These potted plants were placed in a greenhouse with natural day/night conditions. The photosynthetically active radiation was  $700 \pm 28 \mu\text{mol m}^{-2} \text{s}^{-1}$  and the average temperature for day and night was  $23/17 \pm 2 \text{ }^\circ\text{C}$  for plant growth. The experimentation was carried out at the Botany Department, Aligarh Muslim University, Aligarh, India. Soil used was sandy loam of pH 8.0 in 1:2 soil/water mixture. Available  $\text{NO}_3\text{-N}$  in the soil was  $100 \text{ mg kg}^{-1}$  soil. Urea was used as N source and 0 or  $100 \text{ mg N kg}^{-1}$  soil was applied before sowing the seeds. Thus, the available soil N was 100 (low-N, control) and 200 (sufficient-N)  $\text{mg N kg}^{-1}$  soil, respectively. After seedling emergence, four plants per pot were maintained. Drought stress was imposed by withholding water in all pots to 50% of field capacity after 15 days of sowing (DAS). The weight of the pot with the amount of water needed for the desired soil moisture regime was computed. To avoid water entry during rainfall rain-out shelters were used. These shelters were removed after rainfall to provide pots with maximum sunlight. During night, the pots were regularly covered with rainout shelters to protect pots from rainfall or any moisture entry from outside. SA was supplemented after dissolving in absolute ethanol then it was added drop wise to water so that ethanol/water was maintained at 1/1000 v/v. The amount of SA applied was 30 ml per plant and was applied at 20 DAS on the plant's foliage. The concentration of SA was 0 or 0.5mM and was applied with a hand sprayer. The experiment was carried out following a randomized complete block design. Four replicates were taken for each treatment and the parameters were studied after 30 days of sowing (DAS). Leaves of the same age were taken for determinations.

The leaves on which gas exchange parameters, water, and osmotic potential measured 10 days after SA application were collected to form a composite sample from the four replicates of each treatment to estimate other parameters.

## Determination of photosynthetic parameters

Gas exchange parameters as net photosynthetic rate, stomatal conductance and intercellular  $\text{CO}_2$  concentration were determined using Infra-Red Gas Analyzer (CI-340 Photosynthesis system, CID Bioscience, USA) in fully extended leaves. The measurements were done between 10.00-11.30 a.m. at light saturating intensity on a bright sunlight day. The atmospheric conditions at the time of measurements were PAR,  $\sim 740 \mu\text{mol m}^{-2} \text{s}^{-1}$ , air temperature,  $\sim 22 \text{ }^\circ\text{C}$  and relative humidity,  $\sim 70\%$ .

With the help of chlorophyll fluorometer (Os-30p, USA), chlorophyll fluorescence (Fv/Fm) of fully expanded leaf was measured *in vivo* after 0.5 h dark adaptation of leaves.

The ratio of photosynthetic rate to N content per unit leaf area was measured as PNUE, where leaf area was measured using leaf area meter (LA 211, Systronic).

According to Von Caemmerer and Farquhar (1981), the WUE was computed as the ratio of net photosynthetic rate to stomatal conductance to avoid effects of small changes in vapour pressure between measurements.

Chlorophyll content was measured with the help of SPAD chlorophyll meter (SPAD 502 DL PLUS, Spectrum Technologies, USA).

After adding enzyme extract to the assay medium, the activity of ribulose 1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39) was determined spectrophotometrically using the method of Usuda (1985) by monitoring NADH oxidation at 30°C at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate.

### **Abscisic Acid Determination**

The content of ABA was estimated by adopting the method of Hung and Kao (2003) with small modifications. The details are given in Fatma et al. (2021) and provided in the Supplementary File 1.

### **Measurement of ethylene evolution**

Ethylene was measured according to the process of Fatma et al. (2021) and details are provided in the Supplementary File 1.

### **Determination of H<sub>2</sub>O<sub>2</sub> and TBARS Content**

H<sub>2</sub>O<sub>2</sub> and TBARS content were determined using Okuda et al. (1991) and Dhindsa et al. (1981) techniques, respectively. The details are described in Iqbal et al. (2017) and provided in the Supplementary File 1.

### **Activity of glutathione reductase and content of reduced and oxidized glutathione and redox state**

Glutathione reductase (GR) activity was measured using Foyer and Halliwell (1976) method which involved the measuring the GSH-dependent oxidation of NADPH at 340 nm. The assay mixture contained phosphate buffer (25 mM, pH 7.8), 0.5 mM oxidized GSH, 0.2 mM NADPH, and the enzyme extract. The extinction coefficient at  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$  was used for the determination of GR activity. The amount of enzyme required for breakdown 1  $\mu\text{mol}$  of NADPH/min at 25°C.

Content of reduced glutathione (GSH) and oxidized glutathione (GSSG) was examined through the procedure described by Griffith (1980). Details are there in the Supplementary File 1. Redox state as the ratio of GSH/GSSG was calculated.

## **Leaf water potential and osmotic potential**

Using a water potential apparatus, the water potential of the plant's second leaf (completely expanded youngest leaf) was measured (Psypro, WESCOR, USA). The leaf used for water potential testing was frozen in liquid nitrogen and then thawed in sealed polythene bags, with cell sap removed using a disposable syringe. The extracted sap was used for the determination of osmotic potential using a vapour pressure osmometer (5520, WESCOR, USA).

## **Determination of nitrate reductase activity and N content**

The method of Kuo et al. (1982) was utilized to estimate the nitrate reductase (NR) activity (EC, 1.6.6.1) in leaf by making enzyme extract, which was then spectrophotometrically measured as the rate of nitrite formation at 28°C using the process of Nakagawa et al. (1984). The detailed process has been described (Iqbal et al. 2012). Lindner's (1944) approach was used to determine the leaf N content in a plant. Detailed analysis provided in the Supplementary file 1.

## **Determination of glucose content**

The glucose content of leaves was determined using the Krishnaveni et al. (1984) method and glucose as the standard. Details of the process was given in Iqbal et al. (2021) and provided in the Supplementary File 1.

## **Determination of relative growth rate**

Relative growth rate (RGR) was calculated at 20-30 d intervals using the formulae of Watson (1952).

## **Statistical analysis**

Data were assessed statistically using analysis of variance (ANOVA) by SPSS statistics (ver. 17.0) and represented as treatment mean  $\pm$  SE (n=4). Least significant difference (LSD) was calculated for the significant data at  $P < 0.05$ . Bars showing the same letter are not significantly different by LSD test at  $p < 0.05$ .

# **Results**

## **Influence of SA on biosynthesis of ABA and ethylene with N availability under drought**

Treatment of drought stress with low-N increased ABA content which decreased by addition of sufficient-N. Sufficient-N decreased ABA content by 28.0% under drought stress compared to low-N. SA applied to low-N grown plants under drought stress, a decrease of 9.45% in ABA content was observed, while plants

receiving sufficient-N and SA maximally decreased ABA content by 38.1% compared to plant that had low-N and no SA (control) (Fig. 1).

Ethylene was maximally present in the drought stressed low-N plants. Addition of N decreased ethylene evolution by 22.6%. Ethylene evolution decreased by 10.7% in plants receiving SA and low-N, but sufficient-N and SA was most promising in reducing stress ethylene. SA with sufficient-N decreased stress ethylene by 33.5% compared to control (Fig. 1).

### **Influence of SA on photosynthetic characteristics of low and sufficient-N grown plants under drought**

The interaction between SA and N under drought stress increased photosynthetic characteristics more promisingly when N was sufficient. Photosynthetic characteristics of plants with low-N (soil native N as  $100 \text{ mg kg}^{-1}$ ) were the lowest. Addition of N (sufficient-N) increased net photosynthetic rate by 36.4%. However, SA supplementation to low-N and sufficient-N grown plants increased photosynthetic rate by 17.7% and 58.3% in comparison to the low-N and no SA control (Table 1). Similar trend was observed in stomatal conductance which increased from 15.2% to 53.3% when SA was applied to plants receiving low-N and sufficient-N, respectively compared to low-N and no SA control. Without SA, sufficient-N increased stomatal conductance by 32.4% but with SA maximum increase in stomatal conductance was observed in comparison to control. The intercellular  $\text{CO}_2$  concentration increased when SA was given to plants and higher increase was observed in sufficient-N treated plants compared to plants receiving low-N and SA. Intercellular  $\text{CO}_2$  concentration increased from its lowest concentration of  $\sim 183 \mu\text{mol mol}^{-1}$  for the control to  $\sim 286 \mu\text{mol mol}^{-1}$  for the plants receiving sufficient-N and SA (Table 1) which was 55.9% increase compared to low-N and no SA control.

The chlorophyll fluorescence parameters increased with SA, N and interaction of SA and N. Maximum increase in the values of maximum efficiency of PSII was found with the application of SA to sufficient-N grown plants. However, in comparison to the low-N and no SA control, application of either SA and/or N increased the chlorophyll fluorescence, but lesser than the plants supplied with SA and sufficient-N.

SA application was effective in increasing chlorophyll content by 55.6% and Rubisco activity by 68.3% in sufficient-N grown plants, and lower increase of 13.2% and 29.3% in low-N grown plants. Increased chlorophyll content and Rubisco activity by 32.1% and 43.9% was found with plants grown with sufficient-N compared to low-N grown plants which increased by the addition of SA.

Sufficient-N grown plants exhibited more prominent increase in PNUE and WUE by 35.1% and 38.1% compared to low-N grown plants. SA applied to plants with low-N, an increase of 18.2% and 29.7% in PNUE and WUE was observed, however a higher increase of 53.0% and 52.0% was observed when SA was given to sufficient-N grown plants in comparison to the control (Table 1).

### **Influence of SA on water relations under drought condition and N availability**

Application of SA under drought stress increased water potential and osmotic potential in comparison to control (no SA or low-N). Application of sufficient-N increased water potential and the osmotic potential by 33.3% and 35.20% compared to control. SA application to low-N receiving plants increased water potential and osmotic potential by 16.7% and 23.5%, while SA application to sufficient-N plants showed maximum increase in water potential and osmotic potential (Table 1).

### **Impact of SA with sufficient-N on glucose content under drought stress**

Glucose content decreased with the addition of N under drought stress. Salicylic acid treatment to low-N or sufficient-N also decreased the glucose content compared to without SA control. Application of SA reduced glucose content in sufficient-N grown plants by 40.9% and in low-N grown plants by 18.2% compared to low-N and no SA control (Table 1).

### **Salicylic acid maintains NR activity and N content under drought stress on N availability**

NR activity and content of N were influenced with SA application and more positively when SA was applied to sufficient-N supplemented plants. Nitrogen alone increased NR activity and N content by 43.5% and 52.0% compared to the respective control. SA increased NR activity and N content by 14.6% and 22.6% in low-N grown plants, it maximally increased by 63.6% in NR activity and 67.3% in N content when N was sufficient in comparison to control (Fig. 2).

### **Influence of SA on oxidative stress in plants with varying N levels**

Under conditions of drought stress and low-N, plants exhibited maximum oxidative stress. Presence of sufficient-N decreased H<sub>2</sub>O<sub>2</sub> and TBARS content by 54.6% and 70.0%, respectively compared to the control plants. However, SA application profoundly decreased H<sub>2</sub>O<sub>2</sub> and TBARS content by 22.6% and 61.3% in plants that received low-N, while maximal decrease of 67.4% and 77.3% was observed in plants on sufficient-N availability (Fig. 3).

### **Salicylic acid modulates GR activity, glutathione pool and redox ratio in presence of sufficient- N under drought stress**

Plants grown with sufficient-N showed increased activity of GR by 28.0% (Fig. 4) compared to low-N grown plants (Fig. 4). SA application increased the GR activity by 16.0% in low-N and 52.0% in sufficient-N grown plants compared to control.. SA supplementation to low-N grown plants increased GSH content by 42.3% and maximum increase in GSH content was noted when SA was applied to sufficient-N which was 1.4 times of low-N and SA treatment signifying the importance of N in SA-mediated responses. The GSSG content decreased with the application of N. Maximum GSSG content was observed in plants which received low N. Supplementation of SA to low N plant decreased GSSG content by 53.4% while in the presence of sufficient N it was decreased by 57.5% compared to low N and no SA control (Fig. 4).

The redox state increased with sufficient-N and maximally when sufficient-N grown plants were supplemented with SA. Application of SA increased redox state by 5 times with sufficient-N, and by 3

times with low-N compared to the low-N and no SA control (Fig. 4).

### **Effect of SA and N availability on leaf area and relative growth rate under drought stress**

The effect of SA was prominently observed in the presence of sufficient-N compared to low-N. SA increased leaf area in both low-N and sufficient-N grown plants but more conspicuously in plants receiving sufficient-N. SA supplementation increased leaf area by 33.9% in low-N plants and by 113% in plants receiving sufficient-N. Similarly, SA increased RGR by 38.5% in low-N receiving plants while it maximally enhanced the RGR when sufficient-N was present compared to the control. In plants with sufficient-N, SA increased the RGR by 2 times compared to low N and no SA control signifying the importance of SA with sufficient-N in improving plant growth rate (Fig. 5).

## **Discussion**

Drought stress is an inevitable factor that exists in various environments and hampers photosynthetic capacity and plant biomass production (Seleiman et al. 2021). In response to drought plants stomatal closure, according to Galle et al. (2009), is the first and most noticeable barrier to CO<sub>2</sub> uptake because CO<sub>2</sub> transport from the atmosphere to the sites of carboxylation in the chloroplast is hampered. The closure of stomata leads to reduced WUE. In case of drought and stomatal closure, plants maximize the use efficiency of the most limited resource to adjust to stress situations. The present study discusses the importance of SA with low or sufficient-N to enhance the use efficiency of most limited resources. The favorable effects of SA application on photosynthesis and WUE in the present study (Table 1) may be attributed to increased stomatal conductance with lower oxidative stress, higher GR activity and redox state, and enhanced accumulation of nitrogen with lower glucose-mediated photosynthetic repression.

In both natural and agricultural situations, nitrogen regulates the number, structure, and composition of the photosynthetic apparatus, and hence plays a critical role in defining the photosynthetic capacity of plants (Abrol et al. 1999; Kumar et al. 2002). Nazar et al. (2015) reported the influence of SA in enhancing the photosynthetic NUE in mustard under drought. Kumar et al. (2010) found that SA increased chlorophyll content, total non-structural carbohydrate and total nitrogen content. It also increased NR activity and nitrate assimilation in isolated cucumber cotyledons. Kaya (2020) found that in pepper under drought, nitrate reductase is required for SA-induced drought tolerance through upregulation of ascorbate-glutathione pathway and glyoxylase system. Gunes et al. (2007) reported that SA stimulated the concentrations of mineral nutrient such as phosphorus (P), potassium (K), magnesium (Mg), and manganese (Mn) in *Zea mays* under water deficit and boron stress. Supplementation of SA enhanced the activities of the enzymes of the N metabolism pathway and alleviated chromium (Cr) stress (Sangwan et al. 2015). Recently, Hussain et al. (2021) reported that both SA and S helped in alleviating salinity stress by enhancing N metabolism and increasing antioxidative machinery.

Thus, SA plays a prominent role in regulating N assimilation. SA enhanced both the content of N and the activity of NR under drought stress and more prominently when sufficient-N was available. The higher increase in photosynthesis with SA application in sufficient-N supplied plants in the present study was attributed to the enhancement in PNUE (Table 1) allowing greater allocation of N to Rubisco. By increasing N allocation to the photosynthetic machinery, SA can increase photosynthesis. The increase in WUE with both SA and N application may be due to increased binding of CO<sub>2</sub> and less binding of O<sub>2</sub> to the active site of Rubisco as CO<sub>2</sub> concentration increased. The increased CO<sub>2</sub> concentrations could improve the drought resistance of mulberry seedling leaves by increasing their WUE and improving their PSII function (Liu et al. 2019). Thus, under conditions of drought stress, SA increased stomatal conductance and WUE resulting in increased photosynthesis. Moreover, plants with low-N under drought showed reduced photosynthesis perhaps due to glucose mediated photosynthetic repression. Endogenous glucose levels are reported to inhibit Rubisco transcript levels reducing photosynthesis and growth (Tholen et al. 2007). Hussain et al. (2021) reported that SA and S under salt stress reduced the glucose content and enhanced Rubisco activity to enhance the photosynthetic efficiency. SA increased GSH synthesis which reduced the oxidative stress and increased growth which consumed the available glucose and decreased its content. Iqbal et al. (2012, 2021) and Sehar et al. (2019) reported that higher glucose content represses photosynthesis and decrease in glucose with nitric oxide under stress was because of its greater utilization in growth; our findings also show similar results. The relative growth rate was found to increase with SA application suggesting that SA is utilizing the glucose to improve growth under stress. Sufficient-N and SA combination had lowest glucose content but highest photosynthesis and leaf area and growth.

The increased activity of GR, GSH content and redox ratio was responsible for the reduction of oxidative stress and enhancement of photosynthesis and growth. GSH behaves as an antioxidant to reduced oxidative stress and its formation is associated with SA supplementation. Plants require antioxidants to protect them from the oxidative damage caused by abiotic stress. Glutathione (GSH; -glutamyl-cysteinyl-glycine) is a non-enzymatic antioxidant that plays a role in ROS detoxification, either directly or indirectly (Foyer and Noctor 2005). The activity of GR, which catalyzes the regeneration of GSH from GSSG, is linked to its antioxidant efficacy. It may be said that SA may alter GSH content in plant cells, which reduces oxidative stress, because N is a constituent of GSH and SA enhances NR activity and N content. Li et al. (2013) reported that SA enhanced the GSH level by temporally regulating the transcript levels of the concerned genes in wheat for salt tolerance. Nazar et al. (2015) studied the influence of SA in modulating GSH content under drought.

Thus, SA supplementation to drought stressed plants receiving sufficient-N maximally enhanced antioxidative enzymes activity and stress tolerance to increase photosynthesis. To further study the mechanism of SA in the presence of sufficient-N, its effect on ABA and ethylene was studied. Application of SA resulted in reduced ethylene and ABA content more prominently in the presence of N, resulting in higher stomatal conductance and photosynthesis. Hormonal interaction is not an independent process

rather a cascade of complex *interactions* occurs to control a wide range of physiological processes. The influence of SA on ethylene and ABA was analyzed to completely understand the SA-mediated drought tolerance mechanism. In plants, ABA works as a major regulatory component to govern stomatal movement in response to drought (Schroeder et al. 1992; Niu and Liao 2016; Sehar et al. 2020) and stimulates stomatal closure via second messengers such as reactive oxygen species, nitric oxide, calcium, and protein kinases (Schroeder and Hagiwara 1989; Negi et al. 2008). The ROS generated during drought are reduced by SA more prominently when N was sufficient and concomitantly reduced ABA content. However, stomatal regulation is not an independent process but is influenced by hormonal interaction (Fatma et al. 2021). Lee et al. (2019) reported that ABA content was largely augmented by drought stress however, in plants pre-treated with SA, ABA content was comparatively lesser and both were antagonistic to each other in sugar regulation. Fatma et al. (2021) reported that ethylene antagonizes the ABA effect in stomatal closure under salt stress. SA is reported to inhibit stress ethylene formation by reducing ethylene level and sensitivity. Li et al. (2019) reported that the interaction between SA and ethylene-mediated defense response pathways are mutually antagonistic to each other. Similar response was observed when SA was provided to heat stressed wheat. SA treatment restricted the formation of stress ethylene under heat stress and brought ethylene to an optimal range by inhibiting 1-aminocyclopropane carboxylic acid synthase activity (Khan et al. 2013). Salicylic acid is one of the most significant ethylene inhibitors, and it was utilized to modulate the physiological processes in sweet pepper to counteract stress ethylene produced during salinity stress (Ahmed et al. 2020). The crosstalk between SA and ethylene in plant defense has been shown (Li et al. 2019). Stress ethylene and stress generated ABA are plants inherent ability to adapt to stress situation while compromising plants photosynthesis and growth. In a strategy to negotiate this compromise, SA plays a pivotal role by reducing both ethylene and ABA content while at the same time increasing stomatal conductance, water use efficiency and photosynthesis. It is interesting to observe that under condition of drought stress when N is limiting then there is higher ABA and ethylene content. Addition of SA to sufficient-N grown plants enhanced the utilization of N by increasing NR activity, N content and PNUE, GSH content and reduced the formation of stress ethylene and ABA to inhibit stomatal closure thereby increasing intercellular CO<sub>2</sub> concentration, stomatal conductance and photosynthesis. Fatma et al. (2021) reported that ethephon supplementation to the plants formed optimum ethylene evolution and which inhibited ABA-mediated stomatal closure under salt stress. The present study provides a good example of the interaction between these three phytohormones to regulate stomatal conductance and photosynthesis. Figure 6 shows the mechanisms of interaction of SA, ethylene and ABA in regulation of photosynthesis under drought stress.

In conclusion, the study provides evidence that SA increases photosynthetic capacity of mustard by enhancing the use efficiency of water and N. SA application to sufficient-N receiving plants enhanced nitrogen assimilation that was incorporated into GSH to maintain the redox state and reducing oxidative stress. SA reduced stress ethylene and ABA content and glucose-mediated photosynthetic repression to open the stomata and increase photosynthesis and growth. The strategy of providing sufficient-N together with SA may be used for optimizing photosynthesis and growth of drought stressed plants.

# Declarations

**Conflict of Interest:** There is no conflict of interest.

**Author Contributions:** Conceptualization: N.I., N.A.K.; Investigation and data curation: M.F., N.I., H.G, Z.S. Physiological analysis: N.I., Z.S., F.R. M.I.R.K; Original draft preparation: F.R., M.F. N.I.; Editing and content improvement: N.I., A.S. N.A.K.; Supervision: N.A.K. All authors have read and agreed to the published version of the manuscript.

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## Table

**Table 1.** Net photosynthetic rate ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), intercellular CO<sub>2</sub> concentration ( $\mu\text{mol CO}_2 \text{ mol}^{-1}$ ), chlorophyll content (SPAD value), Rubisco activity ( $\mu\text{mol CO}_2 \text{ mg protein min}^{-1}$ ), water use efficiency (WUE,  $\mu\text{mol mol}^{-1}$ ), photosynthetic nitrogen use efficiency (PNUE;  $\text{gm m}^{-2}$ ), water potential (-MPA), osmotic potential (-MPA) and glucose content ( $\text{mg glucose g}^{-1} \text{ DM}$ ) in mustard at 40 DAS. Data are mean  $\pm$  SE (n=4). Data followed by the same letter are not significantly different by LSD test at ( $p < 0.05$ ). N, nitrogen; DAS, days after sowing; DM, dry mass.

Parameters	Low	Sufficient N	Low N + SA	Sufficient N + SA
Net photosynthetic rate	9.6 ± 0.29d	13.1 ± 0.52 b	11.3 ± 0.51c	15.2 ± 0.47a
Stomatal conductance	297.5 ± 7.49d	394 ± 13.51b	342.6 ± 10.71c	456.2 ± 16.12a
Intercellular CO <sub>2</sub> concentration	183.4 ± 4.61d	249.4 ± 8.57b	210 ± 6.21c	286 ± 10.72a
Chlorophyll content	21.2 ± 0.53d	28.2 ± 1.21b	24 ± 0.82c	33 ± 1.39a
Rubisco	0.41 ± 0.03d	0.96 ± 0.03b	0.80 ± 0.05c	1.26 ± 0.07a
WUE	27.3 ± 1.22d	43.2 ± 2.51b	37.1 ± 1.76c	56.2 ± 2.81a
PNUE	30.2 ± 0.96d	40.8 ± 1.87b	35.7 ± 1.16c	46.2 ± 2.92a
Water potential	1.7 ± 0.09a	1.1 ± 0.07c	1.3 ± 0.06b	0.96 ± 0.04d
Osmotic potential	1.8 ± 0.95a	1.2 ± 0.05c	1.5 ± 0.07b	0.75 ± 0.04d
Glucose	2.2 ± 0.89a	1.6 ± 0.06c	1.8 ± 0.07b	1.3 ± 0.048d

## Figures

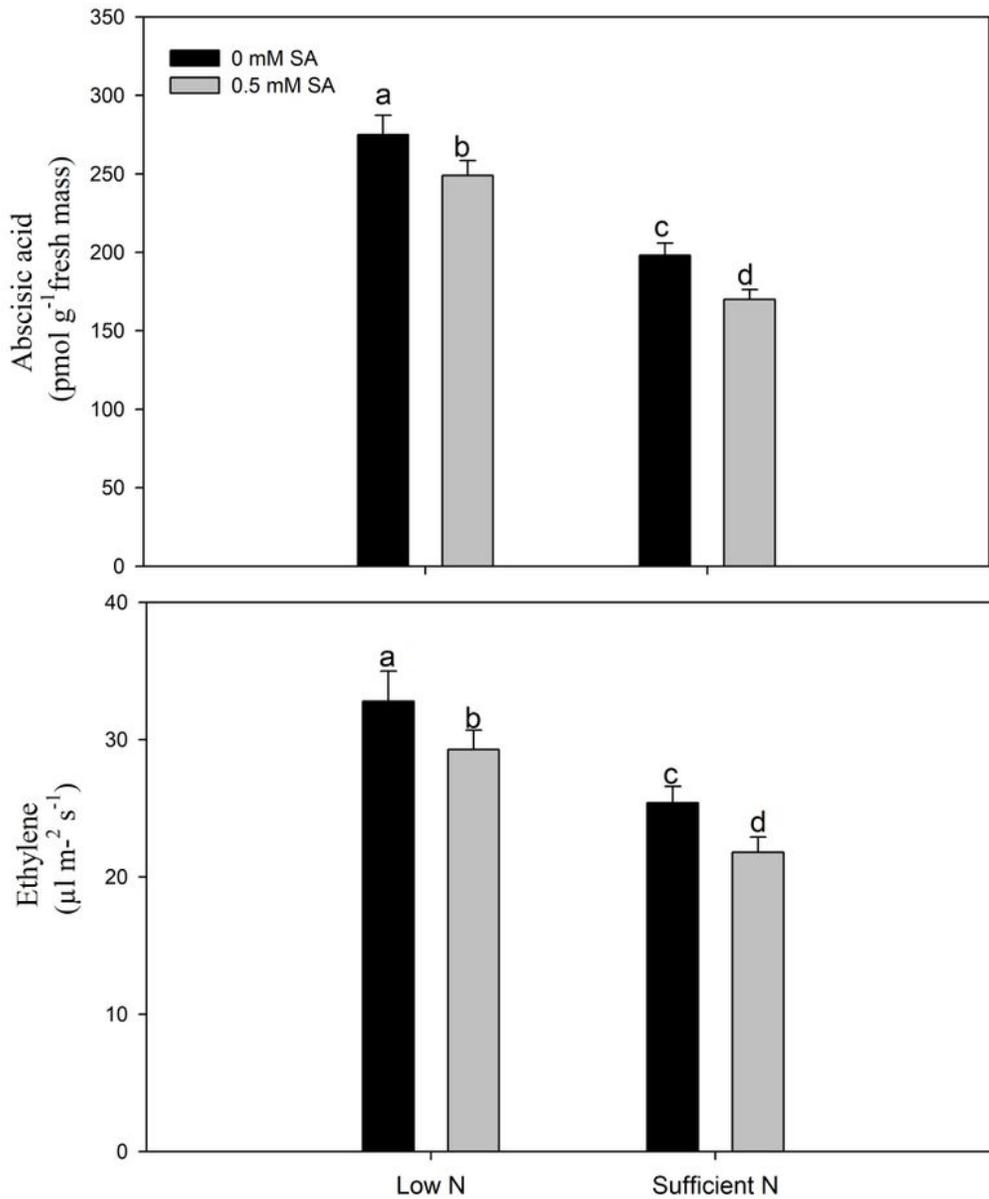


Fig. 1

## Figure 1

Effects of salicylic acid (0 or 0.5 mM) and N [100 (low-N) and 200 (sufficient-N) mg N kg<sup>-1</sup> soil] on ABA content (A) and ethylene evolution (B) of mustard (*Brassica juncea* L.) at 40 d after sowing (DAS). Data are mean  $\pm$  SE (n=4). The data were declared significant if values were higher than F values at p<0.05. ABA, abscisic acid; DAS, days after sowing; N, nitrogen

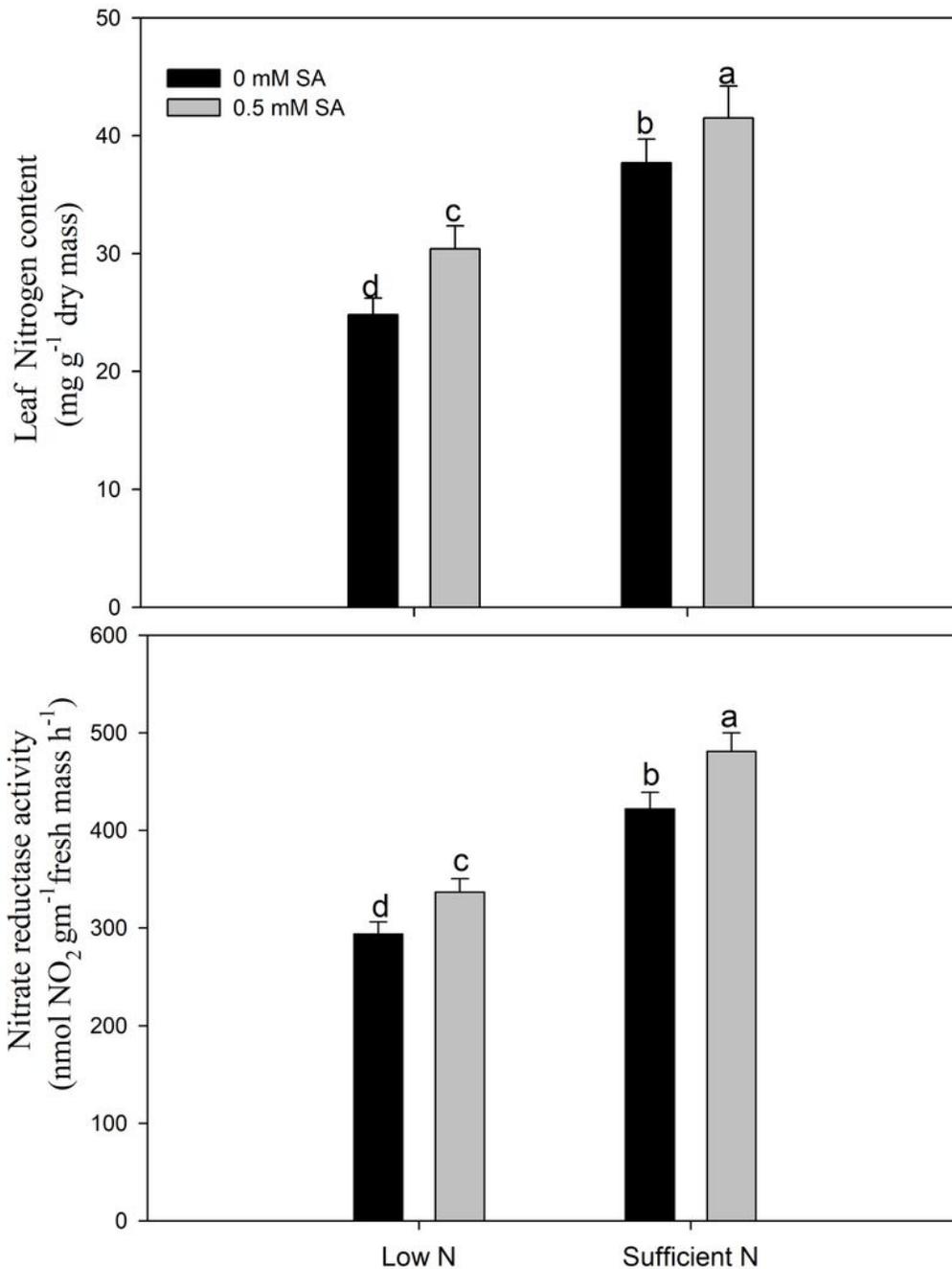


Fig. 2

## Figure 2

Fig. 2 Effects of salicylic acid (0 or 0.5 mM) and N [100 (low-N) and 200 (sufficient-N) mg N kg<sup>-1</sup> soil] on leaf nitrogen content (A) and nitrate reductase activity (B) of mustard (*Brassica juncea* L.) at 40 d after sowing (DAS). Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at p<0.05. DAS, days after sowing; N, nitrogen.

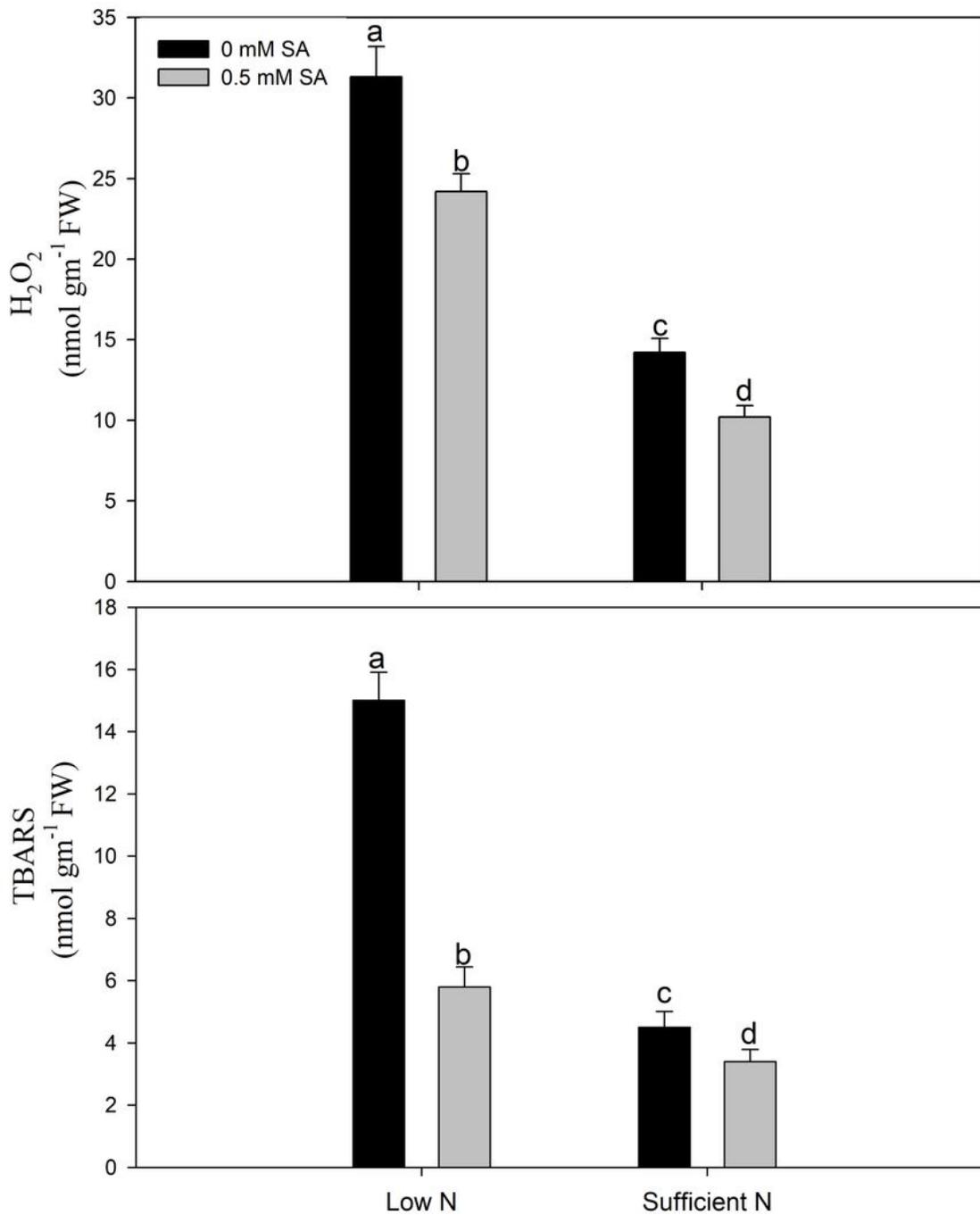


Fig. 3

### Figure 3

Fig. 3 Effects of salicylic acid (0 or 0.5 mM) and N [100 (low-N) and 200 (sufficient-N) mg N kg<sup>-1</sup> soil] on H<sub>2</sub>O<sub>2</sub> (A) and TBARS (B) contents of mustard (*Brassica juncea* L.) at 40 d after sowing (DAS). Data are mean  $\pm$  SE (n=4). The data were declared significant if values were higher than F values at p<0.05. DAS, days after sowing; N, nitrogen.

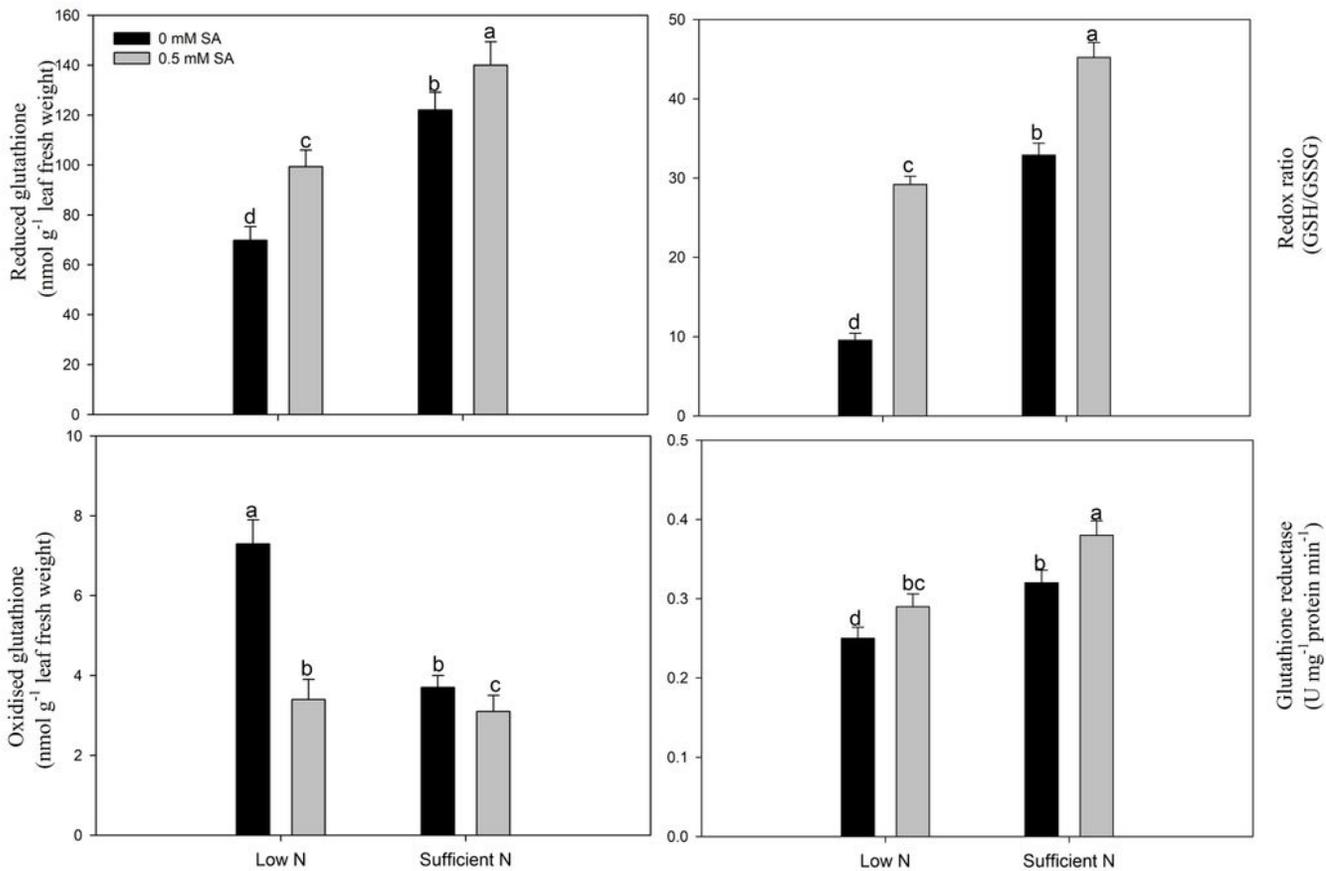


Fig. 4

Fig. 2

## Figure 4

Fig. 4 Effects of salicylic acid (0 or 0.5 mM) and N [100 (low-N) and 200 (sufficient-N) mg N kg<sup>-1</sup> soil] on reduced glutathione (A, GSH), oxidised glutathione (B, GSSG) redox state (C, GSH/GSSG) and glutathione reductase activity (D) of mustard (*Brassica juncea* L.) at 40 d after sowing (DAS). Data are mean  $\pm$  SE (n=4). The data were declared significant if values were higher than F values at p<0.05. DAS, days after sowing; N, nitrogen

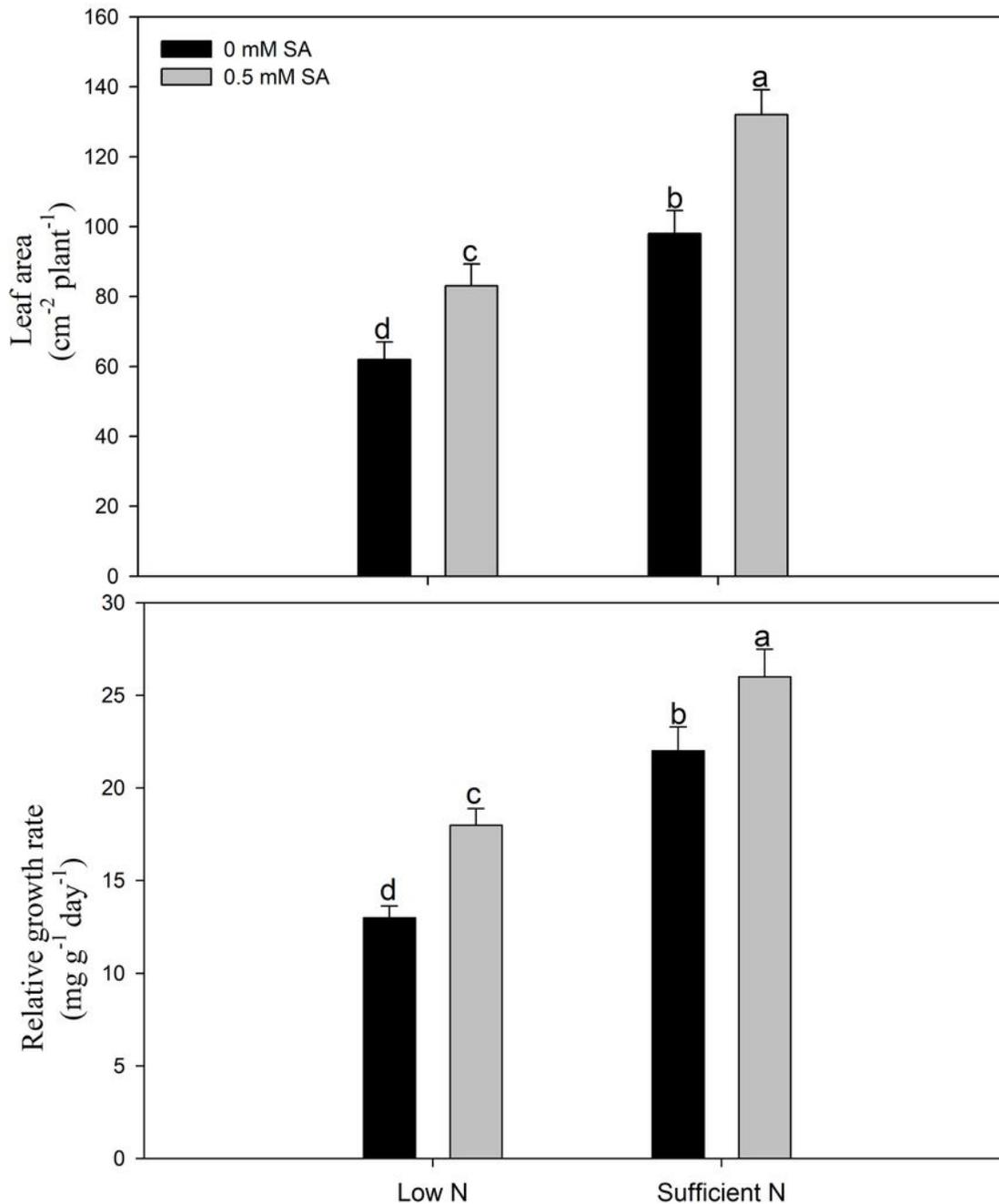


Fig. 5

### Figure 5

Fig. 5 Effects of salicylic acid (0 or 0.5 mM) and N [100 (low-N) and 200 (sufficient-N) mg N kg<sup>-1</sup> soil] on leaf area (A) relative growth rate (B) of mustard (*Brassica juncea* L.) at 40 d after sowing (DAS). Data are mean  $\pm$  SE (n=4). The data were declared significant if values were higher than F values at  $p < 0.05$ . DAS, days after sowing; N, nitrogen

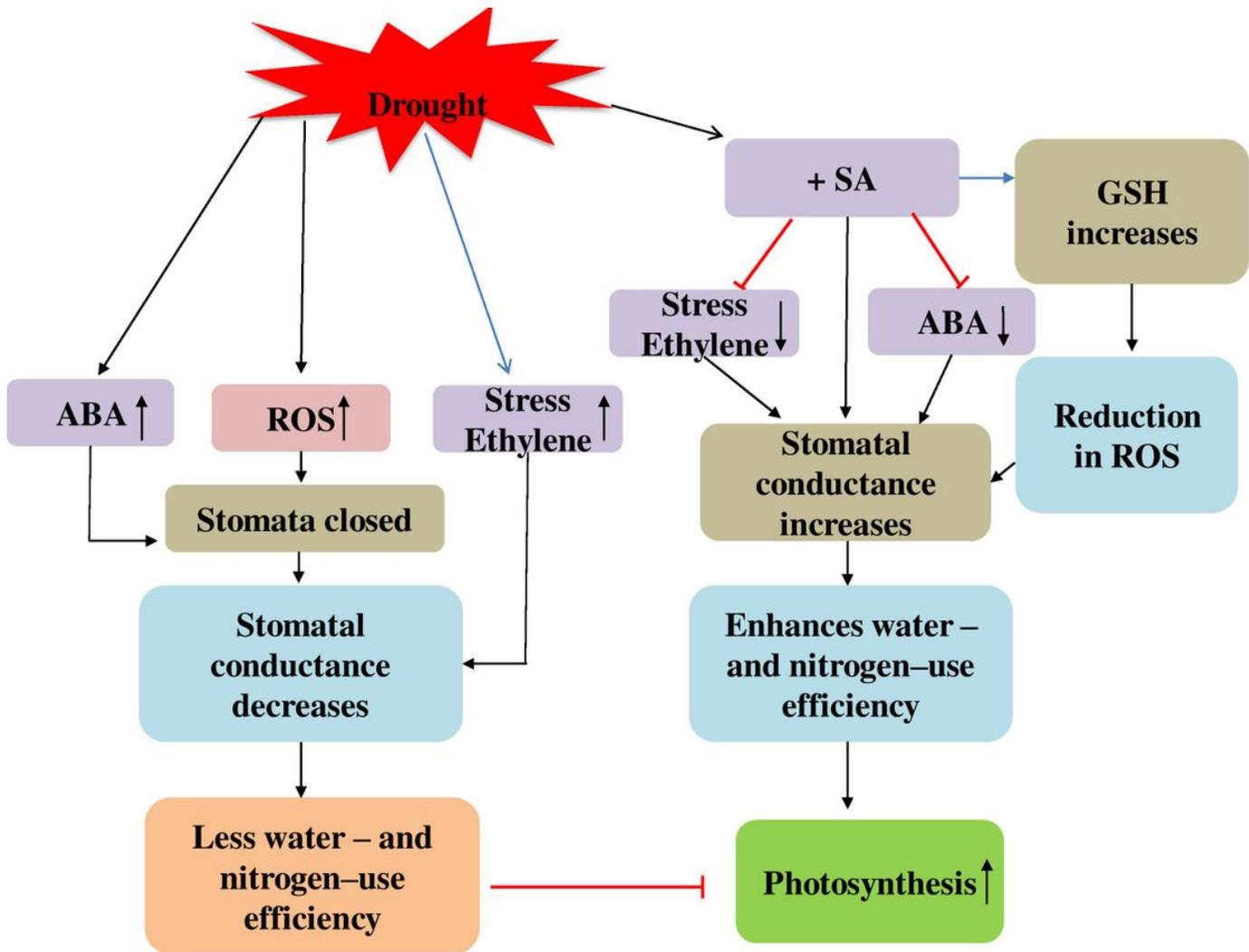


Figure 6

Fig. 6 Schematic representation of action of SA in regulating stomatal response and use efficiency of water and N in Brassica juncea L. under drought stress.

## Supplementary Files

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