



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

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Abstract

An essential approach to reduce drought in plants is to maximize the use of most limited available 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⁻¹ soil) and sufficient-N (200 mg N kg⁻¹ soil) and sprayed with 0 or 0.5 mM salicylic acid (SA). Application of SA increased water potential, osmotic potential, WUE, and the incorporation of soil N into photosynthetic machinery by enhancing PNUE. It also increased photosynthesis of plants maximally by increasing stomatal conductance and intercellular CO₂ 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 acid (ABA) synthesis. It could be inferred that SA enhanced N utilization through increase in NUE when it is sufficiently available, increasing the GSH content to maintain redox ratio and inhibit ABA-mediated stomatal closure to enhance the resource utilization and photosynthesis. SA also enhanced glucose utilization which prevented glucose-mediated photosynthetic repression under stress. Thus, SA application may impart a potential management tool for increasing photosynthetic NUE, WUE, and photosynthesis under drought.

Keywords Abscisic acid · Ethylene · Mustard · Nitrogen · Photosynthesis · Salicylic acid

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 respond to environmental signals by changing their physiology 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 lesser 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. Under

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drought, plants maximally utilize 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 through its presence in proteins of the Calvin cycle and thylakoids, Rubisco, and chlorophyll resulting in increased photosynthesis. Iqbal et al. (2011) reported 100 mg N kg⁻¹ soil to be deficient N for mustard growth, while supplementation of 80 mg N kg⁻¹ soil increased growth through reduction in stress ethylene evolution. Jahan et al. (2020) reported that supplementation of 100 mg N kg⁻¹ soil was beneficial under salt stress in the split application. Moreover, a relationship between N and photosynthesis has also been reported to be influenced by water availability and plant growth regulators (PGRs) (Lawlor 2001).

Plant growth regulators 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 and affects proline metabolism and photosynthesis (Khan et al. 2015). Thus, increased SA synthesis under drought stress might play a role in the acclimation of plant through maximal utilization of the available water resources (Kaya 2020; Hussain et al. 2021; Khalvandi et al. 2021).

It is noteworthy that hormonal signals crosstalk under drought condition may cooperate to govern drought stress responses, such as ethylene and ABA accumulation, and regulate many developmental processes and adaptive stress responses (Nazar et al. 2015; Khan et al. 2020; 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 WUE in several plant species, while mutations in *nced3* reduced drought tolerance (Iuchi et al. 2001; Tung et al. 2008). The review of Ali et al. (2020) and

references therein summarized that 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 the optimum level to initiate maximum utilization of available resource and avoid stress-induced senescence. 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 H₂O₂ 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 through its coordination with ethylene and ABA was studied on the use efficiency of water, N, and glucose accumulation to regulate stomatal conductance to protect photosynthetic functions of plants under drought. We hypothesized that SA promotes photosynthesis of plants under drought through 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 ratio 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 silt as 70:20:10 on a dry weight basis. These potted plants were placed in a naturally illuminated net house (with natural day/night) conditions. The photosynthetically active radiation was $700 \pm 28 \mu\text{mol m}^{-2} \text{s}^{-1}$, the average temperature for day and night was $23/17 \pm 2 \text{ }^\circ\text{C}$, and the relative humidity was

at $68 \pm 5\%$ for plant growth. The experiment was carried out at the Department of Botany, Aligarh Muslim University, Aligarh, India. Soil used was sandy loam of pH 8.0 in 1:2 soil/water mixture. The native available soil NO_3^- (N) was at a concentration of 100 mg kg^{-1} soil. Urea was used as N source and 0 or 100 mg N kg^{-1} soil was applied before sowing the seeds to both no stress and drought-stressed plants. Thus, the available soil N was 100 (low-N) and 200 (sufficient-N) mg N kg^{-1} soil, respectively. Plants with 100 mg N kg^{-1} soil under no stress served as control. 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 rain-out shelters to protect pots from rainfall or any moisture entry from outside. SA was supplemented after dissolving in absolute ethanol and then it was added dropwise 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 0.5 mM 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. Two plants per pot were maintained with four replicates for each treatment. The treatments were control (no stress, no SA, 100 mg N kg^{-1} soil N), sufficient-N (200 mg N kg^{-1} soil), SA (0.5 mM), SA + sufficient-N, drought, drought + sufficient-N, drought + SA, and drought + sufficient-N + SA.

At 10 days after spray, i.e., at 30 DAS, one plant from each pot was selected for non-destructive sampling of parameters, afterward these plants were used harvested for the determination of other parameters.

Determination of Relative Growth Rate and Leaf Area

Relative growth rate (RGR) is calculated at 20–30-day interval using formulae of Watson (1952). Leaf area was measured using leaf area meter (LA 211, Systronics, New Delhi, India).

Determination of Photosynthetic Parameters

Gas exchange parameters as net photosynthetic rate, stomatal conductance, and intercellular CO_2 concentration were determined using Infra-Red Gas Analyzer (CI-340

Photosynthesis system, CID Bio-science, Camas, WA, USA) in fully extended leaves. The measurements were done between 10.00 and 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, $\sim 22^\circ\text{C}$, and relative humidity, $\sim 70\%$.

With the help of chlorophyll fluorometer (Os-30p, Opti Sciences Inc., Hudson, 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.

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 vapor pressure between measurements.

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

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, UT, USA). The leaf used for water potential testing was frozen in liquid N 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 vapor pressure osmometer (5520, WESCOR, UT, USA).

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 is given in Iqbal et al. (2021) and provided in the Supplementary File 1.

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 (1944) approach

was used to determine the leaf N content in a plant. Detailed analysis provided in the Supplementary file 1.

Determination of H₂O₂ and TBARS Content

H₂O₂ and TBARS contents 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 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 mM⁻¹ cm⁻¹ was used for the determination of GR activity. The amount of enzyme required for breakdown 1 μmol of NADPH/min at 25 °C.

Content of reduced glutathione (GSH) and oxidized glutathione (GSSG) was examined through the procedure described by Anderson (1985). Details are there in the Supplementary File 1. Redox state as the ratio of GSH/GSSG was calculated.

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

Statistical Analysis

Data were assessed statistically using analysis of variance (ANOVA) by SPSS statistics (ver. 17.0) and represented as treatment mean ± 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 $P < 0.05$.

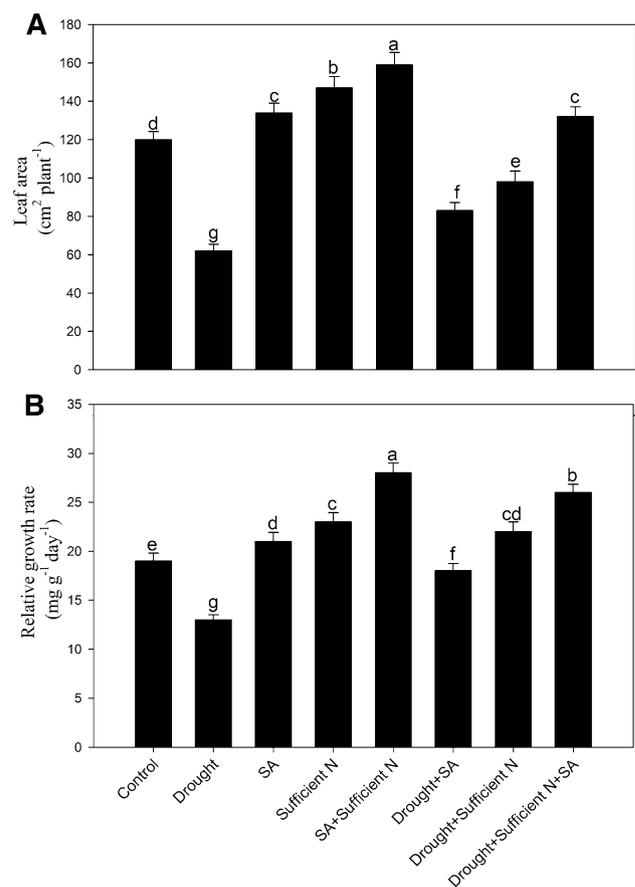


Fig. 1 Effects of salicylic acid (0 or 0.5 mM) and nitrogen [N, 100 (low-N) and 200 (sufficient-N) mg N kg⁻¹ soil] on leaf area (**A**) and relative growth rate (**B**) of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Data are mean ± SE ($n=4$). The data were declared significant if values were higher than F values at $p < 0.05$

Results

Effect of SA and N Availability on Leaf Area and Relative Growth Rate Under Drought Stress

The effect of SA under drought was prominently observed in the presence of sufficient-N compared to plants with no N under drought. SA increased leaf area in both low-N and sufficient-N grown plants under drought or without drought stress conditions but more conspicuously in plants receiving sufficient-N. SA supplementation increased leaf area by 33.9% in drought-stressed plants and by 113% in plants receiving sufficient-N under drought. Similarly, SA increased RGR by 38.5% in no external N-receiving plants subjected to drought while it maximally enhanced the RGR when sufficient-N was present under drought stress compared to drought-stressed plants alone. In plants with sufficient-N, SA increased the RGR by 2 times compared to drought and no SA control signifying the importance of SA

with sufficient-N in improving plant growth rate (Fig. 1). Plants that were not subjected to drought stress showed increase in leaf area and RGR with both SA and N but maximally of 32.5% in leaf area and 47.4% in RGR with their combined application compared to control.

Influence of SA on Photosynthetic Characteristics of Low- and Sufficient-N Grown Plants Under Drought

The effect of SA treatment at different soil N contents under drought stress increased photosynthetic characteristics more promisingly when N was sufficient. Photosynthetic characteristics of plants under drought with low level of N (soil native N as 100 mg kg⁻¹) were the lowest. Addition of N (sufficient-N) increased net photosynthetic rate by 36.4% compared to drought stress and low-N grown plants. However, SA supplementation to low-N and sufficient-N grown plants under drought stress increased photosynthetic rate by 17.7% and 58.3% in comparison to the low-N and no SA receiving drought-stressed plants (Table 1). Similar trend was observed in stomatal conductance which increased from 15.2% to 53.3% when SA was applied to drought-stressed plants having low-N and sufficient-N, respectively, compared to drought stress. Without SA, sufficient-N increased stomatal conductance by 32.4%

but with SA maximum increase in stomatal conductance was observed in comparison to drought stress. The intercellular CO₂ 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 under drought. Intercellular CO₂ concentration increased from its lowest concentration of ~183 μmol mol⁻¹ for drought-stressed plants to ~286 μmol mol⁻¹ for the plants receiving sufficient-N and SA under drought (Table 1) which was 55.9% increase compared to low-N and no SA containing drought-stressed plants.

The chlorophyll fluorescence parameter 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 under drought stress. However, in comparison to the low-N and no SA drought-stressed plants, application of either SA and/or N increased the maximum efficiency of PSII, but lesser than the plants supplied with SA and sufficient-N under drought.

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 under drought compared to drought stress. Increased chlorophyll content and Rubisco activity by 32.1% and 43.9% was found with plants grown

Table 1 Net photosynthetic rate (μmolCO₂ m⁻² s⁻¹), stomatal conductance (mmol CO₂ m⁻² s⁻¹), intercellular CO₂ concentration (μmolCO₂ mol⁻¹), chlorophyll content (SPAD value), Rubisco activity (μmol CO₂mg protein min⁻¹), water-use efficiency (WUE, (μmol

mol⁻¹), photosynthetic nitrogen-use efficiency (PNUE, (g m⁻²), water potential (-MPa), osmotic potential (-MPa), and glucose content (mg glucose g⁻¹dry weight) in mustard at 30 DAS

Parameters	Control	SA	Sufficient N	Sufficient N+SA	Drought	Drought+SA	Drought+Sufficient N	Drought+Sufficient N+SA
Net photosynthetic rate	11.0±0.54 ^d	12.9±0.59 ^c	14.6±0.66 ^b	16.6±0.581 ^a	9.6±0.297 ^e	11.3±0.508 ^d	13.1±0.521 ^c	15.2±0.471 ^b
Stomatal conductance	351±8.8 ^d	401±9.4 ^c	462±10.2 ^b	478.2±18.01 ^a	297.5±7.49 ^e	342.6±10.7 ^d	394±13.5 ^c	456.2±16.12 ^b
Intercellular CO ₂ concentration	219.4±6.61 ^d	253±8.2 ^c	298.4±9.57 ^b	306±12.2 ^a	183.4±4.61 ^e	210±6.2 ^d	249.4±8.57 ^c	286±10.7 ^b
Chlorophyll content	24.9±0.88 ^d	29.0±0.96 ^c	34.2±1.41 ^b	36.4±1.59 ^a	21.2±0.53 ^e	24.0±0.82 ^d	28.2±1.21 ^c	33.1±1.39 ^b
Rubisco	0.86±0.037 ^d	1.08±0.061 ^c	1.32±0.033 ^b	1.51±0.069 ^a	0.41±0.029 ^e	0.80±0.051 ^d	0.96±0.033 ^c	1.26±0.069 ^b
WUE	38.2±1.72 ^d	43.9±2.4 ^c	56.3±2.9 ^b	61.4±3.21 ^a	27.3±1.22 ^e	37.1±1.76 ^d	43.2±2.5 ^c	56.9±2.81 ^b
PNUE	36.2±1.24 ^d	41.7±1.16 ^c	47.0±2.81 ^b	52.8±3.31 ^a	30.2±0.96 ^e	35.7±1.16 ^d	40.8±1.87 ^c	46.2±2.9 ^b
Water potential	1.41±0.068 ^b	1.17±0.069 ^c	0.87±0.06 ^d	0.66±0.033 ^e	1.7±0.098 ^a	1.3±0.062 ^b	1.1±0.067 ^c	0.96±0.041 ^d
Osmotic potential	1.43±0.753 ^b	1.17±0.08 ^c	0.7±0.041 ^d	0.61±0.034 ^e	1.8±0.953 ^a	1.5±0.07 ^b	1.2±0.0502 ^c	0.75±0.045 ^d
Glucose	1.89±0.86 ^b	1.52±0.04 ^c	1.26±0.04 ^d	1.1±0.0478 ^e	2.2±0.898 ^a	1.8±0.0704 ^b	1.6±0.057 ^c	1.3±0.048 ^d

Data are mean ± SE (n=4). The data were declared significant if values were higher than F values at P<0.05

Data followed by same letter were not significantly different by LSD test at P<0.05

N nitrogen

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 under drought. SA applied to drought-stressed plants with low-N caused an increase of 18.2% and 29.7% in PNUE and WUE; 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 drought-stressed plants (Table 1). All photosynthetic characteristics increased with both SA and N under no stress and maximally with the combined application of SA and N under control conditions.

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 drought-stressed plants with no SA or low-N. Application of sufficient-N increased water potential and the osmotic potential by 33.3% and 35.20% compared to drought-stressed plants. SA application to low-N-receiving plants under drought increased water potential and osmotic potential by 16.7% and 23.5%, while SA application to sufficient-N plants under drought showed maximum increase in water potential and osmotic potential compared to drought-subjected plants (Table 1). Osmotic and water potential showed similar trends under control conditions and increased by both SA and N applications and maximally with combined SA and N under no stress. Their combined application increased osmotic potential by 57.3% and water potential by 53.2% compared to control.

Impact of SA with Sufficient-N on Glucose Content Under Drought Stress

Glucose content decreased with the addition of N or SA under drought stress or no stress. Salicylic acid treatment to low-N or sufficient-N also decreased the glucose content compared to without SA control both under no stress or stress. Application of SA under drought 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 drought control. Similarly, under no stress SA decreased glucose content by 19.6% with low N and 41.8% with sufficient N compared to 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 by 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 drought and by % and % compared to no drought low-N control. SA increased NR activity and N content by 14.6% and 22.6% in low-N grown plants and it maximally increased by 63.6% in NR activity and 67.3% in N content when N was sufficient under drought in comparison to drought-stressed plants (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 H₂O₂ and TBARS content by 54.6% and 70.0%, respectively, compared to the drought-stressed plants.

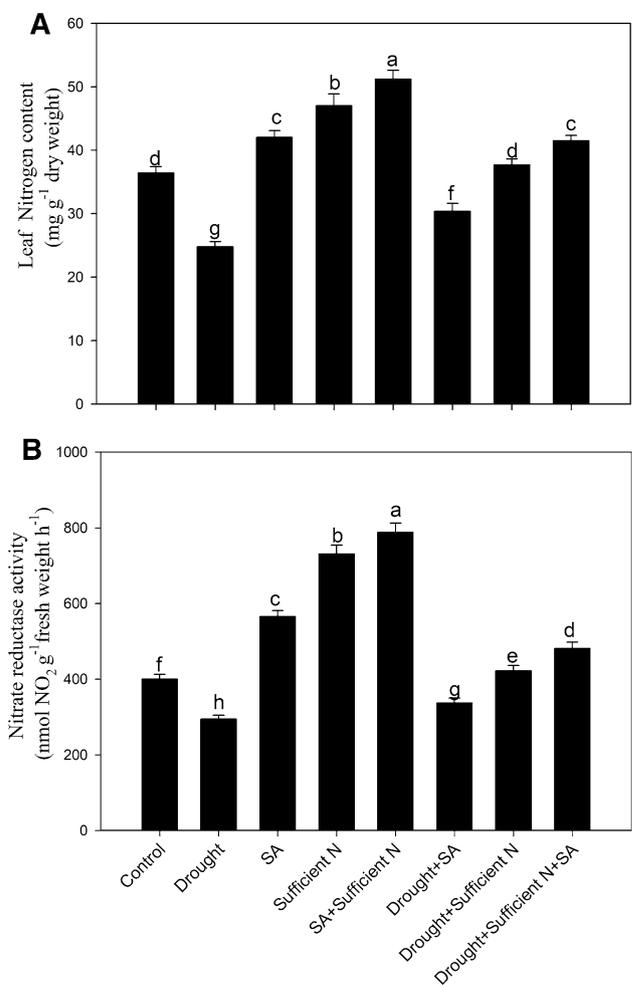


Fig. 2 Effects of salicylic acid (0 or 0.5 mM) and nitrogen [N, 100 (low-N) and 200 (sufficient-N) mg N kg⁻¹ soil] on leaf N content (A) and nitrate reductase activity (B) of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Data are mean \pm SE ($n=4$). The data were declared significant if values were higher than F values at $p < 0.05$

However, SA application profoundly decreased H_2O_2 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 under drought compared to plants under drought with low N and no SA (Fig. 3). Content of H_2O_2 and TBARS decreased with SA, N, and maximally with their combined application under no stress conditions compared to control.

Salicylic Acid Modulates GR Activity, Glutathione Pool, and Redox Ratio in Presence of Sufficient-N Under Drought Stress

Plants grown with sufficient-N under drought showed increased activity of GR by 28.0% (Fig. 4) compared to low-N drought grown plants (Fig. 4). SA application increased the GR activity by 16.0% when supplied to drought-stressed plants and 52.0% in sufficient-N grown

plants under drought stress compared to drought-stressed plants. SA supplementation to low-N grown drought-stressed 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 availability 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).

Influence of SA on Biosynthesis of ABA and Ethylene with N Availability Under Drought

Drought-stressed plants with low-N showed increased ABA content which decreased by addition of sufficient-N. Sufficient-N decreased ABA content by 28.0% under drought stress and SA applied under drought stress exhibited decreased of 9.45% in ABA content, while plants receiving sufficient-N and SA maximally decreased ABA content by 38.1% compared to plant that had low-N and no SA under drought (Fig. 5).

Ethylene was highest in the drought-stressed low-N plants. Addition of N decreased ethylene evolution by 22.6% compared to drought stress. Ethylene evolution decreased by 10.7% in plants receiving SA under drought, but sufficient-N and SA were most promising in reducing stress ethylene. SA with sufficient-N decreased stress ethylene by 33.5% compared to drought-stressed plants with low-N and no SA (Fig. 5).

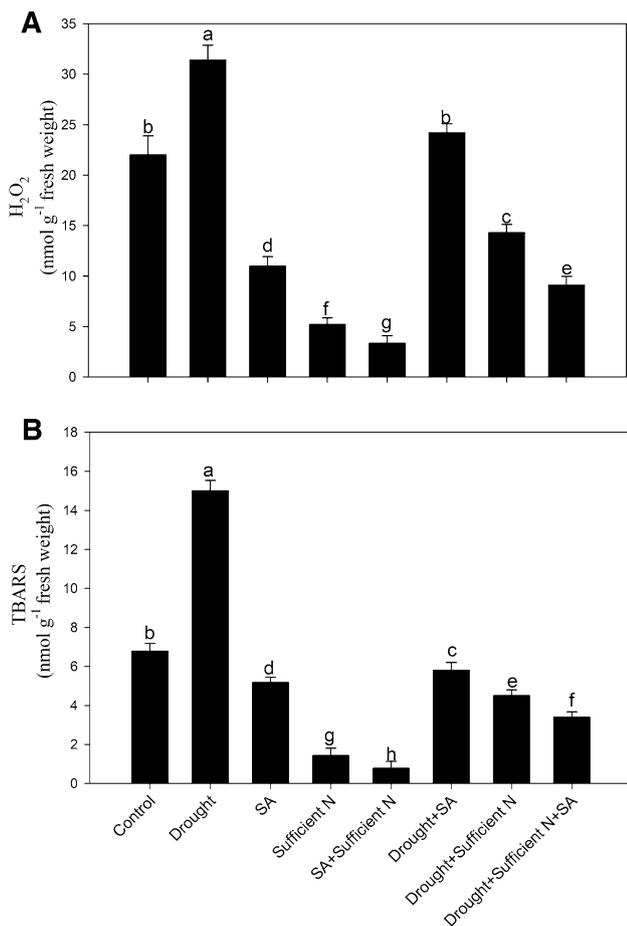


Fig. 3 Effects of salicylic acid (0 or 0.5 mM) and nitrogen [N, 100 (low-N) and 200 (sufficient-N) mg N kg⁻¹ soil] on H_2O_2 (A) and TBARS (B) contents of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Data are mean \pm SE ($n=4$). The data were declared significant if values were higher than F values at $p < 0.05$

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, stomatal closure is the first and most noticeable barrier to CO_2 uptake and restricts CO_2 transport from the atmosphere to the sites of carboxylation in the chloroplast (Galle et al. 2009). 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

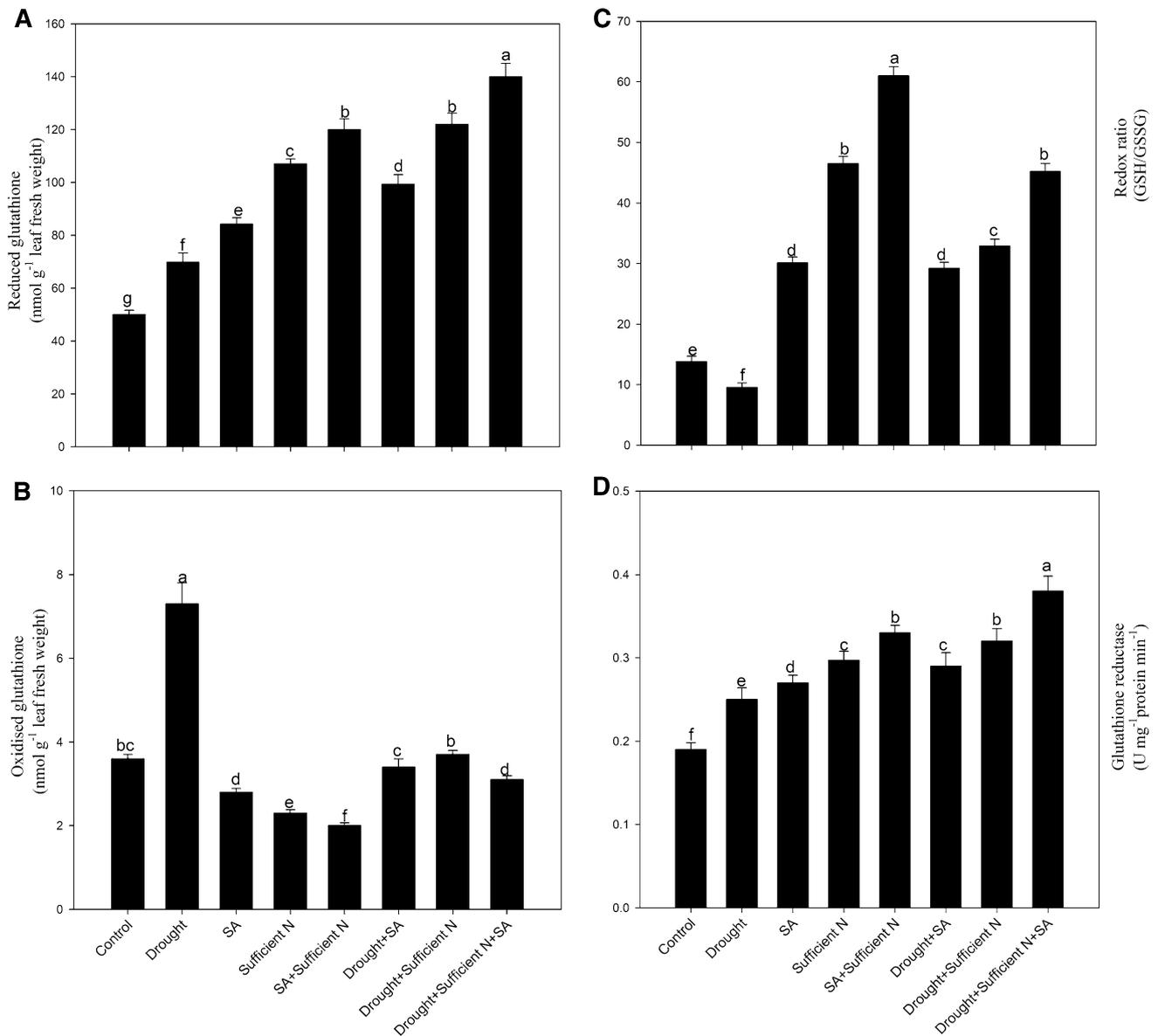


Fig. 4 Effects of salicylic acid (0 or 0.5 mM) and nitrogen [N, 100 (low-N) and 200 (sufficient-N) mg N kg⁻¹ soil] on reduced glutathione (A, GSH), oxidized glutathione (B, GSSG), redox ratio (C, GSH/GSSG), and glutathione reductase activity (D) of mus-

tard (*Brassica juncea* L.) at 30 days after sowing (DAS). Data are mean \pm SE ($n=4$). The data were declared significant if values were higher than F values at $p < 0.05$

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 N with lower glucose-mediated photosynthetic repression.

In both natural and agricultural situations, N 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 N 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. Recently, Hussain et al. (2021) reported that both SA and S helped in alleviating salinity stress by enhancing N metabolism and increasing antioxidative machinery.

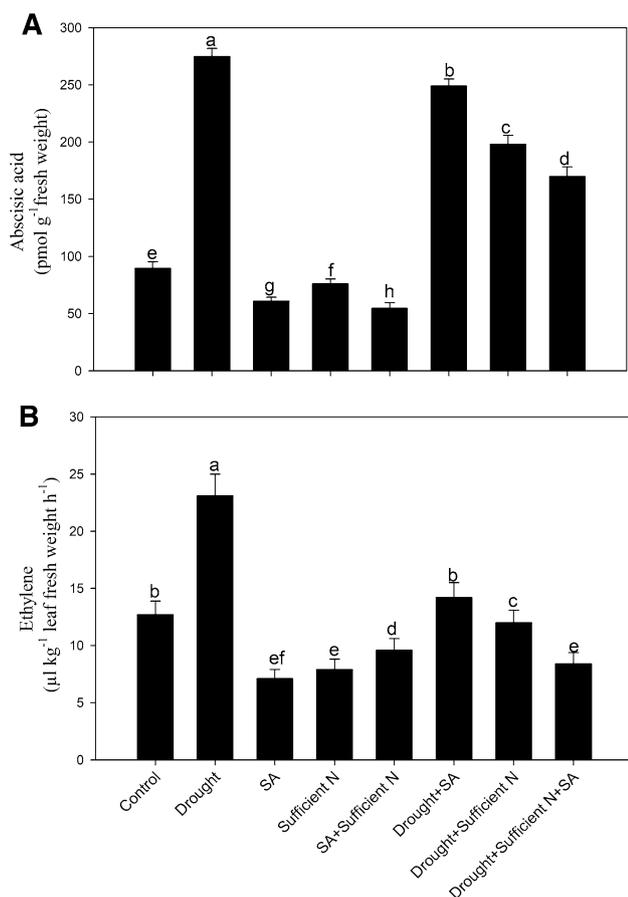


Fig. 5 Effects of salicylic acid (0 or 0.5 mM) and nitrogen [N, 100 (low-N) and 200 (sufficient-N) mg N kg⁻¹ soil] on abscisic acid (ABA) content (**A**) and ethylene evolution (**B**) of mustard (*Brassica juncea* L.) at 30 days after sowing (DAS). Data are mean \pm SE ($n=4$). The data were declared significant if values were higher than F values at $p < 0.05$

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). The WUE increased with both SA and N applications which could probably be due to increased binding of CO₂ and less binding of O₂ to the active site of Rubisco as CO₂ concentration increased. The increased CO₂ 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 maximum quantum yield of PSII and 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 modulates pathways that help in 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. Perhaps, reduction in ABA content by SA could increase in growth and thus better glucose utilization.

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 reduce 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-cysteinylglycine) is a non-enzymatic antioxidant that plays a role in reactive oxygen species (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) reported the influence of SA in enhancing GSH content under drought.

Thus, SA supplementation to drought-stressed plants receiving sufficient-N maximally enhanced GR activity, GSH content, 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. In plants, ABA works as a major regulatory component to govern stomatal movement in response to drought (Schroeder and Keller 1992; Niu and Liao 2016; Sehar et al. 2020) and stimulates stomatal closure via second messengers, such as ROS, nitric oxide, calcium, and protein kinases (Schroeder and Hagiwara 1989; Negi et al. 2008). 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 by Li et al. (2019). Stress ethylene and stress-generated ABA are plant's 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, WUE, 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, and GSH content and reduced the formation of stress ethylene and ABA to inhibit stomatal closure, thereby increasing intercellular CO₂ concentration, stomatal conductance, and photosynthesis. Increased photosynthesis resulted in increase in growth that led to relieve of glucose-mediated photosynthetic repression. Increased chlorophyll content also contributed to increased photosynthesis and growth with SA supplementation to sufficient-N. 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 N 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

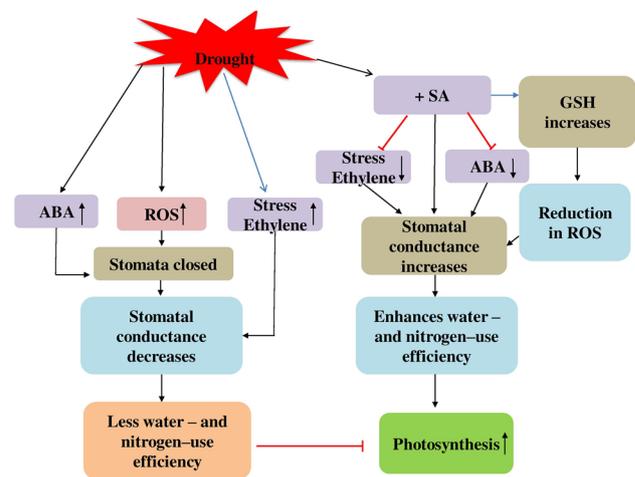


Fig. 6 Schematic representation of salicylic acid action in regulating stomatal response and use efficiency of water and nitrogen (N) in *Brassica juncea* L. under drought stress

SA may be used for optimizing photosynthesis and growth of drought-stressed plants.

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Declarations

Conflict of interest There is no conflict of interest.

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