



ISSN: 2075-6240

Salicylic acid and thiourea ameliorate the negative impact of salt stress in wheat (*Triticum aestivum* L.) seedlings by up-regulating photosynthetic pigments, leaf water status, and antioxidant defense system

Sumaiya Farzana^{1, 2}, Md. Rasel^{3*}, Md. Tahjib-Ul-Arif²,
Mohammad Anwar Hossain^{3*}, Md. Golam Azam⁴, Md. Asadulla Al Galib⁵,
A.G.M. Sofi Uddin Mahamud⁶ and Mohammad Afzal Hossain²

¹Graduate Training Institute, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, ²Department of Biochemistry and Molecular Biology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, ³Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, ⁴Pulses Research Center, Bangladesh Agricultural Research Institute, Ishurdi, Pabna-6620, Bangladesh, ⁵Agronomy Division, Bangladesh Rice Research Institute, Gazipur-1701, Bangladesh, ⁶School of Food Science and Engineering, South China University of Technology, Guangzhou, 510641, China

ABSTRACT

Salinity is one of the most important abiotic stresses that inhibit wheat (*Triticum aestivum* L.) growth, development and yield. Therefore, finding efficient strategies to prevent salt-induced growth retardation and yield loss is crucial for modern agriculture to sustain production. This study was conducted to explore the roles of exogenous salicylic acid (SA) and thiourea (TU) in regulating salt tolerance by evaluating morpho-physiological traits and antioxidant response in two wheat genotypes at the seedling stage. Imposition of salt stress resulted in reduced growth characteristics, leaf water status, and photosynthetic pigments, while the Na⁺/K⁺ ratio, hydrogen peroxide (H₂O₂), and malondialdehyde (MDA) content were increased significantly in both of the wheat genotypes. In contrast, exogenous application of SA or TU in the salt-stressed plants significantly reduced the negative effects of salt stress and improved growth performance by up-regulating photosynthetic pigments, leaf water status, and proline content in both wheat genotypes. Besides, when compared to the seedlings treated with salt stress only, SA and TU were found to play an important role in maintaining lower Na⁺/K⁺ levels and reducing oxidative stress by lowering MDA and H₂O₂ levels in salt-stressed plants through boosting the activities of antioxidant enzymes such as catalase, ascorbate peroxidase, and peroxidase. In addition, hierarchical clustering and principal component analysis revealed a significant interaction among growth characteristics, chlorophyll and carotenoid content and antioxidant activities with the salt, SA or TU treatments. The findings suggested that exogenous application of SA or TU could be a useful strategy for reducing the negative effects of salinity on wheat growth and development.

KEYWORDS: Antioxidant enzymes, oxidative damage, proline, salinity, *Triticum aestivum*

Received: July 18, 2021
Revised: August 23, 2021
Accepted: August 30, 2021
Published: September 19, 2021

Corresponding Authors:
Md. Rasel,
E-mail: rasel.gpb@bau.edu.bd;
Mohammad Anwar Hossain,
E-mail: anwargpb@bau.edu.bd

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the major cereal crops in Bangladesh, with a global production of about 700 million tons, providing 20% of the daily protein and calorie requirements for over 4.5 billion people worldwide (Arzani & Ashraf 2017). As a staple food grain for more than 35% of the

total populations of Bangladesh, it has emerged as the country's second most profitable cereal crop after rice (Golder *et al.*, 2013). Wheat cultivation has recently received a lot of attention from Bangladeshi farmers as the demand has increased in the bakery industry (Mottaleb *et al.*, 2018). However, outrageous environmental change as a result of dangerous atmospheric deviation and global warming has recently caused a drastic shift

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in salinity dominated areas of Bangladesh, putting the present wheat cultivars' productivity at risk (Siddiqui *et al.*, 2017). Saline soil is characterized by the procurement of sodium ions (Na^+) with dominant anions of chloride and sulphate leading to the high electrical conductivity of more than 4 dSm^{-1} (Ali *et al.*, 2013). As a result of climate change, soil salinity is becoming a major issue in coastal areas, particularly in developing countries (Nicholls *et al.*, 2007) and over 800 million hectares of land are expected to be adversely affected by soil salinity globally (Munns & Tester 2008). Soil salinity is a growing menace all over the world because of its noxious effect on reducing soil fertility and water uptake in the crop plants (Yuvaraj *et al.*, 2021). In recent years, soil salinity has severely hampered agricultural production by affecting plant growth and development at various phases of plant growth (James *et al.*, 2012; Konuşkan *et al.*, 2017). In Bangladesh, different levels of salinity have already impacted approximately 0.87 million hectares which grasped almost 30% of cultivable lands causing 30-50% yields loss every year (Khatun *et al.*, 2019). As a result, successful and cost-effective strategies should undoubtedly be introduced in the saline belt of Bangladesh to minimize salinity-induced yield reduction and meet the increasing population's demand for wheat.

Wheat is a salt-sensitive crop that exhibits high salt susceptibility throughout its entire life cycle, especially during the vegetative and early reproductive phases (Kumar *et al.*, 2013; Uddin *et al.*, 2017). Salinity exerts its cellular toxicity by instigating ionic, osmotic, and oxidative stresses (Akramet *et al.*, 2017; Kamran *et al.*, 2020). Demonstration of over aggregation of soluble salt most likely Na^+ restricts the plant development and advancement by upsetting numerous physiological and biochemical procedures, for example, osmotic modification, water parity, membrane integrity, alteration of growth regulators levels, and metabolic failure going with ionic and osmotic pressure as a result of hyper-ionic and osmotic stresses (Nieves-Cordones *et al.*, 2016; Negrão *et al.*, 2017; Mathur *et al.*, 2019). High amount of Na^+ accumulation in soils lowers the potassium (K^+) ions uptake into plant cells. As a result, excessive Na^+ damages the cell membrane and plant organelles, resulting in plant cell death (Ma *et al.*, 2016). Therefore, plants activate various mechanisms to reduce Na^+ aggregation in the cytoplasm, thereby preventing Na^+ intrusion into the cell, especially by removing Na^+ from the cell or categorizing Na^+ into the vacuole (Shi *et al.*, 2003; Mostofa *et al.*, 2015). Plants also regulate the expression and activity of various membrane transporters, such as high-affinity K^+ transporters, which help cation transport throughout the membrane and uphold the standard Na^+/K^+ ratio (Waters *et al.*, 2013). In general, salinity resistance in crop plants is connected with the upkeep of a low Na^+/K^+ ratio (Omisun *et al.*, 2018). Thus, maintaining the proper Na^+/K^+ ratio is critical for the development of salt-tolerant wheat cultivars under stress conditions (Sun *et al.*, 2014). Besides ion homeostasis, different osmoprotectant like proline, also demonstrate a very crucial role under salinity stress to maintain lower levels of lipid peroxidation in plants (Khan *et al.*, 2019). Thus, the increase of proline accumulation levels in plants under saline conditions is correlated with the improvement of salt tolerance (Gharsallah *et al.*, 2016). The noxious effects of salt stress also lead to metabolic changes that increase reactive oxygen species

generation (ROS) in plants (Chawla *et al.*, 2013; Mostofa *et al.*, 2015; AbdElgawad *et al.*, 2016; Hossain *et al.*, 2021). When one, two, or three O_2 electrons exchange, hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\cdot-}$), or hydroxyl radical (OH^{\cdot}) are formed, which usually results in the formation of ROS (Rodrigo-Moreno *et al.* 2013). The overproduction of ROS disrupts normal plant metabolism by targeting proteins, DNA, and membrane lipids (Parihar *et al.*, 2015). To counteract the toxic effects of stress-induced ROS, plants itself develop an inherent protective mechanism that includes enzymatic antioxidants such as catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) (Choudhury *et al.*, 2017; Tanveer & Shabala, 2018). Plants trigger this enzymatic antioxidant metabolism to reduce ROS-induced toxic effects under stress conditions because they detoxify H_2O_2 and inhibit lipid peroxidation through specific mechanisms (Aghaleh *et al.*, 2009; Dugasa *et al.*, 2019). Previously, few plant species showed a significant positive correlation between enzymatic antioxidants and salinity tolerance level (Cicek & Cakirlar, 2008; Ashraf, 2009).

Recent studies revealed that the priming of seeds or plants by certain exogenous protecting agents such as plant hormone is potentially a significant in modulating resistance in plants against various abiotic stresses (Borges *et al.*, 2014; Ismail *et al.*, 2016). It has also been reported that treatment with exogenous substances like organic extracts and lipoic acid significantly improved the antioxidant defense system of the plants, thereby reducing ROS levels and increasing wheat growth and production under saline conditions (Elrys *et al.*, 2020). Among the potential endogenous hormones, salicylic acid (SA), and thiourea (TU) has been marked assuming a very noteworthy role in plant growth and development and impact a wide assortment of plant physiological processes, ion uptake and transportation, proline metabolism, and plant water relations by minimizing salt toxicity under stress situations (Wakchaure *et al.*, 2018; Kaya *et al.*, 2019). The shoot and root length, chlorophyll content, relative water content was found to increase whereas malondialdehyde, proline and ion leakage were decreased after application of SA in Triticale under saline condition (Erkan and Aras Aşci 2021). Furthermore, both TU and SA serve as a critical signalling molecule for counteracting the negative effects of ROS induced oxidative stress by the enhancement of antioxidant enzyme activity such as CAT, APX, and POD (Patade *et al.*, 2012; Yadu *et al.*, 2017; Kaya *et al.*, 2020). In addition, numerous reports demonstrated that salt stress caused a significant increase in MDA content on rice and maize which was prevented by SA treatments (Erkan & Aras Aşci, 2021; Pandey *et al.*, 2021). Several previous studies also reported that priming with exogenous SA and TU was found to mitigate the salt stress in rice (*Oryza sativa*), onion (*Pisum sativum*), potato (*Solanum tuberosum*), faba bean (*Vicia faba*), mung bean (*Vigna radiata*), and lentil (*Lens culinaris*) by intensifying the activities of antioxidant enzymes and osmotic adjustment and by promoting root and shoot formation and by stimulating flowering (Mani *et al.*, 2012; Mostofa & Fujita 2013; Khan *et al.*, 2014; Ahmad *et al.*, 2017; Nooren *et al.*, 2017; Nie *et al.*, 2018; El-Kinany, 2020; Yadav *et al.*, 2020; Pandey *et al.*, 2021). While the stress alleviating roles of SA and TU are largely analysed in several crops, however many aspects of

exogenous SA and TU-mediated salt tolerance in wheat remain elusive. The present research work was therefore executed to analyse the potential roles and possible mechanisms of SA and TU-mediated salt stress tolerance in wheat at the seedling stage. Different agronomic and biochemical determinants of salinity tolerance were assessed to scrutinize the salt stress relief mechanism by SA and TU.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was carried out using two salt-sensitive high yielding wheat genotypes namely BARI Gom-25 and BARI Gom-26 under hydroponic conditions following a two factorial randomized complete block design with three replications. The salinity factor (NaCl) is comprised of one level (150 mM), and the SA and TU were exogenously applied at 0.75 mM and 15 mM levels respectively. Thus, the treatment combinations were as follows: control, C (T0, nutrient solution); salt, S (T1, nutrient solution with 150 mM NaCl); S+SA (T2, 150 mM NaCl with 0.75 mM SA); and S+TU (T3, 150 mM NaCl with 15 mM TU). The concentrations of SA and TU were selected based on previously published literature on different crops (Khan *et al.*, 2015; Perveen *et al.* 2016; Waqas *et al.*, 2019).

Initially, seeds were treated for 25 minutes with 5% sodium hypochlorite + 2% Tween-20, followed by three washes with distilled water. Approximately hundreds of seeds were pre-soaked for 24 hrs in distilled water (dH₂O) and incubated in a petri-dish with one sheet of moistened filter paper containing 8 ml of dH₂O water for 3 days in dark condition at 25°C to induce germination. Afterward, three days old evenly sprouted seeds were placed into the holes of circular plates in a Styrofoam sheet (28 cm × 32 cm × 1.25 cm) having nylon net at the bottom and fitted in a 12-L plastic tray containing nutrient solution. The modified cooper's nutrient solution was used to supply the nutrients for the growth and development of wheat seedlings (Cooper, 1988). After 5 days of seedling establishment, the salt treatment (T1, 150 mM NaCl) was applied in six steps for 42 days employing 25 mM in each time in each tray at an interval of 6 days. The first and the last salt stress imposition was applied on the day 5th and day 35th, respectively after planting. Simultaneously, the exogenous SA (T2, 0.75 mM) and TU (T3, 15 mM) treatments were sprayed individually for 6 times to the leaf surface of salt treated wheat seedlings at 5-day intervals for the first time on day 20 following planting and the last time on day 45. From the 36th day of planting, the salinized nutrient solution was altered with nutrient solution only, and this setup was maintained until harvesting (day 55). The control (T0) plants were grown on the nutrient solutions only with dH₂O without the addition of NaCl. The nutrient solutions were renewed at an interval of 15 days. The pH (6.5) and EC of the nutrient solution kept constant throughout the experimental period measured by pH meter (Hanna HI2211) and EC-meter (Hanna HI 4321) respectively. Different growth traits were measured from 10 wheat seedlings in each replication after 55 days of crop life span and afterward, the average value was taken. Furthermore, leaf samples were collected from the

55-days old wheat seedlings for the analyses of photosynthetic components, leaf water parameters, proline accumulation, Na⁺/K⁺ ratio, H₂O₂, and MDA content, and the activity of antioxidant enzymes (APX, CAT, and POD).

Determination of Growth Parameters

The number of live leaves was divided by the total number of leaves and multiplied by a hundred to get percentages of the leaf live (%). Using a centimetre (cm) scale, root length was measured from shoot initiation to root tip, and shoot length was calculated by subtracting plant length from root length. The shoot samples were separated from the root samples immediately after harvesting, and the fresh weight of root and shoot were meticulously measured using an electric balance. Following that, plant samples were individually wrapped in a brown envelope (20 cm x 10 cm) and oven-dried for 3 days at 60°C. After that, an electric balance was used to determine the dry weight of the root and shoot.

Determination of Leaf Water-related Parameters

Different leaf water-related traits *viz.*, relative water content (RWC), excised leaf water retention (ELWR), and relative water loss (RWL) were estimated from the representative fresh leaves samples of 55-days old wheat seedlings. For the measurement of RWC, fresh weight (FW) of leaves were measured, and subsequently submerged in distilled water (dH₂O) for 4 h and reweighed to obtain turgid weight (TW). Then, leaf samples were oven-dried at 70°C for 48 h and dry weight (DW) was recorded. Finally, RWC was calculated using the formula of Mostofa and Fujita (2013). In the case of RWL and ELWR measurement, FW of leaves was recorded and oven-dried at 30°C for 4 hours and reweighed (WW4h). Afterwards, RWL and ELWR were estimated following the previously described protocols of Gavuzzi *et al.* (1997) and Lonbani and Arzani (2011) respectively.

Determination of Photosynthetic Pigments

Chlorophyll a (Chl a), Chlorophyll b (Chl b), total chlorophyll content, and carotenoids were determined on the 55th day of planting from the 2nd leaf. An amount of 0.05 g fresh leaf sample was taken into a small vial containing 10 ml of 80% acetone and was covered by aluminum foil, and preserved in the dark for 7-10 days. The absorbance was measured at 663 for Chl a, 645 for Chl b, 663 for total chlorophyll, and 470 nm wavelengths for carotenoids by using a spectrophotometer (Shimadzu UV-2550, Kyoto, Japan). Afterwards, the concentrations of Chl a, Chl b and total chlorophyll content were calculated using a standard method developed by Lichtenthaler and Buschmann (2001) and expressed as mg g⁻¹FW.

Determination of Na⁺/K⁺ Ion Concentration and Proline Content

After harvesting, shoot samples were oven-dried at 60°C for 3 days and the finely powdered plant material was digested

with HNO_3 : HClO_4 (2:1v/v) mixture at 220°C for 1.5 - 2 hours according to the method of Tahjib-Ul-Arif et al. (2018a). Na^+ and K^+ contents were quantified by flame photometry (Jencon PFP 7, JENCONS-PLS, UK) following to Brown and Lilleland (1946).

The proline content of the leaves was estimated using the guidelines described by Bates et al. (1973), with some adjustments as stated by Rasel et al. (2019).

Determination of H_2O_2 and MDA Content

The level of H_2O_2 was determined from leaf tissues using the procedure of Velikova et al. (2000), with an extinction coefficient of $0.28 \mu\text{M}^{-1} \text{cm}^{-1}$, and the values expressed as $\text{mmol g}^{-1} \text{FW}$.

Lipid peroxidation, defined as MDA content, was determined from the seedlings' leaf tissues using Khan and Panda's (2008) technique, with an extinction co-efficient of $155 \text{mM}^{-1} \text{cm}^{-1}$ and values converted in $\text{mmol g}^{-1} \text{FW}$.

Determination of Antioxidant Enzyme Activities

A pre-cooled mortar and pestle was used to homogenize approximately 0.5 g of fresh leaf tissues in 3 mL of 50 mM ice-cold potassium phosphate buffer (pH 8.0). The supernatant was separated and utilized to analyse various antioxidant enzymatic reactions after centrifugation of the homogenate at $11,500 \times g$ for 10 minutes at 4 °C.

The activity of CAT (EC: 1.11.1.6) was measured using the Aebi (1984) technique, which involved measuring the reduction in absorbance at 240 nm with an extinction value of $39.4 \text{M}^{-1} \text{cm}^{-1}$ and expressed the results in $\text{mmol min}^{-1} \text{g}^{-1} \text{FW}$.

According to the method of Nakano and Asada (1981), the activity of APX (EC: 1.11.1.11) was estimated by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. The specific activity was calculated using a $2.8 \text{mM}^{-1} \text{cm}^{-1}$ extinction coefficient and expressed as $\mu \text{mol min}^{-1} \text{g}^{-1} \text{FW}$.

The activity of POX (EC: 1.11.1.7) was determined using the approach of Nakano and Asada (1981), with slight modifications. The extinction coefficient of H_2O_2 was $40 \text{M}^{-1} \text{cm}^{-1}$, the activity of POX was determined as $\text{mmol min}^{-1} \text{g}^{-1} \text{FW}$ from the increase in absorbance per minute.

Statistical Analysis

To assess the effects of salt treatments, data were analysed following one-way analysis of variance using the Statistical Tool for Agricultural Research (STAR) (IRRI, Los Baños, Laguna, Philippines). The statistical differences between mean values were assessed using the least significant differences (LSD) test at a significance level of $P < 0.05$, and different alphabetical letters in the same column were used to denote them. Biovinci was used to carry out the hierarchical clustering. The STAR was

used to perform the principal component analysis (PCA), and bi-plots were created using the first two PCA components, PC1 and PC2, which explained the maximum variations throughout the datasets.

RESULTS

Effect of NaCl, SA, and TU on Plant Growth Traits

The results related to the effects of NaCl, SA, and TU on morphological parameters of wheat genotypes are presented in Table 1. Salt stress significantly decreased the growth traits in both genotypes, however, the exogenous application of SA and TU successfully mitigate the toxic effect of salt stress and thereby, considerably improved the growth performance in wheat seedlings under salt-stressed conditions (Table 1; Figure 1). In the present investigation, the reduction of leaf live (%) was recorded by 13% and 18% in the varieties named BARI Gom-25 and BARI Gom-26 respectively when plants exposed to salt stress in comparison with control. On the other hand, when the exogenous SA or TU applied on stressed wheat seedling, leaf live (%) was substantially increased by 36% and 42% in BARI Gom-25 and BARI Gom-26 in comparison with salt-treated plants only (Table 1). Salt treatment considerably curtailed the shoot length and root length in both genotypes. However, the supplementation of salt-stressed seedlings with exogenous TU and SA enhanced the shoot length by 10% in BARI Gom-25, and 13% BARI Gom-26 respectively as compared to the salt-treated plants in the absence of SA and TU (Table 1). Similarly, the foliar application of SA also significantly improved the root length in both genotypes and more evidently in BARI Gom-26 (54%) (Table 1). The results of the study confirmed that salt treatment manifested the noteworthy reduction of root fresh weight and shoot fresh weight in BARI Gom-25 (26% and 17%, respectively) and BARI Gom-26 (49% and 38%, respectively) were manifested under salt treatment than the level of control plants (Table 1). But the greatly amelioration of the harmful effect of salinity more pronouncedly done by foliar application of SA in BARI Gom-25 and increased root fresh weight and shoot fresh weight by 91%, 46% as compared to the seedlings treated with salt-stress without SA application, whereas the foliar spray of TU to salt-stressed wheat seedlings also caused the moderate enhancement of root and shoot fresh weight in BARI Gom-26 (13% and 15% respectively) as compared to the NaCl treated plants only (Table 1). Salt stress also remarkably decreased the dry biomass of root and shoot significantly in BARI Gom-26 (48% and 42% respectively). In the contrast, the greater increment of root dry weight and shoot dry weight were observed in stressed BARI Gom-26 (38% and 53% respectively) upon the exogenous application of TU to salt-stressed plants in comparison with the seedlings treated with stress only (Table 1). Similarly, the foliar application of SA on the salt-treated plant also substantially improved the dry weight of root and shoot by 77% and 20% respectively in BARI Gom-26. However, the exogenous application of SA and TU didn't substantially increase the dry biomass in salt-stressed BARI Gom-25 (Table 1).

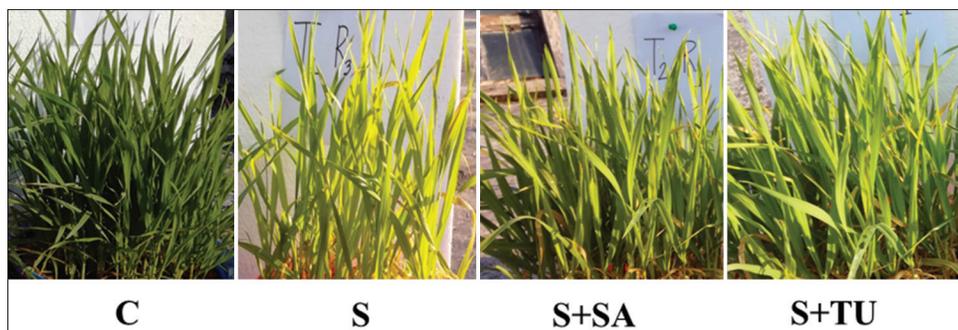


Figure 1: Effects of SA and TU on the phenotype of wheat seedling's grown under salt stress conditions. Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU).

Table 1: The effects of exogenous salicylic acid (SA) and thiourea (TU) on growth parameters of the two wheat cultivars under non-salinized and salinized conditions.

Genotypes	Treatment	LL (%)	SL	RL	SFW	RFW	SDW	RDW
BARI Gom 25	C	4.31±0.04 ^c	44.47±0.19 ^a	32.62±0.39 ^a	3.036±0.09 ^b	1.58±0.05 ^b	0.33±0.01 ^a	0.13±0.01 ^a
	S	3.75±0.19 ^d	28.42±0.41 ^d	22.19±0.31 ^c	2.246±0.02 ^c	1.316±0.03 ^c	0.31±0.01 ^a	0.11±0.03 ^a
	S+SA	5.58±0.14 ^b	29.89±1.09 ^c	26.40±0.22 ^b	3.28±0.04 ^a	2.52±0.02 ^a	0.33±0.04 ^a	0.14±0.03 ^a
	S+TU	5.91±0.06 ^a	31.27±0.26 ^b	26.85±0.2 ^b	2.35±0.18 ^c	1.65±0.03 ^b	0.32±0.02 ^a	0.14±0.01 ^a
BARI Gom 26	C	4.44±0.02 ^c	47.24±0.53 ^a	38.67±0.02 ^a	3.23±0.07 ^a	1.20±0.1 ^a	0.36±0.03 ^a	0.14±0.02 ^a
	S	3.63±0.1 ^d	27.19±0.11 ^c	21.41±0.11 ^d	1.65±0.02 ^d	0.74±0.02 ^d	0.21±0.01 ^d	0.07±0.02 ^b
	S+SA	5.38±0.34 ^a	30.82±0.36 ^b	32.95±2.16 ^b	2.03±0.03 ^b	0.91±0.07 ^b	0.25±0.02 ^c	0.12±0.03 ^a
	S+TU	4.85±0.1 ^b	30.14±0.15 ^b	24.30±0.1 ^c	1.86±0.03 ^c	0.85±0.02 ^c	0.32±0.02 ^b	0.10±0.01 ^{ab}

Here, LL(%) leaf live (%), SL Shoot length (cm), RL Root length (cm), SFW Shoot fresh weight (g), RFW Root fresh weight (g), SDW Shoot dry weight (g), and RDW Root dry weight (g). Data are presented with mean values of three independent replicates ± standard errors (n = 3) indicated by different alphabetical letters in the same column at the p < 0.05 level using the least significant difference (LSD) test. Different letters indicate significant differences and the same letter indicates no significant differences between the treatments. Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75mM SA); and S+TU (150 mM NaCl with 15 mM TU)

Estimation of Leaf Water-related Parameters

To examine the alleviating effect of SA and TU on leaf water status in wheat seedlings under salt stress, some leaf water-related parameters viz., RWC, ELWR, and RWL were measured (Table 2). Salt intrusion caused a significant reduction of RWC and ELWR by 25% and 33% in BARI Gom-25 and by 21% and 40% respectively in BARI Gom-26 compared to the salt-free plants. In contrast, the plants supplemented with exogenous SA demonstrated the highest increment of RWC and ELWR in the leaves of BARI Gom-25 (30% and 49%, respectively) and BARI Gom-26 (21% and 46%, respectively) compared to the salt-stressed plant in the absence of SA (Table 2). The imposition of salinity stress also had a remarkable effect on RWL in the leaves of BARI Gom-25 and BARI Gom-26 in the level of control, whereas when the plants were sprayed with exogenous SA, RWL was considerably decreased by 27% BARI Gom-25 and by 41% in BARI Gom-26 compared to the salt-treated plants only. On the other hand, the application of TU didn't exhibit significant improvement of RWC and ELWR as well as the reduction of RWL in both salt-stressed wheat genotypes (Table 2).

Determination of Photosynthetic Pigment under NaCl, SA, and TU Treatments

Wheat seedlings subjected to salt stress caused the severe curtailment of chl a, and chl b content in both genotypes and

Table 2: The effects of exogenous salicylic acid (SA) and thiourea (TU) on relative water content (RWC), relative water loss (RWL), and excised leaf water retention (ELWR) of the two wheat cultivars under non-salinized and salinized conditions.

Genotypes	Treatment	RWC (%)	RWL (%)	ELWR (%)
BARI Gom 25	C	83.67±0.62 ^a	0.32±0.02 ^b	0.38±0.04 ^a
	S	63.30±1.96 ^c	0.44±0.08 ^a	0.22±0.03 ^b
	S+SA	81.93±0.84 ^a	0.32±0.03 ^b	0.35±0.02 ^a
	S+TU	71.57±0.25 ^b	0.39±0.06 ^{ab}	0.27±0.02 ^b
BARI Gom 26	C	65.14±0.65 ^a	0.27±0.03 ^c	0.36±0.03 ^a
	S	51.41±0.26 ^c	0.39±0.08 ^a	0.21±0.01 ^b
	S+SA	61.96±0.08 ^a	0.23±0.02 ^c	0.31±0.06 ^a
	S+TU	61.45±0.05 ^a	0.30±0.03 ^b	0.27±0.02 ^b

Data are presented with mean values of three independent replicates ± standard errors (n = 3) indicated by different alphabetical letters in the same column at the p < 0.05 level using the least significant difference (LSD) test. Different letters indicate significant differences and the same letter indicates no significant differences between the treatments. Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU)

the reduction is much pronounced in BARI Gom-26 (16%, chl a and 42% chl b) (Figure 2A and B). The alleviation effect of TU on salt-stressed plants was considered best in compared with SA treatment alone in this case. When the wheat plants were treated with NaCl+TU, the chl a and chl b content was substantially increased in wheat genotypes viz., BARI Gom-26 (33% and 53% respectively) and BARI Gom-25 (15% and 36% respectively) as compared to seedlings treated with

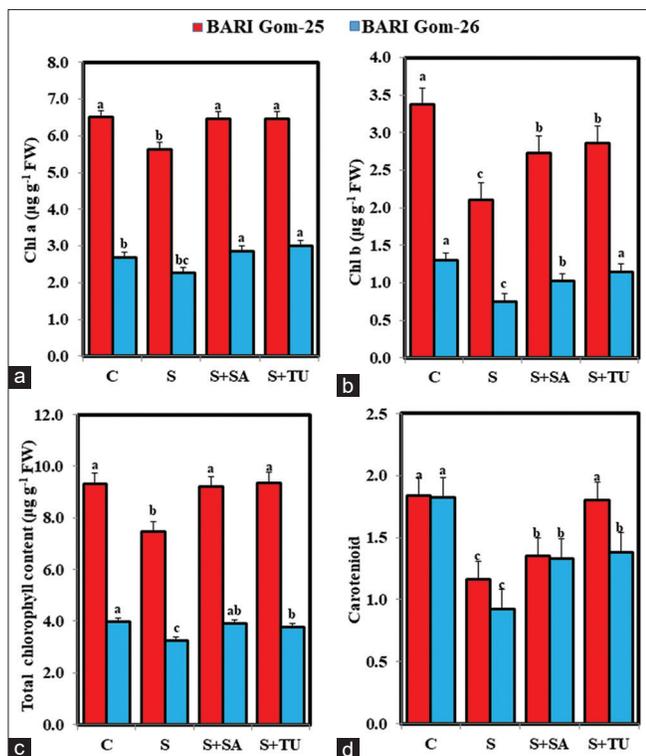


Figure 2: The effects of exogenous salicylic acid (SA) and thiourea (TU) on (a) Carotenoid, (b) Chl a, (c) Chl b and (d) Total chlorophyll content of the two wheat genotypes under non-salinized and salinized conditions. Data are presented with mean values of three independent replicates indicated by different alphabetical letters in the same column at the $p < 0.05$ level using the least significant difference (LSD) test. Different letters indicate significant differences and the same letter indicates no significant differences between the treatments. Vertical bars indicate standard errors. Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU).

salt stress only (Figure 2A and B). On the other hand, chl b content substantially improved only by 30% in BARI Gom-25 and 36% in BARI Gom-26 due to the foliar application of SA particularly. Carotenoid concentrations reduced in both BARI Gom-25 (37%) and BARI Gom-26 (49%) under salt stress condition compared to control-treated plants. However, S+SA and S+TU treatments displayed the greater increment of carotenoid content by 44% and 49% respectively in BARI Gom-26. Moreover, only 'S+TU' treatment promoted the carotenoid content BARI Gom-25 by 55% in as compared with the plants treated with salt only (Figure 2D).

Determination of Na^+/K^+ under NaCl, SA, and TU Treatments

The Na^+/K^+ in the leaves of both genotypes enhanced after imposition of salt treatment with a 15% increment in BARI Gom-26 and 2% increment in BARI Gom-25 compared to control condition plants (Figure 3A). However, when the salt-stressed plants were supplemented with SA and TU, Na^+/K^+ was markedly lowered in BARI Gom-25 by 33% and 17% respectively due to the mitigation effects of SA and TU in compared with

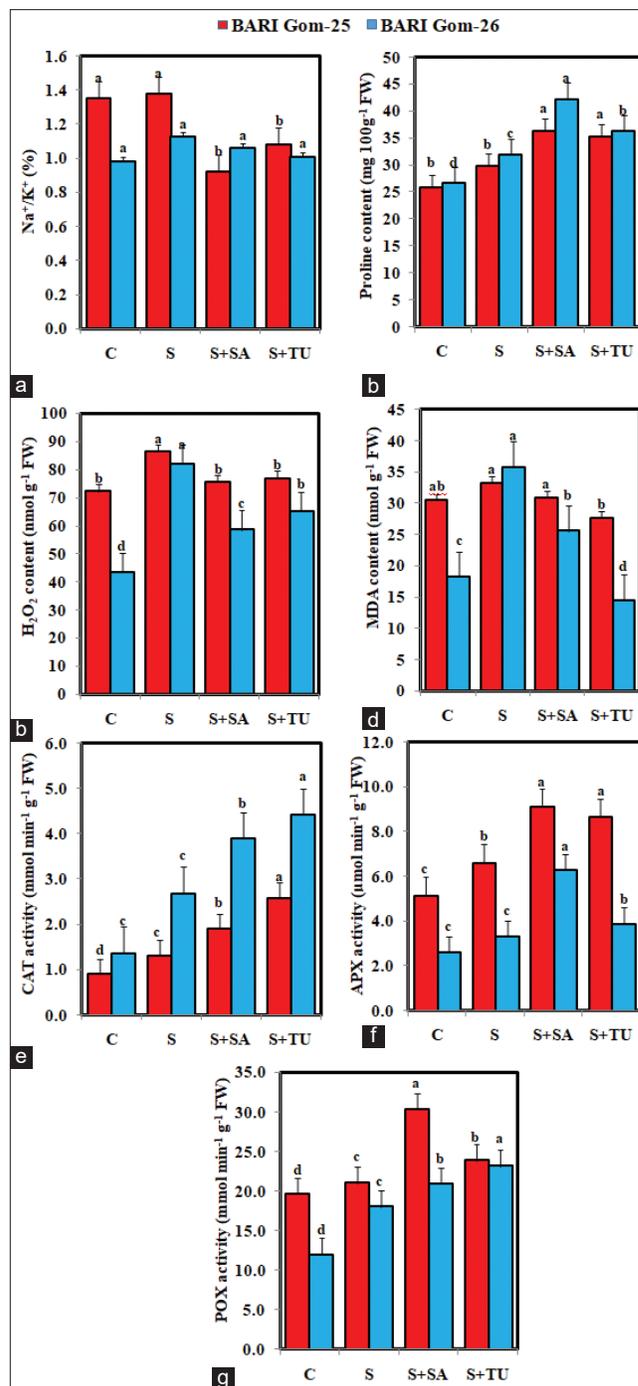


Figure 3: The effects of exogenous salicylic acid (SA) and thiourea (TU) on (a) Na^+/K^+ , (b) Proline content, (c) Hydrogen peroxide (H_2O_2), (d) Malondialdehyde (MDA) content, (e) Catalase (CAT) activity, (f) Ascorbate peroxidase (APX) activity and, (g) Peroxidase (POX) activity of the two wheat genotypes under non-salinized and salinized conditions. FW denotes fresh weight. Data are presented with mean values of three independent replicates indicated by different alphabetical letters in the same column at the $p < 0.05$ level using the least significant difference (LSD) test. Different letters indicate significant differences and the same letter indicates no significant differences between the treatments. Vertical bars indicate standard errors. Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU).

that of the salt-stressed plants only. Besides, the exogenous SA or TU application did not cause significant changes of Na^+/K^+ in salt-stressed BARI Gom-26 as compared to that of salt-treated plants (Figure 3A).

Determination of Proline Content under NaCl, SA, and TU Treatments

Salt stress caused a marked change in proline accumulation in the leaves of wheat seedlings (Figure 3B). The application of salt treatment resulted in an increase of proline content resulted in BARI Gom-25 and BARI Gom-26 by 16% and 19% respectively due to salt application as compared to control (Figure 3B). Moreover, exogenous application of both SA and TU significantly increased the proline level in salt-stressed wheat genotypes namely BARI Gom-25 (22% and 3%, respectively) and BARI Gom-26 (32% and 14%, respectively) in comparison with the seedlings treated with salt stress only (Figure 3B).

Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) Content, under NaCl, SA, and TU Treatments

To investigate the alleviating effect of SA and TU on salt-induced oxidative damage in the wheat genotypes leaves H_2O_2 and MDA contents were measured under salt stress. The results of the study demonstrated that the application of 150 mM salt stress increased the production of H_2O_2 by 89% as well as a substantial rise of MDA content by 96% in BARI Gom-26 in comparison with the salt-treated plant in the absence of SA or TU (Figure 3C and D). In contrast, spraying of SA and TU on salt-stressed plants defended the cell membrane from salt commenced oxidative injury which is indicated by lower H_2O_2 (29% and 20%, respectively) and MDA content (29% and 59%, respectively) markedly in BARI Gom-26 as compared with the seedlings treated with salt-stressed only (Figure 3C and D). However, the generation of H_2O_2 and MDA were not significantly varied in the leaves of BARI Gom-25 under different treatments in this study (Figure 3C and D).

Different Antioxidant Enzyme Activities under NaCl, SA, and TU Treatments

The enzymatic activities of antioxidants *viz.*, CAT, APX, POX, were determined in salt exposed wheat seedlings which are as vital players and give protection to plant from membrane damage mediated by H_2O_2 . Salt stress increased the activities of antioxidant enzymes in wheat genotypes; however, more enhancements were found when the salt-treated plants were supplemented with SA or TU (Figure 3). In the present study, CAT activities were expressed with 46% increment in BARI Gom-25 and 94% increment in BARI Gom-26 under salt stress conditions whereas APX activity displayed moderate increment in both BARI Gom-25 (28%) and BARI Gom-26 (26%) with regard to control plant (Figure 3E and F). However, the further higher increment of CAT with the application of 'S+TU' combinations was remarkably noted in BARI Gom-25 by 35% and BARI Gom-26 by 65%. Then again, to salt-stressed wheat

plants with individual foliar application of SA revealed greater enhancement of the APX activity in both genotypes such as BARI Gom-25 (38%) and BARI Gom-26 (90%) in compared with the salt-treated plants only (Figure 3E and F). The consequences of the study also exposed that increased activities of POX by 7% in BARI Gom-25 and 50% BARI Gom-26 under salt stress as compared to the control plants (Figure 3G). Moreover, salt-stressed seedlings displayed further enhancement of POX activity in BARI Gom-25 (44% and 21%, respectively) and BARI Gom-26 (16% and 28% respectively) when supplemented with 'S+SA' and 'S+TU' treatments in comparison with seedlings treated with salt-stress only (Figure 3G).

Treatment-Variable Interaction Measurement Using Hierarchical Clustering and Principal Component Analysis (PCA)

The heat map and hierarchical cluster analysis based on the mean value of different morpho-physiological, and biochemical traits of two wheat genotypes under both non-salinized and salinized conditions and all the traits were grouped into three main clusters (Cluster-I, -II and -III) (Figure 4). The cluster distribution design showed that the variables *viz.*, H_2O_2 , CAT, LL (%), APX, PRO, and POX were grouped in Cluster- I. Among these traits, H_2O_2 was significantly increased in salt stress conditions, however, declined upon the foliar spray of SA or TU on salt-stressed wheat plants. On the other hand, other traits namely PRO, and POD showed unchanged activities

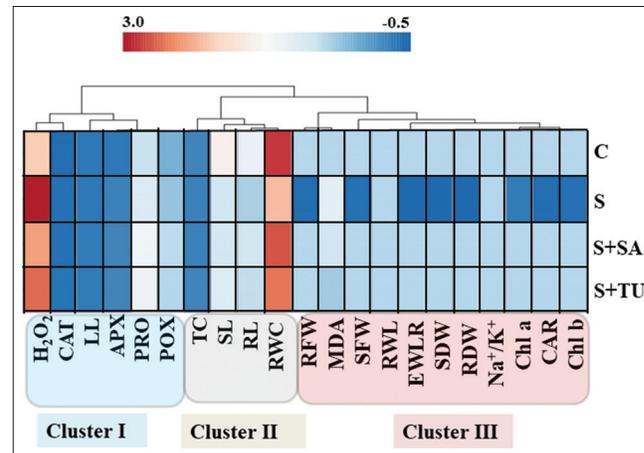


Figure 4: Hierarchical clustering to understand treatment-variable relationships of two wheat genotypes, BARI Gom-25 and BARI Gom-26 under different treatment combinations including Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU). Three distinct clusters (I, -II and -III) were obtained at the variable level. The colour scale shows the intensity of the normalized mean values of different traits. The variables included LL (%) leaf live (%), SL Shoot length, RL Root length, SFW Shoot fresh weight, RFW Root fresh weight, SDW Shoot dry weight, RDW Root dry weight, RWC Relative water content, RWL Relative water loss, ELWR excised leaf water retention, CAR Carotenoid, Chl a Chlorophyll a, Chl b Chlorophyll b, TC Total chlorophyll content, Na^+/K^+ Na^+/K^+ ratio, PRO Proline content, H_2O_2 Hydrogen peroxide, MDA Malondialdehyde content, CAT Catalase activity, APX Ascorbate peroxidase activity, and POX Peroxidase activity.

under salinity stress in compared with control condition plants whereas the exogenous application of SA and TU caused the clear increasing trend of PRO, and POX activities in salt-treated plants when compared with 'S' alone (Figure 4). Moreover, CAT, APX, and LL (%) reflected no change in wheat plants upon the addition of S, S+SA, and S+TU treatments in compared with non-stressed treatment. The traits of Cluster-II (TC, SL, RL, and RWC) demonstrated differential reaction due to the application of NaCl and exogenous SA or TU on wheat plants with regards to the salt-free control plants (Figure 4). The TC content was unchanged and SL, RL, and RWC were decreased when salt stress was imposed, while the supplementation of salt-treated plants with SA and TU displayed the increased level of these parameters except TC content (Figure 4). The heat map also exhibited different response in Cluster-III variable's where some traits namely RWL, and Na^+/K^+ were enhanced and other traits viz., RFW, MDA, SFW, RWL, EWLR, SDW, RDW, Na^+/K^+ , Chl a, Chl b, and CAR were declined in wheat plants due to salt-induced injury when compared with control-treated plants only. In contrast, these traits showed positive results when the salt-stressed plants were supplemented by SA or TU such as a decreasing trend of RWL and Na^+/K^+ and an increasing trend of RFW, MDA, SFW, RWL, EWLR, SDW, RDW, Na^+/K^+ , Chl (a, b) as well as CAR in comparison with the plant under salt-stress with no use of SA or TU, indicating that exogenous SA and TU

might play ameliorating role to mitigate the adverse effect of salt stress and thereby, enhanced the growth performance in wheat plants under salt stress situations (Figure 4).

To reveal the association of the different treatments (S, S+SA, and S+TU) with morpho-physiological and biochemical traits, the PCA was also performed (Figure 5). In the present investigation, the PC1 from the PCA analysis explained about 58.75% of the total variation present in these genotypes, whereas PC2 describes 32.28% and the two components (PC1 and PC2) collectively explained 91.03% of data variability. The results of the PCA displayed that the parameters Na^+/K^+ , RWL, MDA, and H_2O_2 were associated salt stress treatment demonstrated that these traits increased under salt stress (Figure 5). Besides, another treatment's 'S+SA' and 'S+TU' were clustered in two different groups of morpho-physiological and biochemical characteristics. The variables PRO, POX, CAT, and APX were closely associated with 'S + TU', whereas LL (%), RFW, Chl a, TC and RDW were associated with 'S + SA'. Another group of traits viz., SL, SDW, SFW, CAR, Chl b, RL, RWC, and EWLR showed significant interaction with 'C' treatment (Figure 5).

DISCUSSION

Salinity stress is considered as one of the major limiting factors for agricultural crop production, which becomes a significant threat to ensure food security for the increasing population of the world (Alhasnawi *et al.*, 2015; Gupta & Huang, 2015). Under high salt stress conditions, different morpho-physiological and biochemical changes occurred in plants resulting in the reduction of growth potential as well as productivity (Bacha *et al.*, 2017; Jahan *et al.*, 2020; Rahman *et al.*, 2020). In recent times, exogenous osmoprotectants such as SA and TU demonstrated the capacity to enhance the plants' growth and development under salinity stress by mitigating the salt-induced damage through osmotic adjustment, scavenging ROS and stabilization of membrane integrity, etc. (Ali & Ashraf, 2011; Hasanuzzaman *et al.*, 2013; Pandey *et al.*, 2021). Thus, the present study revealed the alleviating actions of exogenous application of SA and TU against the negative impacts of saline stress in two wheat genotypes by increasing the growth traits, leaf water status and photosynthetic components, and antioxidant defence systems.

Under stressful conditions, the cell metabolism of plants is inhibited which mandate the dysfunction of cell division as well as cell differentiation (Veylder *et al.*, 2007). Salt stress primarily incites ionic toxicity causing osmotic and oxidative stresses, later on, all of which collectively exert a negative effect on plant growth performance and development (Muchate *et al.*, 2016; Roupheal *et al.*, 2018). The results of our study showed that the exposure of wheat seedlings to salinity stress severely diminished the growth parameters viz., lower live leaf (%), reduced root and shoot length, and reduced root and shoot biomass in both wheat genotypes (Table 1; Figure 1). These outcomes have similarity to the findings of Sanaullah *et al.* (2016) and Chunthaburee *et al.* (2016) who observed a reduced shoot and root growth, and less dry mass production

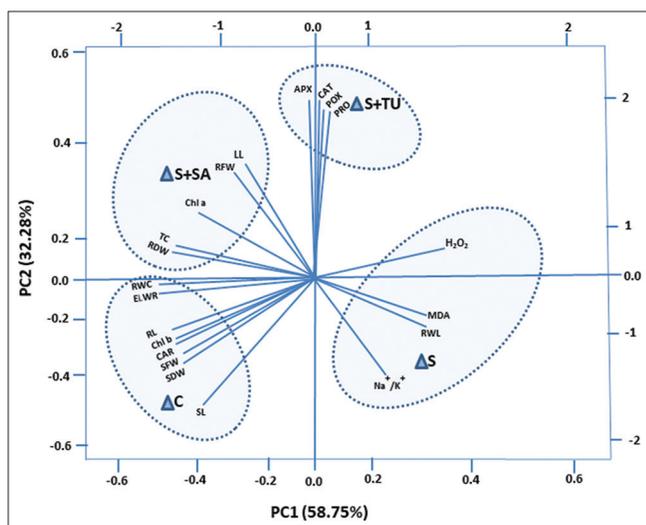


Figure 5: Principle component analysis (PCA) to understand the treatment-variable relationships of two wheat genotypes, BARI Gom-25 and BARI Gom-26, under different treatment combinations including Control, C (nutrient solution); salt, S (nutrient solution with 150 mM NaCl); S+SA (150 mM NaCl with 0.75 mM SA); and S+TU (150 mM NaCl with 15 mM TU). The lines originating from central point of biplots indicate positive or negative correlations of different variables; where their closeness indicates correlation strength with particular treatment. The variables included LL (%) leaf live (%), SL Shoot length, RL Root length, SFW Shoot fresh weight, RFW Root fresh weight, SDW Shoot dry weight, RDW Root dry weight, RWC Relative water content, RWL Relative water loss, ELWR excised leaf water retention, CAR Carotenoid, Chl a Chlorophyll a, Chl b Chlorophyll b, TC Total chlorophyll content, Na^+/K^+ Na^+/K^+ ratio, PRO Proline content, H_2O_2 Hydrogen peroxide, MDA Malondialdehyde content, CAT Catalase activity, APX Ascorbate peroxidase activity, and POX Peroxidase activity.

at the seedling stage of maize (*Zea mays* L.) and rice (*Oryza sativa* L.) plants under saline condition, respectively. The higher level of salt concentration dispenses a toxic effect on shoots resulting lower live leaves (%) because of the exclusion of symplastic xylem accumulation of calcium by salt into the root of plants (Läuchli & Grattan 2007).

As the number of live leaves is reduced, the rate of photosynthesis ultimately decreases hampering the supply of carbohydrates and growth hormones to meristematic tissues and ultimately, decelerates plant biomass production (Hussain *et al.*, 2018). However, foliar treatment of salt-stressed BARI Gom-25 and BARI Gom-26 with SA or TU demonstrated a higher efficiency in the reduction of the adverse impacts of salt stress on plant growth and revealed a protective role of SA and TU in wheat seedlings against salinity stress by exhibiting higher percentage of live leaf, higher weight of root and shoot in both fresh and dry conditions, and improvement in shoot and root length as compared to the plants treated with salt stress only (Table 1) (Nie *et al.*, 2018). The similar findings were also reported in cucumber (*Cucumis sativus*) (Shim *et al.*, 2009), cotton (*Gossypium hirsutum*) (Shaheen *et al.*, 2012), and teak (*Tectona grandis*) (Li *et al.*, 2014). SA-induced growth increase could be related to the enhanced activities of antioxidant enzymes in salt stress conditions allowing plant with a greater proficiency of growth and development by encountering the salt-induced oxidative stress (Horvath *et al.*, 2007; Harfouche *et al.*, 2008). Besides, the growth-flourishing effects of SA might be caused by the positive changes of plant hormonal status leading to the improvement of photosynthesis, transpiration, and stomatal conductance in plants under stress situations (Stevens *et al.*, 2006; Abreu & Munne-Bosch, 2009). On the other hand, the exogenous application of TU might provide the source of C and N to the leaves of salt-stressed wheat seedlings, which was positively correlated to the enhanced production of plant biomass (Anjum *et al.*, 2011). Moreover, TU increased the uptake of essential nutrients and accelerates the metabolic processes under stress conditions, which lead to higher growth and dry matter accumulation in stressed plants (Burman *et al.*, 2004). The maintenance of optimal water status in plants is considered as a key physiological process for keeping normal growth and development under salt stress (Ahanger *et al.*, 2017). Different leaf water-related parameters such as RWC, RWL, and ELWR are commonly used to measure the water status of plant as well as osmotic adjustment in stress conditions (Baisakh *et al.*, 2012). Salinity is often lower the water potential in the soil environment which is found to decline the RWC in root and shoot that ultimately causing the closure of stomata and limits the assimilation of CO₂ mounting, an adverse impact on photosynthetic components in plants (González & González-Vilar 2006; Pattangul & Thitisaksakul 2008). In the present study, RWC, and ELWR were severely diminished when the wheat seedlings were confronted with salt stress (Table 2). This is because the salt-induced injury caused a reduction of water uptake in the leaves by disrupting the cell wall structure (Khaled *et al.*, 2018; Parvin *et al.*, 2019). Moreover, seedlings exposed to salt stress also showed a severe increment of RWL in the leaves in comparison with untreated plants (Table 2), as stress condition forced to more leaf electrolyte leakage in plants

which severely reduced the water content in leaves (Parida & Das 2005; Hnilíčková *et al.*, 2019).

In contrast, the exogenous application of SA dramatically up-regulated leaf RWC and ELWR in wheat leaves, as well as decreased the RWL in salt-stressed wheat seedlings (Table 2), reflecting that SA considerably elevated the membrane damages caused in salinity thereby minimizing water loss through facilitating the adjustment of optimal water status inside the plant tissues by reducing the transpiration and/or up-taking more water from the soil (Tahjib-Ul-Arif *et al.*, 2018b). This result was in agreement with Shi *et al.* (2006) and Khalifa *et al.* (2016) who observed lower electrolyte leakage in salt stress affected lettuce plants when treated with exogenous SA treatments. Moreover, the exogenous SA lowered the membrane damages in salinity stressed plants, which might be related to the up-regulation of antioxidant responses that safeguard the plant from oxidative damage (Khan *et al.*, 2015).

The photosynthetic attributes such as chlorophyll a and b are the vital factors fixing photosynthetic efficiency and leading the mechanism of the photosynthetic process in plants (Shao *et al.*, 2014). Several reports suggested that photosynthetic pigments are highly affected by salt stress which inhibits photosynthesis by worsening the leaf chlorophyll content directly or by feigning photosynthetic apparatus (Sharma *et al.*, 2020). Furthermore, when the high levels of salt accumulate in leaves, the activity of chlorophyll degrading enzyme namely chlorophyllase accelerates the degradation or inhibition of chlorophyll synthesis leading to decrease in chlorophyll content (Ashraf & Harris 2013; Kordrostami *et al.*, 2017).

Our investigation reported that the photosynthetic pigments viz., Chl a, Chl b, total chlorophyll, and carotenoid contents were declined in greater proportions in both salt-stressed wheat genotypes (Figure 2), whereas when stressed plants were supplemented with SA or TU alone, the photosynthetic components were significantly augmented in the seedlings of both genotypes (Figure 2) which indicated that SA or TU might assist photosynthesis process by protecting chloroplast pigments from the salinity induced toxicity probably through the oxidative protection of chloroplasts (Foyer & Shigeoka 2011; Amin *et al.*, 2016). Furthermore, SA and TU might flourish the enzyme's activity by regulating chlorophyll biosynthesis or might protect the photosynthetic system from impairment, thereby decreasing chlorophyll degradation under salt stress situations (Ma *et al.*, 2017). The similar findings to our study were also reported by Amin *et al.* (2014) and Abdelaal *et al.* (2020) who were reported an up-regulation of chlorophyll content in the salt-stressed leaves of barley (*Hordeum vulgare*) and faba bean (*Vicia faba*) upon the addition of exogenous SA and TU.

The prevailing of excess salinity in the soil leads to saline toxicity and nutrient deficiencies in plants through the excessive uptake of individual ions like Na⁺ and inhibiting the absorption of essential plant nutrients, respectively (Assaha *et al.*, 2017; Sa *et al.*, 2019). Salt stress also accelerated the expression OsNHX1, which is associated with the transfer of Na⁺ for

vascular compartmentalization (Mekawy *et al.*, 2015). Plants can reduce the excessive accumulation of Na^+ in the shoots in two different ways, such as preventing the load or maximization of Na^+ retrieval from the xylem by minimizing the Na^+ entry from the root symplast (Davenport *et al.*, 2007), or exporting the Na^+ from the leaf into the phloem (Berthomieu *et al.*, 2003). Ejection of superfluous Na^+ from the shoots is considered as one of the most vital characteristics of salt tolerance ability in plants (Wu, 2018), hence it is crucial to measure Na^+ in plants under saline conditions to understand the mechanisms of salinity tolerance in plants (Waqas *et al.*, 2019). In our study, the imposition of salinity stress significantly increased Na^+ concentration in the leaves of wheat genotypes reflecting a higher Na^+/K^+ ratio (Figure 3A). However, salt-stressed wheat seedlings treated with 'S+SA' or 'S+TU' demonstrated lower Na^+ accumulation in the leaves which destined a lowered Na^+/K^+ ratio (Figure 3A). This result was in agreement with Sanaullah et al. (2016) who observed a lower leaf Na^+/K^+ ratio in the salt-stressed maize (*Zea mays* L.) leaves with the foliar application of TU. The application of SA and/or TU on the leaves of stressed plant could inhibit the uptake of excessive salt by restricting passive Na^+ influx under salt stress conditions and eventually improve the transportation of essential ions from roots to shoots to maintain a balance Na^+/K^+ ratio (Liu *et al.*, 2014). The exogenous SA and TU could modulate the Na^+/K^+ ratio to improve the salt-tolerance mechanisms by facilitating the activity of H^+ -ATPase in the plasma membrane, which plays a major role in the increased absorption of K^+ , Ca^{2+} , and Mg^{2+} under NaCl induced toxicity (Nazar *et al.*, 2011; Kaya *et al.*, 2015; Kaya *et al.*, 2016).

Proline supplementation plays a crucial role to prevent salinity stress-mediated oxidative damage and lowered cell death, as proline can scavenges free radicals and minimizes the production of ROS (Banu *et al.*, 2009; Kibria *et al.*, 2017). Moreover, proline might inhibit stress-induced harmful effects on cellular organelles by stabilizing membrane structures, enzyme functioning, and maintaining water status (Ahanger *et al.*, 2018). In the present investigation, proline content was significantly affected by the application of salt stress, SA, and TU in wheat genotypes (Figure 3B). When plants faced salt stress, the storage of intracellular proline content is enhanced in both genotypes (Figure 3B). Increased accumulation of proline in plants under salinity stress has been also reported by several workers in wheat (*Triticum aestivum* L.), mustard (*Brassica juncea* L.), and canola (*Brassica napus* L.) which is similar to our investigation (Ashrafijou *et al.*, 2010; Ahmad *et al.*, 2011; Ahanger *et al.*, 2017). Moreover, accumulation of proline was demonstrated in the leaves of salt-treated wheat seedlings due to the exogenous application of SA or, TU in comparison with the plants treated with salt stress only (Figure 3B). Khan et al. (2014) have also observed the improvement of salinity tolerance in mung bean (*Vigna radiata* L.) due to increased accumulation proline upon the exogenous application of SA. Kaya et al. (2016) also reported the higher accumulation of proline content in salt-stressed maize when TU sprayed on foliar leaves. This might be due to the involvement of SA or TU in up-regulation of pro-biosynthesis enzymes such as γ -glut amyl kinase and pyrroline-5-carboxylate reductase under salt stress

along with the improved salinity tolerance ability in wheat plants (Misra & Saxena 2009; Khan *et al.*, 2013). The increment of the proline content with the application of SA or TU under saline conditions contributes to maintain osmotic balance, membrane integrity, enzyme and protein stability, and ROS detoxification, which may subsequently facilitate the conferring tolerance of stressed plants (Blum, 2017).

Accumulation of toxic ion (Na^+) in plant cells resulted in oxidative damage as reflected by higher ROS generation in parallel with higher lipid peroxidation (Soundararajan *et al.*, 2018). MDA is considered as a determining factor for measuring the lipid peroxidation level, which is a decomposition product of the thiobarbituric acid (TBA)-induced peroxidised polyunsaturated fatty acid content of the membrane lipid (Alamet *et al.*, 2013; Shen *et al.*, 2014). Salt stress caused an increment of H_2O_2 content in the leaves of plants, thus disrupting its permeability through the peroxidation of the lipid membrane (Hossain *et al.*, 2015). The excessive procurement of H_2O_2 exerts negative impacts on membrane functioning by commencing the peroxidation of lipids. Our investigation showed that the seedlings treated with salt without spraying of SA and TU accumulated higher MDA and H_2O_2 levels in leaves resulted in higher oxidative damage as compared to control (Figure 3C and D). Kadioglu et al. (2011) and Kaya *et al.* (2013) also demonstrated that the prolonged exposure of salt stress to wheat plants resulted in a significant boost of MDA and H_2O_2 content. By contrast, lower level of MDA and H_2O_2 accumulation in salt-stressed wheat leaves were observed in the present study due to the exogenous application of SA and TU (Figure 3C and D). These findings were supported by the other findings recorded in sweet basil (*Ocimum basilicum*) (Delavari *et al.*, 2010), savory (*Satureja hortensis*) (Yazdanpanah *et al.*, 2011), and in barley (*Hordeum vulgare*) (Alamri *et al.*, 2018). This is because the exogenous application of SA and TU decreased lipid peroxidation as well as H_2O_2 production by the induction of antioxidant responses in plants for quenching ROS in stress situations, which protects the plant from oxidative stress-induced membrane damage (Alam *et al.*, 2013). Moreover, SA supplementation was also increased GSH and AsA, and enhanced GSH/GSSG ratio, which causes a marked decline in salt-induced oxidative burst by the reduction of MDA and H_2O_2 content under salt stress (Hasanuzzaman *et al.*, 2014). The procurement of venturesome elements like ROS in plant cells is the most general consequence of saline condition which causes different oxidative damage such as infringement to proteins, lipids, and nucleic acids thereby inciting rapid plant cell death (Sharma *et al.*, 2012; Xie *et al.*, 2019). To safeguard form oxidative stress, the plant adopts a defensive mechanism with the activation of antioxidant defence systems including CAT, APX, and POX, which are played a key role in the scavenging of ROS, and to counteract against lipid peroxidation (Ahmad *et al.*, 2010; Dugasa *et al.*, 2019). One of the most effective antioxidant enzymes is CAT, which detoxifies H_2O_2 by converting it into H_2O and O_2 in the in different cellular organelles of the cells whereas APX plays the central role in the H_2O_2 detoxifying system in plant cells by catalysing the conversion of the H_2O_2 into H_2O and O_2 through using AsA as a specific electron donor (Caverzan *et al.*, 2012; Sofo *et al.*, 2015).

In the current study, a significant increase in CAT, APX, and POX activities was observed in the leaf tissues of the wheat genotypes under salt stress conditions (Figure 3A, B and C). The results observed in this study are in accordance with the results reported in different plant species such as mustard (*Brassica spp.*), wheat (*Triticum aestivum*), and pepper (*Piper nigrum*) (Nazar et al., 2011; Ahanger & Agarwal, 2017; Shams et al., 2019). Moreover, a higher activity of CAT, APX and POX was observed in salt-stressed wheat leaves because of applying exogenous SA and TU (Figure 3A, B and C), which clearly indicates that exogenous SA and TU might have an ameliorating capacity to detoxify excessively generated H₂O₂ and subsequently reduced the MDA accumulation. Hence, the exogenous SA and TU diminished the oxidative damage in the wheat genotypes under salt stress through up-regulating the antioxidant enzymes activities. These findings were consistent with other researchers who observed an enhanced antioxidant response with the application of SA or TU in strawberry (*Fragaria ananassa* L.), cucumber (*Cucumis sativus* L.), and rice (*Oryza sativa* L.) (Faghih et al., 2017; Kim et al., 2017; Zahra et al., 2018). Additionally, exogenously applied SA was found to modulate the transcription of antioxidant genes including, GPX1, GPX2, DHAR, GR, GST1, GST2, MDHAR, and GS, and enhanced the activities of ascorbate-glutathione (AsA-GSH) pathway enzymes under salt stress situations for improving salt tolerance in wheat seedlings (Li et al., 2013).

CONCLUSIONS

The present data clearly demonstrated that salt stress lead to growth inhibition, reduced photosynthetic capacity, leaf water status, and increased Na⁺/K⁺ ratio, and evocation of oxidative damage due to the generation of excessive H₂O₂ and MDA in both wheat genotypes. Salt-stressed wheat plants supplemented with exogenous SA or TU exhibited better growth performance through the enhancement of photosynthetic attributes and intracellular proline content. SA or TU also played an important role in minimizing membrane damage as indicated by lower level of MDA and H₂O₂ accumulation in the leaves of wheat seedlings exposed to salt stress. More evidently, antioxidant enzyme activities were considerably up-regulated in salinity exposed wheat plants upon the addition of SA or TU; representing the protective effects of SA or TU against the salt-induced oxidative stress. However, in-depth studies with different concentration of SA and TU under direct field condition will decipher the molecular mechanisms of SA- and TU-induced salt stress tolerance in wheat.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge the financial support provided by the Ministry of Science and Technology, Government of the People's Republic of Bangladesh.

AUTHOR'S CONTRIBUTION

Mohammad Afzal Hossain conceived the project and planned the experiment. Sumaiya Farzana performed the experiment

and wrote the first draft of the paper. A.G.M. Sofiuddin Mahamud helped to perform biochemical analysis. Md Rasel contributed to the data analysis and wrote the first draft of the paper. Md. Tahjib Ul Arif helped in the experimental works and performed the critical revision of the data. Md. Golam Azam and Md. Asadulla Al Galib contributed to the writing of the manuscript. Mohammad Anwar Hossain wrote the proof-reading of the final draft and edited.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the present study.

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