Promotion of Growth and Physiological Characteristics in Water-Stressed *Triticum aestivum* in Relation to Foliar-Application of Salicylic Acid

Abida Parveen 1,*, Muhammad Arslan Ashraf 1, Iqbal Hussain 1, Shagufta Perveen 1, Rizwan Rasheed 1, Qaisar Mahmood 2,*, Shahid Hussain 1, Allah Ditta 3,4, *, Abeer Hashem 5,6, Al-Bandari Fahad Al-Arjani 5, Abdulaziz A. Alqarawi 7 and Elsayed Fathi Abd Allah 7*

1 Department of Botany, Government College University, Faisalabad 38000, Pakistan; marlsanashraf@gcu.edu.pk (M.A.A.); driqbal@gcu.edu.pk (I.H.); sperveen2@yahoo.com (S.P.); drrizwan@gcuf.edu.pk (R.R.); abc@email.com (S.H.)
2 Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus, Peshawar 24730, Pakistan
3 Department of Environmental Sciences, Shaheen Benazir Bhutto University, Sheringal, Upper Dir, Khyber Pakhtunkhwa, Peshawar 18050, Pakistan; allah.ditta@sbbu.edu.pk
4 School of Biological Sciences, The University of Western Australia, 35 Stirling Highway, Perth, WA 6009, Australia
5 Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; habeer@ksu.edu.sa (A.H.); aarjani@ksu.edu.sa (A.-B.F.A.-A.)
6 Mycology and Plant Disease Survey Department, Plant Pathology Research Institute, ARC, Giza 12511, Egypt
7 Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia; alqarawi@ksu.edu.sa (A.A.A.); eabdallah@ksu.edu.sa (E.F.A.A.)
* Correspondence: abidauaf@yahoo.com (A.P.); mahmoodzju@gmail.com (Q.M.)

Abstract: The present work reports the assessment of the effectiveness of a foliar-spray of salicylic acid (SA) on growth attributes, biochemical characteristics, antioxidant activities and osmolytes accumulation in wheat grown under control (100% field capacity) and water stressed (60% field capacity) conditions. The total available water (TAW), calculated for a rooting depth of 1.65 m was 8.45 inches and readily available water (RAW), considering a depletion factor of 0.55, was 4.65 inches. The water contents corresponding to 100 and 60% field capacity were 5.70 and 1.66 inches, respectively. For this purpose, seeds of two wheat cultivars (Fsd-2008 and S-24) were grown in pots subjected to water stress. Water stress at 60% field capacity markedly reduced the growth attributes, photosynthetic pigments, total soluble proteins (TSP) and total phenolic contents (TPC) compared to cv. S-24, which was moderately drought tolerant. However, water stress enhanced the accumulation in wheat grown under control (100% field capacity) and water stressed (60% field capacity) conditions. The total available water (TAW), calculated for a rooting depth of 1.65 m was 8.45 inches and readily available water (RAW), considering a depletion factor of 0.55, was 4.65 inches. The water contents corresponding to 100 and 60% field capacity were 5.70 and 1.66 inches, respectively. For this purpose, seeds of two wheat cultivars (Fsd-2008 and S-24) were grown in pots subjected to water stress. Water stress at 60% field capacity markedly reduced the growth attributes, photosynthetic pigments, total soluble proteins (TSP) and total phenolic contents (TPC) compared with control. However, cv. Fsd-2008 was recorded as strongly drought-tolerant and performed better compared to cv. S-24, which was moderately drought tolerant. However, water stress enhanced the contents of malondialdehyde (MDA), hydrogen peroxide (H\(_2\)O\(_2\)) and membrane electrolyte leakage (EL) and modulated the activities of antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as accumulation of ascorbic acid and phenolics contents. However, foliar-spray of SA reduced MDA, H\(_2\)O\(_2\)) and membrane permeability in both cultivars under stress conditions. The results of the present study suggest that foliar-spray of salicylic acid was effective in increasing the tolerance of wheat plants under drought stress in terms of growth attributes, antioxidant defense mechanisms, accumulation of osmolytes, and by reducing membrane lipid peroxidation.

Keywords: drought stress; osmolytes; salicylic acid; wheat; proline; photosynthetic pigments
1. Introduction

The distribution of plant-life is largely dependent on water in comparison to other abiotic factors [1]. Important crops show a considerable decline in growth and productivity due to the limited availability of water, which provides a challenge to food security worldwide. In this context, plant crop plants show sensitivity to drought stress at early stages than at other developmental stages [2]. Greater intensity of drought stress severely reduced growth attributes and biomass in maize [3,4]. Drought severely affects key physiological and biochemical processes in crop plants such as photosynthetic rate, stomatal regulation, cell growth, water relations, nutrient metabolism and hormonal regulation [1,5,6]. Scarcity of water affects morphological, physiological and biochemical mechanisms, eventually reducing productivity [3,7–9]. Water stress induces overproduction of reactive oxygen species (ROS), such as singlet oxygen species (1O$_2$), hydrogen peroxide (H$_2$O$_2$), superoxide anions (O$_2^-$) and hydroxyl radical (OH-) which damage membrane lipids, cellular proteins, chlorophyll pigments and nucleic acids [10,11]. Under severe stress, this damage leads to intracellular death and eventually plant death [12]. There is considerable interest in ROS production within organelles such as chloroplasts and vacuoles, as well as in microbes and mitochondria [13]. Plants adopt protective mechanisms, which can quickly detoxify ROS produced due to drought stress [14,15]. From the defense perspective, enzymatic antioxidants neutralize or protect against oxidative damage. Furthermore, superoxide dismutase (SOD) plays a considerable role, detoxifying many cellular organelles, while peroxidase (POD) and catalase (CAT) neutralize potential oxidants, which leads to plant resistance under osmotic stress [13]. If a plant under stressful conditions experiences a decline in antioxidant levels inhibition of photosynthetic machinery and loss of cell functions may occur [16]. Among nonenzymatic antioxidants such as carotenoids, ascorbic acid (AsA) and phenolics play an important role in defensive mechanisms, as well as in the maintenance of key cellular characteristics [17,18]. However, it has been documented by several researchers that antioxidants mitigate osmotic damage and confer stress tolerance in crop species such as maize [19], radish [20] and wheat [21].

To overcome the damaging impacts of drought stress, crop plants develop defensive mechanisms such as the production of osmolyte-assisted proline (PRO), glycine betaine (GB), total soluble proteins (TSP), secondary metabolites and carbohydrates [22–24]. It was found that osmotic adjustment reduced osmotic potential and displayed beneficiaries in terms of increased growth and moderate cell turgor levels, as well as improving metabolic functions of the cell [25]. Similarly, it was observed that glycine betaine accumulated in maize under water deficit conditions, which led to enhanced growth characteristics [26]. In addition to this, proline in cotton plants under water-stressed conditions serves as an efficient osmolyte during osmoregulation [14]. However, plants show variation in response to tolerance of various abiotic stresses, and various strategies have been developed by plants to tolerate abiotic stresses for better growth and yield [6,27,28]. It was found by [29] in Brassica napus that plants under drought-stressed conditions developed stress tolerance mechanisms.

Salicylic acid (SA) is a water-soluble, phenolic compound and an endogenous growth regulator, which participates in the regulation of physiological processes in plants as a signaling molecule. Foliar-spray of SA improved growth characteristics in plants, including shoot length, biomass, chlorophyll pigments and carbohydrate content, as well as yield characteristics [30]. Similarly, SA is involved in activation of physiological processes in plants such as stomatal regulation, nutrient uptake, chlorophyll synthesis, protein synthesis, inhibition of ethylene biosynthesis, transpiration and photosynthesis [31,32]. As a growth regulator, SA is involved in mechanisms to cure diseases after pathogen attack [33]. In addition to this, salicylic acid exerts a growth stimulative impact by direct interfering with enzyme activities by activating growth-promoting cellular activities. SA plays a key role in mitigating numerous abiotic stresses, such as drought tolerance in wheat [34] and cadmium tolerance in barley [35]. Furthermore, SA induces resistance of seedlings to osmotic stress [36], chilling and high temperature by increasing the activities of glutathione
reductase, as well as increasing guaiacol peroxidase [36] activity against the toxicity of heavy metals [37].

Wheat (Triticum aestivum L.) is a major cereal crop, a major source of calories and protein, and is cultivated at a global level. It is estimated that the wheat requirement of humans will increase by up to 60% by 2050 [37]. However, its production is severely hampered by various abiotic stresses, including drought stress [38]. The current investigation indicates that salicylic acid, as an endogenous growth regulator, reduces the adverse effects of drought-induced osmotic stress by modulating antioxidant defense, and physio-biochemical mechanisms. No reports, or very few, are available about the foliar application of salicylic acid, and it being a signaling molecule in terms of activating antioxidant enzymes. This study considered whether or not foliar spray of SA could bring favorable physiological changes to enhance growth in drought-stressed wheat. Thus, the principal objective of the study was to assess whether foliar-applied salicylic acid could alter antioxidant capacity, osmolyte accumulation and photosynthetic pigments to enhance the growth of wheat seedlings exposed to drought stress.

2. Materials and Methods

The present study was conducted to investigate the effect of varying water-limited regimes on different wheat cultivars. Experiments were done in plastic pots placed in the greenhouse of the Botanical Garden of Government College University, Faisalabad, Pakistan to study the effect of foliar-application of a plant growth regulator (SA) on two drought-tolerant wheat cultivars (Fsd-2008, and S-24). A boom sprayer was used with a hollow cone nozzle capable of spraying at a pressure of 50 psi. Fsd-2008 is a strongly drought-tolerant wheat cultivar, and S-24 is a moderately drought-tolerant wheat cultivar, based on various morphological, physiological, and biochemical parameters. Seeds of wheat cultivars were obtained from Ayub Agricultural Research Institutes (AARI), Faisalabad, Pakistan. After surface sterilization of seeds with 0.1% HgCl$_2$ for 2 min seeds were washed with deionized water. Ten seeds were sown per replicate in each plastic pot, filled with 25–30 cm with 10 kg sandy clay loam soil. The design of the experiment was a completely randomized design with three replicates per treatment. Later on, after seedling emergence, five seedlings were maintained in each pot after seven days of germination. The experiment consisted of two sets of pots. One set was supplied with normal irrigation (100% field capacity) and another set was drought-stressed (60% field capacity). Soil moisture level was calculated on a soil dry-weight basis. To check evapotranspiration, pots were weighed at two day-intervals so the soil moisture level inside the pot was kept at 100% and 60% field capacity, precisely, according to treatments, which maintained withholding watering. The total available water (TAW), calculated for a rooting depth of 1.65 m was 8.45 inches, and readily available water (RAW), considering a depletion factor of 0.55, was 4.65 inches. The water contents corresponding to 100 and 60% field capacity were 5.70 and 1.66 inches, respectively. The water contents corresponding to 100 and 60% field capacity were 5.70 and 1.66 inches, respectively. Fertilizer requirements (i.e., N, P, and K) were fulfilled using urea, diammonium phosphate (DAP) and sulfate of potash at 0.03 and 0.025 g/kg of soil, respectively. Soil pH was between 6.6–7.2. Daily climatic conditions on an average basis were recorded showing a day/night length of 14/10 h, minimum and maximum day/night temperatures of 18–9 °C and 25–15 °C, respectively, and a daytime average relative humidity of 50%. Drought stress at 60% field capacity was imposed to seedlings after one week of germination, then seedlings were allowed to grow for two more weeks under varying water regimes. After two weeks of water deficit treatment, the seedlings were subjected to foliarly-applied levels of salicylic acid at 3 mM or 6 mM. The solution of (500 mL) plant growth regulator was prepared in distilled water containing 0.1% Tween-20. After one week of foliar application, data were recorded for various attributes of the seedlings in terms of shoot and root length, and fresh weights were recorded at harvest time. For measuring dry weights, shoots and roots were kept at 70 °C for three weeks. For the estimation of biochemical attributes, fresh leaves were collected and preserved at −20 °C before harvesting.
Photosynthetic attributes. The fresh leaf photosynthetic pigments chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids were estimated according to the method of [39], using acetone as an extract. Fresh leaf material for the sample extract (0.1 g) was put into 10 mL of an 80% acetone containing overnight at 4 °C. The absorbance of the extract was measured on 663 nm, 645 nm and 480 nm (Hitachi U-2001, Tokyo, Japan).

Proline estimation. Proline was estimated by applying the protocol of [40]. Samples were homogenized using sulfosalicylic acid (3% w/v), and after filtration free proline contents were estimated.

Glycine betaine estimation. The method of [41] was employed for the determination of GB content in fresh leaf samples. Leaf samples were ground in 0.5% toluene (10 mL) and the mixture was filtered.

Malondialdehyde (MDA). MDA contents were determined by applying the method of [42]. Wheat leaves (1.0 g) were extracted in 0.1% TCA solution, and the mixture centrifuged for 15 min. Afterwards, 0.5 mL of supernatant was mixed with 3 mL of 0.5% thiobarbituric acid (TBA) solution prepared in 20% TCA. After shaking the mixture, the MDA content of the supernatant was determined at 532 nm and 600 nm using a spectrophotometer.

Hydrogen peroxide (H$_2$O$_2$). Trichloroacetic acid (0.1% w/v) was used for the estimation of H$_2$O$_2$ employing the protocol of [43]. The homogenized material was centrifuged at 15,000 rpm. The supernatant was mixed with 1 mL of KI solution and absorbance measured at 390 nm.

Relative membrane permeability (RMP). The method of [44] was used to determine the membrane stability characteristics of fresh leaves.

Total phenolics. The method of [45] was used to estimate total phenolic content in fresh leaves. Fresh wheat leaves (0.5 g) were extracted in 80% acetone and the extract centrifuged at 1000 rpm for 15 min. An aliquot was mixed with a 5 mL solution of 20% Na$_2$CO$_3$, and the volume maintained at 10 mL using distilled water. After vortexing, the absorbance of the mixture solution was determined at 750 nm.

Ascorbic acid (AsA). The method of [46] was used for the estimation of ascorbic acid content. Fresh wheat leaves were homogenized in 6% trichloroacetic acid (TCA) solution and centrifuged. The supernatant was mixed with 2% dinitrophenyl hydrazine solution. After adding two drops of alcoholic thiourea the mixture was boiled then 5 mL of H$_2$SO$_4$ (80% v/v) was added and the absorbance read using a spectrophotometer at 530 nm.

Total soluble sugars (TSS). Leaf soluble sugars were measured using the method of [47]. The dried and powdered leaf samples were homogenized in a 10 mL solution of 80% ethanol. An aliquot (0.1 mL) of the extract was taken after adding distilled water and 4 mL of anthrone was added. Absorbance of the final solution was measured at 620 nm.

Enzymatic antioxidants SOD, POD, and CAT. Antioxidant activities of POD and CAT were determined by the method of [48], while the activities of SOD were determined according to [49]. Leaf samples were extracted for these measurements in potassium phosphate buffer.

Statistical analysis. The design of the experiment was completely randomized with three replicates. The data on all attributes were subjected to a three-way analysis of variance (ANOVA) and treatment means were compared using least significant design test with Statistix 8.1 (Tallahassee, Florida, USA). The differences among means were calculated with the least significance test level at a 5% probability level.

3. Results

3.1. Growth Attributes

Drought stress (60% field capacity) was found to significantly ($p \leq 0.001$) reduce the shoot length, root length and fresh and dry weights in both wheat cultivars compared with control conditions. Foliar application of both SA levels (3 and 6 mM) significantly ($p \leq 0.001$) alleviated the deleterious effects of drought stress in all growth attributes in
both wheat cultivars, as indicated in Figure 1. However, 6 mM SA foliar spray proved more effective in improving the biomass, shoot and root length in both wheat cultivars.

Figure 1. Effect of foliar-application of salicylic acid on (A) shoot length, (B) root length, (C) shoot fresh weight, (D) scheme 0., FSD-2008 (E) and SD-24 (F) Error bars above the means indicate standard errors (n = 3).

Drought stress decreased the shoot length of wheat by 9.65% and 17.14%, root length by 4.29% and 2.54%, shoot dry weight by 21% and 13%, and root dry weight by 15.87% and 33.34% in S-24 and FSD-2008, respectively. Under drought stress, treatment with 6 mM SA increased the shoot length by 9.5% and 6.62%, root length by 7.32% and 3.13%, shoot dry weight by 9.11% and 2.69% and root dry weight by 28.12% and 26.45% in FSD-2008
and S-24, respectively, as compared with non-stress conditions. As indicated in Figure 1A, the shoot length of the Fsd-2008 cultivar significantly increased with foliar spray of SA. Likewise, root length, shoot fresh weight and shoot dry weight (Figure 1B–D) showed similar linear trends of increase after SA application in both cultivars. However, the growth effects were more prominent in Fsd-2008 in comparison to S-24. The cv. Fsd-2008 cultivar had greater improvement in shoot and root length, as well as biomass, compared with cv. S-24. Therefore, Fsd-2008 was highly drought tolerant and cv. S-24 was moderately drought tolerant regarding all growth parameters (Figure 1A–F; Table 1).

3.2. Photosynthetic Pigments

Results showed that drought stress considerably decreased the Chl a, Chl b and carotenoid levels in both wheat cultivars. Furthermore, foliar spray with SA significantly ($p \leq 0.001$) increased leaf photosynthetic pigments in both cultivars under control and stress conditions. Nevertheless, cultivar difference regarding the accumulation of photosynthetic pigments was also evident in that cv. Fsd-2008 accumulated more photosynthetic pigments compared cv. S-24 under both stress and nonstress conditions. Moreover, the interaction between drought stress, cultivars and SA application was significant ($p \leq 0.5$), ($p \leq 0.01$) for Chl b and carotenoids, respectively. Of the two cultivars, salicylic acid application improved the Chl a content by 22.12% and 12.35%, Chl b content by 29% and 33.50% and carotenoids by 14% and 20.92% under control and drought stress conditions, respectively, as compared with the no SA spray treatment (Figure 2A–C; Table 1).

3.3. Osmolyte Accumulation

A considerable increase in glycine betaine (GB) accumulation was recorded under drought-stress in both wheat cultivars. The exogenous application of SA significantly ($p \leq 0.001$) increased the accumulation of GB in both cultivars under stress and control conditions. Cultivar difference was also apparent, as cv. S-24 accumulated more GB compared cv. Fsd-2008. Of the two levels, SA at 6 mM resulted in a greater increase in the accumulation of GB as compared to 3 mM in both cultivars. (Figure 2F; Table 1). Proline and total soluble sugars were significantly ($p \leq 0.001$) increased in both cultivars under drought stress conditions. However, under water-stress conditions, SA application significantly ($p \leq 0.001$) increased the accumulation of proline and total soluble sugars under both stressed and nonstressed conditions. Between the two treatments, SA 6 mM resulted in a greater increase than 3 mM (Figure 2D,E; Table 1).

3.4. Oxidative Stress Attributes

A significant ($p \leq 0.001$) increase in the activities of CAT and SOD in both the wheat cultivars was recorded when wheat plants were subjected to drought stress. Foliar-application of SA enhanced the CAT and SOD activities in both studied cultivars. The 6 mM SA level was more effective in increasing the activities of CAT and SOD in both wheat cultivars (Figure 3A,B; Table 1). Cultivar response regarding the activities of POD and SOD was also recorded. cv. Fsd-2008 had slightly higher activities of both these antioxidants, and was highly drought-tolerant compared to cv. S-24, which was moderately drought-tolerant (Figure 3B,C; Table 1). Similar to the activities of CAT and SOD, the activity of POD was also significantly ($p \leq 0.01$) increased in both wheat cultivars grown under drought stress conditions, while exogenous application of SA (3 mM and 6 mM) further increased the POD activity in both cultivars under drought stress and control conditions.
Table 1. Mean square values from analysis of variance (ANOVA) of data for foliar-applied salicylic acid with respect to growth, photosynthetic pigments and oxidative defense systems and osmolytes in wheat (*Triticum aestivum* L.) grown under water-stress conditions.

| Source of Variance | df | SL   | RL       | SFW          | SDW          | RFW          | RDW          | Chl. a       | Chl. b       | Carotenoids  | MDA           |
|-------------------|----|------|----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|---------------|
| Varieties (V)     | 1  | 87.11*** | 37.01*** | 73.67***     | 102.35***    | 9.45***      | 3.17***      | 7.40***      | 41.14***     | 0.08***      | 983.28***     |
| Drought Stress (S)| 1  | 35.60*** | 22.2***  | 44.67***     | 8.900***     | 0.51***      | 2.87***      | 13.04***     | 122.57***    | 0.04***      | 1787.99***    |
| Treatments (T)    | 2  | 138.18*** | 23.38*** | 70.86***     | 126.97***    | 2.26***      | 1.48***      | 13.49***     | 28.89***     | 0.08***      | 1392.31***    |
| V × S             | 1  | 4.27 *   | 0.47ns   | 1.17ns       | 1.03 *       | 0.07ns       | 0.27 *       | 0.38 *       | 5.26 ***     | 0.02 *       | 42.09 **      |
| V × T             | 2  | 2.19ns   | 0.4ns    | 0.39ns       | 4.52 ***     | 0.03ns       | 0.01ns       | 0.03ns       | 2.49 ***     | 7.83ns       | 28.08 **      |
| S × T             | 2  | 3.03ns   | 0.32ns   | 2.24ns       | 18.13 ***    | 0.03ns       | 0.04ns       | 0.22ns       | 0.03ns       | 0.001 **     | 347.64 ***    |
| V × S × T         | 2  | 2.29ns   | 0.07ns   | 2.35ns       | 5.64 ***     | 0.01ns       | 0.24ns       | 0.01ns       | 0.22 *       | 8.13ns       | 24.72 *       |
| Error             | 24 | 1.00    | 0.32     | 0.97         | 0.20         | 0.03         | 0.04         | 0.07         | 0.04         | 2.97         | 4.45          |

| Source of Variance | df | H₂O₂   | RMP       | Phenolics    | Ascorbic Acid | Glycine betaine | TSS         | Proline      | CAT          | SOD          | POD          |
|-------------------|----|--------|-----------|--------------|---------------|-----------------|--------------|--------------|--------------|--------------|--------------|
| Varieties (V)     | 1  | 0.36 ** | 157.85ns  | 274.09ns     | 6.65 **       | 82.75 ***       | 86.89 ***    | 4.59 ***     | 3374.62 ***  | 376.62 ***   | 160.94 ***   |
| Drought Stress (S)| 1  | 1.33 ***| 157.85ns  | 4042.84 **   | 75.23 ***     | 182.61 ***      | 393.56 ***   | 10.88 ***    | 2966.28 ***  | 47.60 ***    | 5.84 **      |
| Treatments (T)    | 2  | 0.77 ***| 290.40 ** | 7836.06 **   | 2.59 **       | 86.32 ***       | 122.65 ***   | 38.28 ***    | 2180.47 ***  | 565.74 ***   | 155.29 ***   |
| V × S             | 1  | 0.02ns | 1.17ns    | 25.56ns      | 0.93ns        | 2.65ns          | 10.79 *      | 0.002ns      | 547.68 ***   | 0.01ns       | 19.70 ***    |
| V × T             | 2  | 0.26 ** | 0.94ns    | 4293.44 ***  | 0.24ns        | 1.11ns          | 3.19ns       | 1.56 **      | 11.12ns      | 2.41ns       | 1.52ns       |
| S × T             | 2  | 1.26 ***| 0.94ns    | 613.61ns     | 0.01ns        | 3.25ns          | 3.50ns       | 0.21 **      | 29.92 *      | 8.12ns       | 10.33 ***    |
| V × S × T         | 2  | 0.35 ***| 0.49ns    | 57.12ns      | 0.80ns        | 0.66ns          | 1.31ns       | 2.87 ***     | 10.07ns      | 24.37 **     | 18.51 **     |
| Error             | 24 | 0.03   | 588.81    | 434.98ns     | 0.83          | 2.76            | 1.48         | 0.04         | 8.57         | 4.24         | 3.04         |

*, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant. Abbreviations: SL = shoot length; RL = root length; SFW = shoot fresh weight; RFW = root fresh weight; SDW = shoot dry weight; RDW = root dry weight; Chla = chlorophyll a; Chlb = chlorophyll b; Car = carotenoids; MDA = malondialdehyde; RMP = relative membrane permeability; H₂O₂ = hydrogen peroxide; total phenolics; ASC = ascorbic acid; GB = glycine betaine; TSS = total soluble sugars; Pro = proline; SOD = superoxide dismutase; POD = peroxidase; CAT = catalase.
Figure 2. Effect of foliar-application of salicylic acid on (A) chlorophyll a, (B) chlorophyll b, (C) carotenoids, (D) proline, (E) total soluble sugar contents, (F) glycine betaine contents in two wheat cultivars under water-stress conditions. Means with the same letter (S) do not differ significantly at $p \leq 0.05$. Error bars above the means indicate standard error ($n = 3$).
Figure 3. Effect of foliar application of salicylic acid on (A) catalase, (B) superoxide dismutase, (C) peroxidase, (D) malondialdehyde, (E) hydrogen peroxide, (F) relative membrane permeability, (G) total phenolics, (H) ascorbic acid contents, in two wheat cultivars under water-stress conditions. Means with the same letter (S) do not differ significantly at \( p \leq 0.05 \). Error bars above the means indicate standard error \((n = 3)\).

Compared to control plants, SA application increased the POD activity of S-24 and FSD-2008 by 60.07% and 47.12% under drought stress conditions, respectively. Cultivar differences were also evident, cv. S-24 having less POD activity than cv. Fsd-2008, which was observed as drought tolerant (Figure 3; Table 1).

Leaf MDA and \( \text{H}_2\text{O}_2 \) levels increased considerably in plants of both cultivars under drought stress conditions. Exogenous application of SA (3 and 6 mM) significantly
(p ≤ 0.001) decreased the MDA levels in both cultivars in stressed and control plants. SA 6 mM was more effective in reducing the levels of both MDA and H$_2$O$_2$ in both cultivars. However, a nonsignificant increase was observed in relative membrane permeability (RMP) when plants were grown under drought stress. Exogenous application of SA significantly (p ≤ 0.01) inhibited the RMP level in stressed and nonstressed conditions (Figure 3D–F; Table 1).

Results showed that drought stress significantly (p ≤ 0.01) increased leaf phenolic contents in both wheat cultivars under control and drought stress conditions. However, SA foliar-spray significantly (p ≤ 0.01) increased the phenolic content in both cultivars under drought stress and control conditions. Of the two treatments of SA, 6 mM was more effective in increasing phenolic contents in cv. S-24 (4.88%) and cv. Fsd-2008 (10.42%) (Figure 3G; Table 1). Under drought stress a considerable increase in leaf AsA content was observed in both cultivars. Exogenous application of salicylic acid at 6 mM significantly (p ≤ 0.01) further increased the accumulation of ascorbic acid in stressed and nonstressed plants. Cultivars difference was also apparent in that cv. Fsd-2008 accumulated higher contents of AsA compared to cv. S-24. Interaction between drought stress and salicylic acid spray was also significant (p ≤ 0.01) (Figure 3H; Table 1).

4. Discussion

The exposure of drought stress at 60% field capacity considerably reduced the shoot and root attributes in the range of 9–17% for both wheat cultivars (Figure 1). Drought-induced reduction in growth attributes might be due to alteration in plant water relations, disturbance in photosynthetic pigments, photosynthetic activity and major damage at an oxidative level to macromolecules, as well as membrane deterioration and inhibition of the activity of photosynthetic enzymes [50]. In addition to this, drought stress reduces cell division, cell expansion, the rate of photosynthesis, protein synthesis and nucleic acid metabolism [51]. Previously, it was documented that crop plants use a well-developed root system to avoid desiccation periods. In addition to this, root hydraulic properties and root morphology play vital roles in various responses to drought stress [52]. A healthy shoot requires an extensive root system, which may not available under drought conditions. Eventually shoot and root growth will be affected during periods of reduced water supply because the root system uses many photosynthetic end products for its growth [53].

Foliar-spray with SA improved all growth attributes, such as shoot and root length, as well as fresh and dry weights in both the wheat cultivars grown under drought stress and control conditions (Figure 1). In the two wheat cultivars, 6 mM SA-spray enhanced the shoot length by 11.35% and 20.35% and root length by 7.32% and 3.13% under control and drought stress conditions, respectively. Foliar spray of salicylic acid is potentially effective to exert a suppressive or simulative influence on growth aspects through its direct interference with key enzymatic activities responsible for biosynthesis and metabolism of growth-promoting substances [30,54]. In this context, many researchers found that salicylic acid improved growth in maize [55,56] and wheat [57]. Similarly, the growth-promoting response of salicylic acid in onion was reported earlier by [30,56]. Our results relate to the study of [58] in lemongrass in which the dry weights of shoots and roots were increased by foliar spray of salicylic acid under drought stress.

The present investigation revealed that Chl a Chl b, as well as carotenoid contents, decreased in both wheat cultivars when subjected to drought stress (Figure 2). The drought-induced decrease in chlorophyll pigments might have been due to inhibition in vital processes of photosynthesis, such as chlorophyll biosynthesis, which ultimately decreased chlorophyll contents [59]. Reduction in chlorophyll content could, ultimately, decrease photosynthetic rate [60]. These results relate to the finding of [61] in wheat and [62] in cucumber in which chlorophyll contents decreased under drought stress conditions. Nevertheless, several researchers found that decrease in chlorophyll content was due to over-production of reactive oxygen species (ROS), degradation in nutritional balance and inactivation enzyme activities, as well as a decline in cellular water contents [63–65].
Our results indicate that foliar spray of SA increased chlorophyll pigments in both wheat cultivars under stress and control conditions (Figure 2). The beneficial effects of SA with respect to the deleterious effects of drought on chlorophyll pigments could be attributed to its stimulatory effects on Rubisco activity and, ultimately, to increased photosynthetic rate. Other positive effects of SA could be linked to induction of the synthesis of protein kinases, which have key roles such as in the regulation of cell division and at various cell developmental stages [27,66]. Other reports showed that SA increased photosynthetic rate and stomatal conductance under stressful environmental conditions [67]. Moreover, [31] found that SA improved biosynthesis and enhanced photosynthetic rate in corn and soybean. Compared to controls, an SA application of 6 mM SA increased the Chl a content by 22.12% and 12.35% and Chl. b content by 29.53% and 35.5% in S-24 and FSD-2008, respectively.

In the present investigation, the imposition of drought stress in wheat plants increased osmolytes such as proline, glycine betaine and total soluble sugars in both cultivars (Figure 2). In this respect, to deal with adverse effects of drought stress, plants carried out the process of osmotic adjustment by the synthesis of osmolytes such as GB, proline, and total soluble sugars. Proline and GB are involved in cellular osmotic regulation. Proline, being a secondary metabolite, is an antioxidant as well as an osmoprotectant [68]. As an antioxidant it is has a protective role in the membranes of organelles, and as an osmolyte it plays a vital role in regulating cellular water relations under drought stress conditions [69]. It was documented by Habib et al., [68] that during osmotic stress conditions proline acts as water substituent that stabilizes intercellular structures via hydrophobic interactions and hydrogen bonding, eventually protecting the membrane from dehydration. It was also documented that increased concentration of glycine betaine also plays a role by stimulating novel stress-related genes, detoxifying ROS, as well as a protective role in the photosynthetic machinery and maintenance of protein structures, by acting as a molecular chaperone under stressful conditions [69]. Our results are inconsistent with the investigation of Damghan et al. [70] and Zhang et al. [71] who found that proline acts as an osmolyte rather than in protecting enzymes and other macromolecules, providing a protective mechanism against low water potential conditions via osmotic regulation and acting as an electron acceptor, preventing photosystem injuries by scavenging ROS.

In the present study, foliar-application of SA was found to increase the accumulation of osmolytes under drought stress and control conditions. Between the two levels of SA, 4 mM and 6 mM, 6 mM further increased the osmolyte proline in both wheat cultivars compared to controls (Figure 2). The exogenous application of SA further enhanced proline accumulation in water-stressed plants. A similar study by Umbeese et al. [72] reported that SA-application protects amaranthus plants by enhancing proline accumulation. Foliar application of salicylic acid increased the accumulation of total soluble sugars in onion [73]. Similarly, it was reported that SA provided a protective role against drought stress in tomato and amaranthus [72]. Our results are consistent with the work of Idrees et al. [66] with lemongrass, of Bakry et al., [74] with linseed, and of [75] in with wheat, who reported that SA application increased the proline accumulation under drought stress, and was involved in protective mechanisms mitigating against stress.

The present investigation revealed that the imposition of drought stress increased the contents of MDA and H$_2$O$_2$ in both the wheat cultivars (Figure 3). MDA accumulation was measured in the form of lipid peroxidation. Over-production of MDA is an indicator of oxidative damage [76]. The oxidation of membrane lipids is an indication of the formation of free-radicals producing oxidative stress [77]. Similarly, several researchers showed that increased level of MDA and H$_2$O$_2$ triggered ROS-induced oxidative damage under drought stress [9,59,76]. Similarly, drought-induced oxidative stress enhanced the accumulation of MDA, and H$_2$O$_2$ has been found in wheat [78], cucumber [79] and canola [61]. A nonsignificant increase in the level of relative membrane permeability was observed under wheat cultivars under drought stress conditions (Figure 3). Drought causes deleterious effects such as inactivation of enzyme activities, damage to cell membranes,
ion-leakage and reduced osmotic regulation due to the higher concentration of ROS inside cells [80–82]. Our results indicated that activities of the antioxidant enzymes, SOD, POD and CAT increased when wheat plants were subjected to drought stress conditions. (Figure 3). The formation of the O2-radical is a basic reason for oxidative injury, and many systems are used by plants to mitigate its effects in drought tolerance [83]. Enhanced activity of SOD related to dismutation/modulation of O2 into H2O2 maintains the cellular oxidation state at a normal level and is considered as the protective role of SOD against oxidative stress [84]. In this regard, O2 generation inactivates SOD activity [85]. Mani- vannan et al. [86] found that enhanced SOD activity is a trend towards drought tolerance. Noctor et al., [87] found that during drought stress conditions increased SOD activity is an indicator of drought tolerance in tomato. CAT also has the potential to mitigate oxidative stress [88]. The increased concentration of CAT and SOD converts toxic O2 to H2O2 and ultimately into water and molecular oxygen, reversing damage under adverse conditions, so both these play a role in scavenging ROS at the cellular level [89]. Similar results have been reported by several researchers who found that enhanced CAT and SOD become oxidative scavengers to detoxify radicals under stressful environments [90,91]. Oxidative defense systems comprised of the enzymes CAT, POD, and SOD, and the nonenzymatic ascorbic acid, phenolics, flavonoids, and proline, detoxify stress-induced ROS accumulation, [76,92,93]. Drought-induced increases in the activities of enzymatic antioxidants such as CAT, POD and SOD were reported earlier by Wang et al., [94] in grapes under stress conditions. Ashraf et al., [12] and Akram et al., [61] reported that SOD plays an essential role in detoxification, while POD and CAT are involved in countering several latent oxidants to recover stress tolerance. The upregulation of antioxidants in stressed conditions is an indicator of stress tolerance [95,96]. Canola and radish cultivars were studied under stress conditions and found to be relatively tolerant based on enhanced activities of these antioxidants [61,97].

Foliar application of SA significantly reduced H2O2 and MDA levels, and stabilized membrane stability in wheat seedling compared to control plants, which indicated that ROS production during water-stress conditions was effectively overcome by the SA-foliation spray reducing oxidative damage (Figure 3). Between the two levels (3 mM and 6 mM), 6 mM was more effective in reducing water-stress damage in both cultivars. Foliar-application of SA further enhanced the activities of antioxidant enzymes such as SOD, POD, and CAT under stressed and nonstressed conditions, which indicated lower accumulation of MDA and H2O2 and lesser electrolyte leakage, thereby providing membrane stability (Figure 3). It was reported by Fobert and Despres [98] that SA application plays a key role in the activation of antioxidants such as SOD, POD and CAT to scavenge ROS via activation of a defensive system triggering redox changes in signal transduction pathways. Previously, it was documented that foliar spray of SA upregulated antioxidant activities under stress conditions [99]. It has been shown that SA-application causes an increase in the activity of antioxidants in various stress situations to remove ROS [91].

In the present study, nonenzymatic antioxidants, such as total phenolics and ascorbic acid, were increased under drought stress (Figure 3). These nonenzymatic antioxidants detoxify ROS during oxidative stress [22]. Similarly, it was found that antioxidants increased under drought stress in the work of Ahmad et al., [28] in canola and Shafiq et al. [22] in corn. However, in contrast, a high level was found in carrot [62], while AsC level increased in canola [22]. Furthermore, phenolics and ascorbic acids help in the regulation of metabolism and in reducing oxidative-damage [13]. SA-application increased phenolic and ascorbic acid contents [53,73,74] and enhanced the level of all antioxidant enzymes (enzymatic and nonenzymatic) resulting in detoxification of oxidative stress in wheat seedlings (Figure 3). In conclusion, foliar-spray improved the resistance of plants against drought stress by upregulating the antioxidant defense system of all the antioxidant enzymes and reducing the oxidative damage caused by reactive oxygen species (ROS) [5,66,100]. Further research will be focused on the influence of a synthetic form of salicylic acid on Triticum aestivum under field conditions [101].
5. Conclusions

It can be concluded that foliar spray with salicylic acid was very effective in mitigating the adverse effects of water-stress in wheat. Foliar-application, particularly at 6 mM, significantly enhanced growth attributes which were associated with increased biosynthesis of photosynthetic pigments, as well as cellular osmotic adjustment by the accumulation of osmolytes. Induction of water stress tolerance in wheat plants after foliar spray with SA was also associated with the antioxidative defense mechanism through increases in the activities of antioxidant enzymes (SOD, POD, and CAT) and accumulation of nonenzymatic antioxidants, which actively reduced membrane lipid peroxidation by lowering the level of MDA and H$_2$O$_2$ in both cultivars. Future research may be focused on confirming the obtained results under uncontrolled (field) conditions.

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