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Exogenous Application of Salicylic Acid Improves Physiological and Biochemical Attributes of *Morus alba* Saplings under Soil Water Deficit

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Abstract: *Morus alba* L. is a multipurpose and fast-growing tree species. However, its growth and productivity are susceptible to water stress. Therefore, a study was conducted to check the effectiveness of foliar application of salicylic acid (SA) in improving the water stress tolerance of *M. alba*. A pot experiment was conducted and the morphological, physiological and biochemical attributes of young *M. alba* saplings were assessed under control (CK, 90% of field capacity (FC)), moderate (MS, 60% of FC) and high soil water deficits (HS, 30% of FC), along with MS and HS + foliar application of SA 0.5 and 1.0 mM (MS + 0.5; HS + 0.5; MS + 1.0, and HS+1.0, respectively). Results demonstrated that the highest decrease in plant growth, leaf, stems and roots' dry biomass, chlorophyll *a*, *b*, carotenoid contents and leaf gas exchange parameters was observed under HS, whereas the lowest decrease was evidenced for HS + 1.0 mM SA. Electrolyte leakage, malondialdehyde contents, hydrogen peroxide and superoxide radicals significantly increased under HS, while the lowest increase was evidenced for HS + 1.0 mM SA. The highest increase in proline content, total soluble sugar, total phenolic content, soluble protein and superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase was also found under HS + 1.0 mM SA. Based on the results, it can be concluded that foliar application of SA can help improve the water deficit tolerance of *Morus alba* saplings, especially under high soil water deficit.

Keywords: dry biomass production; CO_2 assimilation rate; water use efficiency; osmolytes; antioxidants

Citation: Zafar, Z.; Rasheed, F.; Mushtaq, N.; Khan, M.U.; Mohsin, M.; Irshad, M.A.; Summer, M.; Raza, Z.; Gailing, O. Exogenous Application of Salicylic Acid Improves Physiological and Biochemical Attributes of *Morus alba* Saplings under Soil Water Deficit. *Forests* **2023**, *14*, 236. https://doi.org/10.3390/f14020236

Academic Editor: Rosana López Rodríguez

Received: 4 January 2023
Revised: 13 January 2023
Accepted: 16 January 2023
Published: 27 January 2023

1. Introduction

Water deficit is among the important environmental challenges across the world, especially for species thriving in arid to semi-arid areas [1]. In such regions, climatic changes have resulted in reduced water availability and enhanced evaporative demands. Furthermore, warm climate and changing summer rainfall patterns have resulted in water shortages [2]. Resultantly, by the end of the 21st century, an acute shortage of water is inevitable due to rises in temperature [3,4]. Climate modeling experts have predicted that the temperature could increase by 3–9 °C by the end of the century [IPCC 2014]. Globally, 36% of global area is classified as having an arid to semi-arid climate, with yearly rainfall of about 55–150 mm [5]. South Asia is ranked 5th amongst the most-affected areas around the

world due to climate change [6]. Mostly, the area of Pakistan is categorized as an arid to a semi-arid climate, where mean annual rainfall is between 200–320 mm, thus, water shortage complications must be confronted [7]. Drought stress in forest ecosystems has gained significant attention in the scientific community during the past decade, particularly due to an unprecedented shift in climate [8]. Drought stress has stimulated concern regarding the reduction of forest productivity and the enhancement of tree mortality [9,10]. During drought, tree mortality is normally caused by hydraulic failure [11,12], which is triggered by a large difference in water uptake and loss though stomata; for example, water uptake by roots is less than water transpired from leaves, which leads to cavitation in the xylem vessels [13,14]. To halt such hydraulic failure, plants can regulate transpiration by opening and closing the stomata [14]. In addition to this, trees often adjust their morphological, physiological, and biochemical properties in response to numerous abiotic stresses. [15]. In this context, a decrease in stomatal conductance permits plants to adjust water loss; however, under extreme conditions, such alterations can result in CO_2 starvation, reduction in leaf area, enhancement in root: shoot ratio, and a reduction in plant productivity under stressful environments [16,17]. Studies have shown that drought stress also instigates production of ROS such as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen radicals (OH^-), hydrogen peroxide (H_2O_2), and alkoxy radicals (RO) in chloroplasts and mitochondria [17,18] that are destructive to the cell membranes, cell proteins and lipids, chlorophyll, and nucleic acids [19]. Increased production of ROS enhances lipid peroxidation, which can be determined by measuring malondialdehyde [20]. Generally, osmolytes such as proline, soluble sugar and carbohydrate increase under drought stress, and have been related to the withholding of water in the cytoplasm, thus protecting protein from denaturation and cell membranes from injury. These osmolytes are also involved in quenching free radicals and maintaining subcellular structures [21,22]. In previous studies, increased protein contents and soluble sugar, total carbohydrate, and phenolic contents were demonstrated in different tree species under a water-deficit environment [23–25]. Plants also produce various antioxidants to cope with the overproduction of ROS. The most significant antioxidant enzymes include peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) [26,27]. Many previous studies have demonstrated that the over production of ROS can be controlled via maintaining a balance between the synthesis of ROS and antioxidant enzymes in response to different abiotic stresses [28]. In this regard, different methods such as the exogenous application of growth regulators have been instrumental in alleviating the damaging effects of drought stress.

Salicylic acid (SA) is an important hormone that is naturally produced in plants and is classified as a phenolic growth regulator that regulates plant growth under various types of abiotic stress (salinity and drought) [29]. Studies have demonstrated the function of SA in plant physiology, for example, in photosynthetic activity, osmolyte accumulation and antioxidant enzyme activities which thus increase stress tolerance in plants [24,30]. The effective role of SA is closely related to various aspects, such as the plant's developmental stage, the concentration of SA and the mode of its application [31]. Different studies have demonstrated that the application of SA helps in maintaining cell membrane steadiness by enhancing the concentration of different antioxidants such as SOD, POD CAT and APX, increasing the leaf's photosynthesis capability under various stresses [32,33]. Reductions in electrolyte leakage and lipid peroxidation have been reported after the application of SA [30].

Morus alba L. (common name: mulberry), belonging to *Moraceae* family, is a deciduous, fast-growing multipurpose species with high economic and environmental importance worldwide [34]. *M. alba* is commonly found and planted in Asia, Europe, Africa and North and South America [35]. Various prominent characteristics such as high biomass and fruit production make *M. alba* a desirable species for growing in arid and semi-arid areas [35]. In addition, *M. alba* leaves contain a considerable number of antioxidants, carbohydrates, fats, fibers, minerals, proteins, and vitamins [36]; it is widely used for rearing silkworms, cattle, goat and other animals, but also for making tea and for consumption as a vegetable [37].

However, this species is vulnerable to water stress especially at sapling stage [16]. Therefore, enhancing the water stress tolerance of *M. alba* saplings can be crucial before considering this species in revegetation initiatives in arid and semi-arid areas of the country. Consequently, the goal of the current study is to assess the effectiveness of salicylic acid in increasing the water stress tolerance of *M. alba* saplings. Various morphological, physiological, and biochemical attributes were evaluated under controlled experimental conditions.

2. Materials and Methods

2.1. Planting Material and Treatment

The present study was conducted in a greenhouse at the Department of Forestry, University of Agriculture, Faisalabad 38040, Pakistan. A maximum and minimum temperature of 25 ± 5 °C and relative humidity 55 ± 5% were maintained throughout the experiment. At 90 to 120 days old, healthy and vigorous saplings of *M. alba* were collected from a single tree progeny at Punjab Forest Research Institute Gatwala Faisalabad, Pakistan and grown under natural environmental conditions. Plastic pots (34 cm × 26 cm) were filled with 10 kg of peat-sand mixture (1:1 ratio). The experimental soil was analyzed for nitrogen (0.78%), phosphorus (12 ppm), organic matter (8%), electric conductivity (2 dS m^{-1}) and pH (6.6). To optimize the nutrient balance NPK fertilizer (15% N, 5% P_2O_5, 5% K_2O) was added at the rate of 5g/kg of soil. A total of 70 saplings (10 saplings per treatment) were assigned to the following combination of water deficit and salicylic acid: control (CK at 90% of field capacity, FC); moderate stress (MS, 60% of FC); high stress (HS, 30% of FC); moderate stress + 0.5mM of salicylic acid (MS + 0.5); high stress + 0.5 mM of salicylic acid (HS + 0.5); moderate stress + 1.0 mM of salicylic acid (MS + 1.0) and high stress + 1.0 mM of salicylic acid (HS + 1.0). Three to five soil cores of 100g samples were produced and dried in an oven to determine that the soil moisture content at field capacity was 25.5g. Using this data, a pot weight of 30%, 60% and 90% of FC was determined, and each pot was watered back to 8215g under CK, 8980g under MS and 9745g under HS, respectively. Each pot was watered back daily to the reference weight by weighing the pots and adding the amount of water lost during evapotranspiration. The water deficit treatments were sustained throughout the experiment, as confirmed by Rasheed et al. [38]. Sodium salicylate was used for salicylic acid solution by dissolving 0.069g for 0.5 mM and 0.138 g for 1.0 mM in 1000 mL of distilled water. The saplings under water deficit were sprayed twice (on the 7th and 45th day) during the experiment. The duration of the whole experiment was about 90 days.

2.2. Growth and Dry Biomass Production

Different growth attributes, for example, plant height (cm), stem diameter (mm) and number of leaves, were measured. At the end of experiment, all saplings were removed from their pots, the soil around the roots was washed away and each sapling was separated into leaf, stem and roots; then, fresh weight was calculated immediately. To measure the dry weight of leaves, the stems and roots were packed into paper bags, dried at 80 °C for 70 h in a heat oven (DGH-9202 Series Thermal Electric Thermostat drying oven) and weighed; the root:shoot ratio (R:S) was also calculated.

2.3. The Physiological Parameters, Chlorophyll a, b and Carotenoid Contents

The chl *a*, *b* and carotenoid contents were determined from healthy and undamaged leaves. Leaf samples weighing 0.5 g were mixed with 4.5 mL of acetone, and the mixture was centrifuged at 13,000 rpm for 11 min. The supernatant was collected and transferred into test tubes to measure the absorbance at 460, 645 and 663 nm with a spectrophotometer (Perkin Elmer, 40 Winter Street Waltham, MA 02451, USA). The chl *a*, *b* and carotenoid contents were measured using the method demonstrated by Arnon [39].

2.4. The Gas Exchange Measurements

The gas exchange measurements such as CO_2 assimilation rate (*A*, μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (*gs*, μmol m^{-2} s^{-1}) were measured on preselected, healthy and

mature leaves of plants, before the end of the experiment, using an infrared Gas Exchange Analyzer (CIRS-3 Amesbury, USA). The temperature of the leaf chamber was set at 27 °C, and relative humidity was kept at 65%; the reference CO_2 was adjusted to 400 µmoL moL^{-1}. The measurements were taken between = 12:00 pm and 1:00 pm. Intrinsic water use efficiency (WUE_i) was calculated as the net ratio of CO_2 assimilation rate and stomatal conductance according to Rasheed et al. [38].

2.5. Proline, Soluble Sugar, Soluble Protein and Total Phenolic Contents

The proline content was measured using the method demonstrated by Bates et al. [40] using ninhydrin. The reaction mixture was extracted with 5 mL toluene and cooled to room temperature, and absorbance was read at 520 nm. Soluble sugar was determined using the anthrone methods described by Yemm and Wills [41]. An 0.5 g leaf sample was added to 5 mL of distilled water; the water was then boiled for 30 min, and the constant volume of the reaction was 25 mL. A total of 0.5 mL of sample reaction was added to 0.5 mL anthrone-ethyl acetate and 5 mL of 98% sulfuric acid, with heat preservation in boiling water performed for 1 min, immediately. The mixture then cooled at room temperature, 26 °C. The extract solution was replaced with distilled water in blank. The absorbance was measured at 630 nm. Soluble protein was calculated using the method followed by Bradford [42]. The total phenolic content was measured following the procedure of the Folin–Ciocalteu (FC) reagent demonstrated by Ainsworth and Gillespie [43]. The 0.5 g leaf sample was homogenized with 5 mL acetone (80%). The reaction mixture was composed of 20 µL extract, 7.9 mL distilled water and 100 µL of FC reagents. The mixture was left at 40 °C for 30 min or at 25 °C for 2 h after adding and mixing the 300 µL sodium carbonate. The absorbance was measured at 750 nm against the blank (80%).

2.6. Lipid Peroxidation, MDA Contents and Electrolyte Leakage (EL %)

Lipid peroxidation was estimated by the concentration of MDA according to the methods of Valentovic [44]. The 0.5 g of leaf sample was mixed with 0.5% thiobarbituric acid solution containing 20% Trichloroacetic acid. The mixture was heated at 95 °C for 25 min, and the reaction was stopped by quickly placing it in an ice bath. The absorbance of the supernatant was read at 532 nm. Electrolyte leakage (EL%) was measured by taking 200 mg of a fresh weight leaf sample that was placed in 20 mL of deionized water; the tube was then put in a water bath at 32 °C. After 120 min, the initial electrical conductivity (EC_1) was determined. All samples were placed in a heated oven at 100 °C for 20 min, and the final electrical conductivity (EC_2) was measured. EL% was measured according to the following equation, as demonstrated by Nayyar [45].

$$EL\% = (EC_1/EC_2) \times 100 \quad (1)$$

2.7. Oxidants, Hydrogen Peroxide (H_2O_2) and Superoxide Radical (O_2^-) Measurements

The hydrogen peroxide H_2O_2 content was estimated according to the method of Velikova [46]. A total of 5 mL of 0.1% TCA (Trichloroacetic acid) was added to 200 mg of leaf samples and placed in an ice bath. After that, all the samples were centrifuged at 12,000× g for 15 min, and 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M potassium iodide were added to 1 mL of the supernatant. All the samples were placed in a dark room for 60 min, and the absorbance of the supernatants was measured at 390 nm with the help of a spectrophotometer (Perkin Elmer, 40 Winter Street Waltham, MA 02451, USA). The superoxide radical O_2^- was determined following the method described by Bai [47]. 1.0 g of leaf samples were homogenized in 4 mL of 65 mM phosphate buffer and centrifuged at 5000 × g for 10 min; 1 mL of supernatant, 0.1 mL of 10 mM hydroxylamine chlorine and 0.9 mL of 65 mM phosphate buffer (pH 7.8) were added, and the mixture kept for 20 min at 25 °C in the water bath. A volume of 0.1 mL of 17 mM sulfanilic acid and 1.0 mL of 7 mM α-naphthylamine were added to 1.0 mL of the solution and held for 20 min at 25 °C. The absorbance was measured at 530 nm.

2.8. Antioxidants' Enzyme Activities

The activity of superoxide dismutase (SOD) was measured by photochemical reduction of NBT (nitroblue tetrazolium) using the method demonstrated by Bayer [48]. One unit of SOD was determined via the 50% photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Perkin Elmer, 40 Winter Street Waltham, MA 02451, USA). The peroxidase (POD) activity was measured following the protocol of Maehly [49]. The enzyme activity was estimated by absorption at 470 nm for 1 minute using a spectrophotometer. The activity of catalase (CAT) was determined as reported by Knörzer [50]. The 0.5 g leaf samples were mixed with phosphate buffer (2.85 mL of 100 mM), the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was obtained. The mixture was prepared with 1.5 mL 1000 mM of phosphate buffer (pH 7.8) and 200 µL of H_2O_2 µL and 200 µL enzyme extract. The CAT activity was measured through the quality of H_2O_2 consumed at 240 nm/minute. The activity of ascorbate peroxidase (APX) was measured following the protocol described by Nakano & Asada [51].

2.9. Statistical Analysis

The analysis was conducted in STATISTICA (Version 12.5, Maison Alford, Paris, France). A one-way analysis of variance (ANOVA) was used to analyze the data relating to the growth parameters, dry biomass, physiological attributes, and biochemical parameters, with the treatment effect chosen as a fixed effect (T-effect). To detect pairwise differences between treatments, Tukey's HSD test was utilized. All tests were deemed significant at $p < 0.05$, and all means along with standard errors are shown (±SE)

3. Results

3.1. Water Deficit Treatments and Application of SA on Morphological Attributes

Plant growth and dry biomass production in *Morus alba* decreased significantly under both water deficit and salicylic acid + water deficit treatments. The greatest decrease in plant height, stem diameter and number of leaves was observed under HS (40.1%, 32.7%, and 50.4%, respectively) as compared to CK (Table 1). Whereas, for MS + 1.0, plant height increased by 10.5% and stem diameter decreased only by 12.9% compared to CK (Table 1). Similarly, leaf, stem, root, and total biomass systematically decreased under both water deficit and salicylic acid + water deficit treatments. However, the greatest decrease was observed under HS (48.4%, 69.1%, 49.8%, and 55.8%, respectively), and the lowest decrease was observed under MS + 1.0 (4.86%, 34.4%, 6.50% and 15.5%, respectively) as compared to CK (Figure 1). The R:S ratio of plants increased across all the treatments, with the highest increase observed under MS (17.1%), followed by MS + 1.0, HS + 1.0, MS + 0.5, HS and HS + 0.5, respectively, as compared to CK (Table 1).

Table 1. Effect of soil water deficit and soil water deficit + foliar application of SA on plant height, stem diameter, number of leaves, root:shoot ratio, chl a, b and carotenoid contents in *M. alba* saplings. The small letters represent significant differences between the treatment means (T-effect) at $p < 0.05$.

	Plant Height (cm)	Stem Diameter (mm)	No. of Leaves	Root/Shoot Ratio	Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
CK	53.0 ± 1.60^{b}	4.49 ± 0.24^{a}	40.4 ± 0.50^{a}	$0.64 \pm 0.04^{b,c}$	1.43 ± 0.06^{a}	1.57 ± 0.10^{a}	0.97 ± 0.07^{a}
MS	40.3 ± 1.34^{d}	3.67 ± 0.15^{c}	20.0 ± 0.70^{f}	0.75 ± 0.04^{a}	1.16 ± 0.02^{e}	1.07 ± 0.02^{f}	0.72 ± 0.03^{f}
HS	31.7 ± 1.37^{e}	3.02 ± 0.06^{f}	26.0 ± 0.94^{e}	0.66 ± 0.05^{c}	0.92 ± 0.04^{g}	0.96 ± 0.01^{g}	0.66 ± 0.03^{g}
MS + 0.5	51.0 ± 1.29^{b}	$3.84 \pm 0.30^{b,c}$	33.3 ± 0.86^{d}	0.69 ± 0.05^{b}	1.23 ± 0.00^{d}	1.17 ± 0.01^{d}	0.75 ± 0.03^{e}
HS + 0.5	47.2 ± 0.76^{c}	3.29 ± 0.10^{e}	$34.2 \pm 1.78^{c,d}$	0.63 ± 0.09^{c}	1.13 ± 0.02^{f}	1.13 ± 0.01^{e}	0.78 ± 0.01^{d}
MS + 1.0	58.6 ± 1.47^{a}	3.91 ± 0.08^{b}	35.6 ± 0.87^{c}	$0.74 \pm 0.06^{a,b}$	1.34 ± 0.01^{b}	1.26 ± 0.01^{b}	0.80 ± 0.01^{c}
HS + 1.0	56.1 ± 1.65^{ab}	3.43 ± 0.12^{d}	37.0 ± 0.70^{b}	0.70 ± 0.04^{b}	1.31 ± 0.02^{c}	1.23 ± 0.03^{c}	0.84 ± 0.02^{b}
T-effect	$p < 0.001$	$p = 0.066$	$p = 0.689$	$p = 0.109$	$p < 0.001$	$p < 0.001$	$p < 0.001$

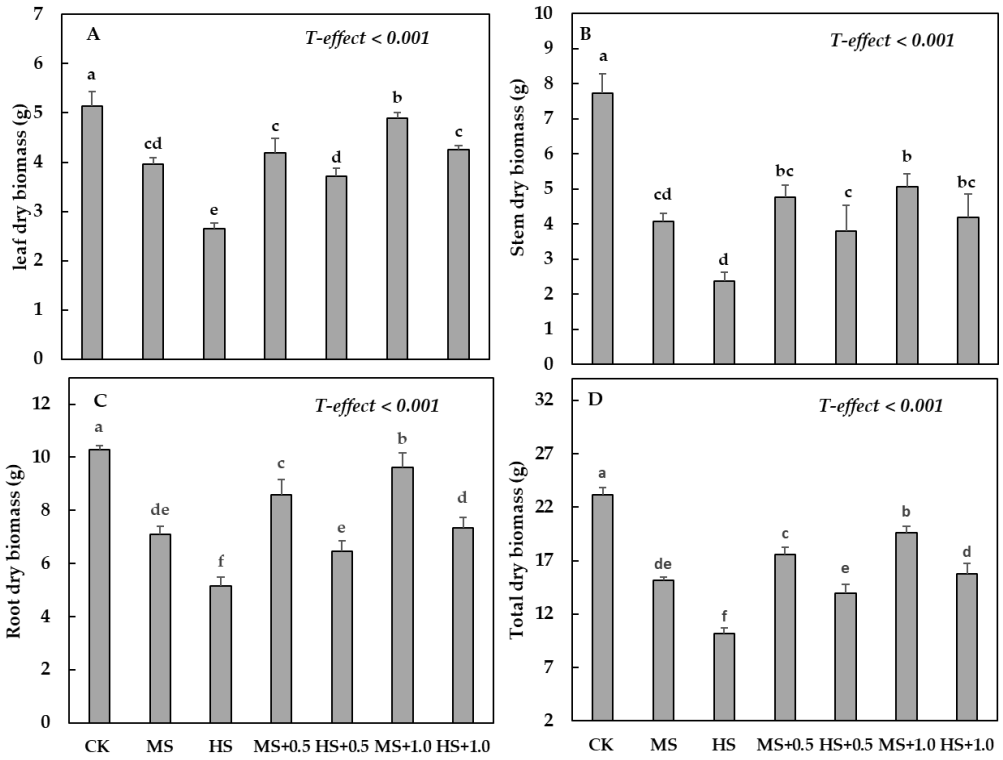

Figure 1. Mean leaf dry biomass (**A**), stem dry biomass (**B**), root dry biomass (**C**) and total dry biomass (**D**) under MS, HS, MS + 0.5, HS + 0.5, MS + 1.0 and HS + 1.0. in *M. alba* saplings. Small letters represent the significant differences between treatment means (T-effect) at $p < 0.05$.

3.2. Water Deficit Treatments and Application of SA on Photosynthetic Pigments

The chl *a*, *b* and carotenoid contents decreased progressively under both water deficit and salicylic acid + water deficit treatments. The highest decrease was evidenced under HS (35.6%, 38.8% and 31.9%, respectively) and the lowest decrease in chl *a*, *b* and carotenoid contents was observed under MS + 1.0 (6.29%, 19.7% and 17.5%, respectively) as compared to CK (Table 1).

3.3. Water Deficit Treatments and Application of SA on Gas Exchange Attributes

In *M. alba* saplings, CO_2 assimilation rate and stomatal conductance also decreased under both water deficit and salicylic acid + water deficit treatments. The greatest decrease in CO_2 assimilation rate and stomatal conductance was observed under HS (16.6% and 33.3%, respectively) and the lowest decrease was observed under MS + 1.0 (3.49% and 16.6%, respectively) as compared to CK (Table 2). Furthermore, the intrinsic water use efficiency (WUE_i) increased significantly under both water stress as well as under salicylic acid + water deficit treatments. The observed increase in WUE_i remained the highest under HS (24.8%) and HS + 0.5 (25.9%), and the lowest under MS (12.9%) as compared to CK.

3.4. Water Deficit Treatments and Effect of SA on Osmolyte Accumulation

Proline, soluble sugar, soluble protein, and total phenolic content increased significantly under both water deficit and salicylic acid + water deficit treatments (Table 2). The increase in concentration of proline, soluble sugar, soluble protein, and total phenolic content was the highest under HS +1.0mM (124%, 20.9%, 47.8% and 56.1%, respectively), and was the lowest under HS treatment (60.1% 13.5%, 32.0% and 23.9%) as compared to CK.

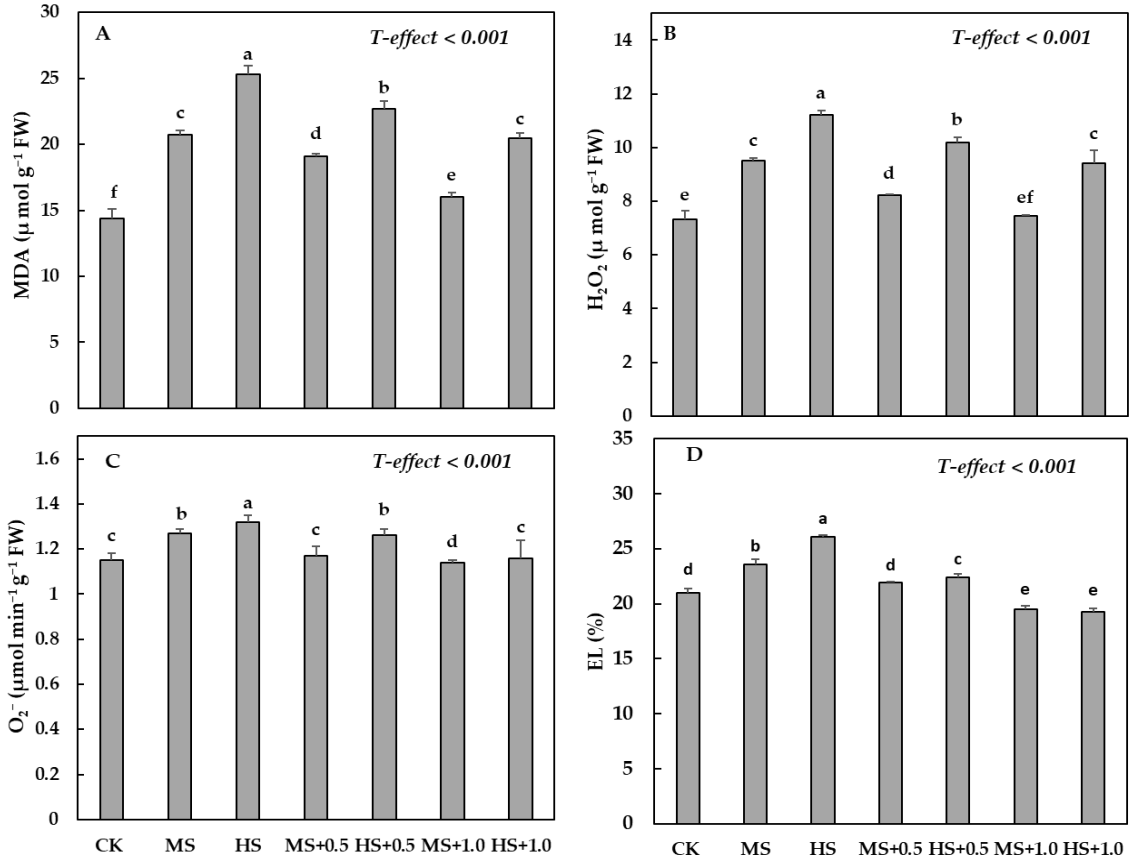

Figure 2. The mean contents/concentration of MDA (**A**), H_2O_2 (**B**), O_2^- (**C**) and EL percentage (**D**) under MS, HS, MS + 0.5, HS + 0.5, MS + 1.0 and HS + 1.0 in *M. alba* saplings. Small letters represent significant differences between the treatment means (T-effect) at $p < 0.05$.

3.5. Water Deficit Treatments and Effect of SA on MDA, H_2O_2, O_2^- and EL%

Overall, the concentration of MDA, H_2O_2, O_2^- and EL% increased significantly in both water deficit and salicylic acid + water deficit treatments (Figure 2). The increase in MDA, H_2O_2, O_2^- and EL% was maximum under HS (75.6%, 53.0%, 14.7% and 24.2%), and the increase in MDA, H_2O_2, and O_2^- was minimum in MS+1.0 (11.1%, 1.63%, and 0.85%, respectively) as compared to CK. The EL% decreased significantly under MA + 1.0 by 7.14% and in HS + 1.0 by 8.09%, respectively.

Table 2. Effect of soil water deficit and soil water deficit + foliar application of SA on proline, soluble sugar, total phenolic, soluble protein contents and CO_2 assimilation rate, stomatal conductance, and intrinsic water use efficiency in *M. alba* saplings. The small letters represent significant differences between the treatment means (T-effect) at $p < 0.05$.

	Proline (μ mol g^{-1} FW)	Soluble Sugar (mg g^{-1} FW)	Total Phenolic Content (mg g^{-1} FW)	Soluble Protein (mg g^{-1} FW)	CO_2 Assimilation Rate (μmoL m^{-2} s^{-1})	Stomatal Conductance (moL m^{-2} s^{-1})	WUEi (μmoL m^{-1})
CK	15.8 ± 0.44 ^{g}	70.6 ± 0.99 ^{e}	1.46 ± 0.02 ^{f}	25.3 ± 0.47 ^{d}	10.0 ± 0.02 ^{a}	0.54 ± 0.01 ^{a}	18.5 ± 1.09 ^{c}
MS	19.7 ± 0.97 ^{f}	76.4 ± 0.65 ^{d}	1.63 ± 0.01 ^{e}	29.7 ± 1.21 ^{c,d}	9.22 ± 0.02 ^{b,c}	0.44 ± 0.02 ^{b,c}	20.9 ± 1.31 ^{b,c}
HS	25.3 ± 0.41 ^{e}	80.2 ± 0.29 ^{c}	1.81 ± 0.02 ^{c}	33.4 ± 0.14 ^{b,c}	8.34 ± 0.06 ^{d}	0.36 ± 0.01 ^{d}	23.1 ± 1.11 ^{a}
MS + 0.5	27.0 ± 0.77 ^{d}	79.3 ± 0.47 ^{c,d}	1.77 ± 0.03 ^{d}	31.6 ± 0.81 ^{c}	9.44 ± 0.09 ^{a,b,c}	0.44 ± 0.03 ^{b,c}	21.4 ± 1.22 ^{a,b}
HS + 0.5	30.6 ± 0.63 ^{c}	83.2 ± 0.49 ^{b}	1.98 ± 0.02 ^{b}	35.2 ± 0.26 ^{a,b,c}	8.65 ± 0.08 ^{c,d}	0.37 ± 0.01 ^{d}	23.3 ± 1.23 ^{a}
MS + 1.0	33.8 ± 0.60 ^{b}	82.4 ± 0.41 ^{b,c}	2.11 ± 0.04 ^{a,b}	36.5 ± 0.37 ^{a,b}	9.66 ± 0.09 ^{a,b}	0.45 ± 0.02 ^{b}	21.4 ± 1.12 ^{a,b}
HS + 1.0	35.4 ± 0.48 ^{a}	85.4 ± 0.37 ^{a}	2.28 ± 0.01 ^{a}	37.4 ± 0.85 ^{a}	8.71 ± 0.01 ^{c}	0.39 ± 0.01 ^{c}	22.3 ± 1.18 ^{a,b}
T-effect	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

3.6. Water Deficit Treatments and Effect of SA on Antioxidant Enzymes Activities

The activity of antioxidant enzymes such as SOD, POD, CAT and APX increased significantly under both water deficit and salicylic acid + water deficit treatments as compared to CK (Figure 3). The activity of SOD, POD, CAT and APX was the highest under HS + 1.0 (56.2%, 35.0%, 41.9%, and 39.5%, respectively) followed by MS + 1.0, HS + 0.5, MS + 0.5, HS, and the least activity was evidenced in MS (56.2%, 35.0%, 41.9%, and 39.5%, respectively) as compared to CK (Figure 3).

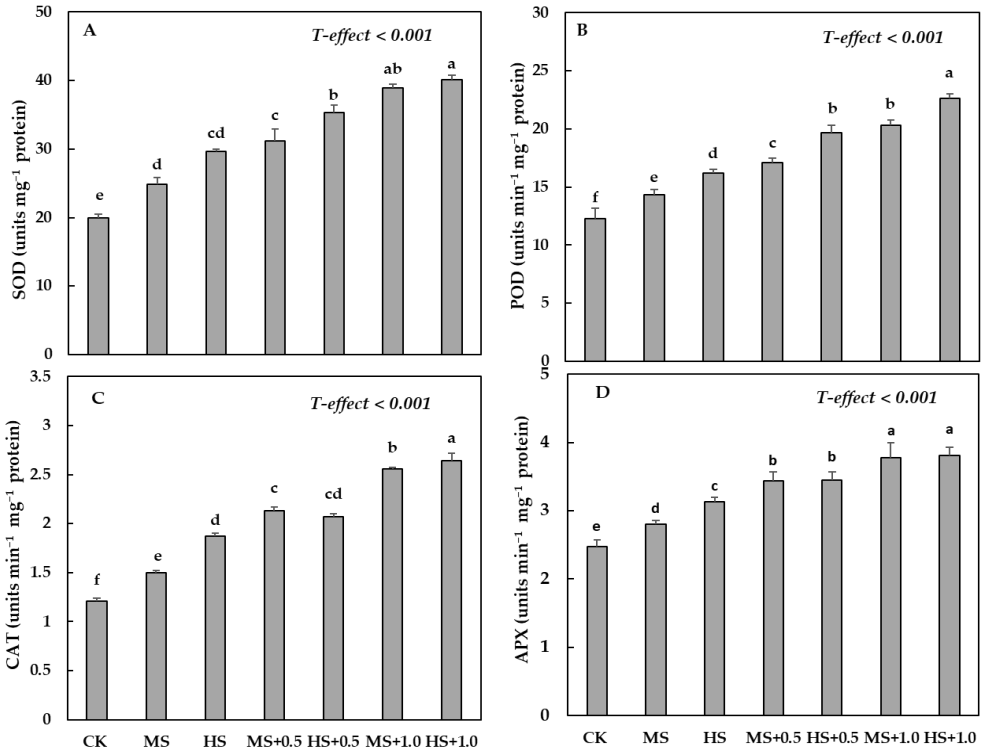

Figure 3. The average values of SOD (**A**), POD (**B**), CAT (**C**), and APX (**D**) under MS, HS, MS + 0.5, HS + 0.5, MS + 1.0 and HS + 1.0 treatments and foliar spray of SA in *M. alba* saplings. Small letters represent significant differences between treatment means at (T-effect) $p < 0.05$.

4. Discussion

4.1. Plant Growth and Dry Biomass Production

Reductions in plant biomass and productivity are the most common effects of soil water deficits in plants. In this study, the soil water deficit had a significant negative impact on the of *M. alba* saplings (Table 1 and Figure 1). Similar observations have been made where reduction in plant growth and development has been reported in various species *Conocarpus*, *Populus*, *Ziziphus*, *Ficus* and *Fagus* species under water deficit respectively [23,25,52,53]. Various scientists have reported that a decrease in plant growth and development is closely related to the reduction in meristematic activity brought about by a decrease in turgor pressure that limits cell growth, division, and development [54]. Development of the root system is also taken as a strong indicator which determines species' ability to survive under water stress [1,54]. In this experiment, the R:S ratio increased significantly in *M. alba* saplings under water deficit (Table 1). Our findings concur with previous studies, where R:S ratio increased under a limited supply of water [23,25,52]. Salicylic acid (SA) is known as an important plant hormone that is naturally present in plants and plays a significant role in reducing the harmful effects of different abiotic stresses [40]. In this study, foliar application of SA reduced the harmful impact of soil water deficit on the plant growth and biomass production of *M. alba* saplings (Table 1 and Figure 1). Our findings were in accordance with different other studies on *Torreya grandis* and *Eucalyptus globulus*, *Conocarpus erectus*, *Populus*

deltoides, *Olea europaea* and *Syzygium sumini* [23,24,55–57], in which a similar increase in growth and biomass production was reported in response to SA under water deficit treatments, respectively. The increment in growth parameters after the foliar application of SA has been associated previously with enhanced cell division in tissues in the meristematic parts of the plants which thus promotes growth and development [58].

4.2. The Physiological Parameters, Chlorophyll a, b and Carotenoid Contents

A decrease in chlorophyll content is a normal response to different abiotic stresses, especially under high stress conditions [54,59] it ultimately causes a decrease in photosynthetic activity and assimilation under a limited supply of water. In this experiment, a significant decrease in stomatal conductance and CO_2 assimilation rate was observed under soil water deficit treatments (Table 2), which agrees with previous findings on various species where in stomatal conductance and CO_2 assimilation rate, along with chl *a*, *b* and carotenoid contents, decreased under a limited supply of water [23,25,52,55,60]. Plants frequently experience stomatal closure in response to water shortage, which reduces both the rate of transpiration and the CO_2 concentration in intercellular spaces because of extreme stomatal closure under severe water stress [18]. A multitude of studies reported that during water deficit, plants balance water losses through transpiration by regulating the stomatal aperture, which helps to maintain high water potential under a limited supply of water [61]. The stomatal aperture is adjusted to maintain the ideal leaf water potential; however occasionally, in severe circumstances, this adjustment may result in a reduction in photosynthesis. Low CO_2 concentration in the intercellular spaces, which lowers the rate of CO_2 assimilation, is one of the detrimental impacts of lowered stomatal aperture on photosynthesis. Due to this condition, photosystem-2 and photochemical activity is disrupted, and the electron supply is hindered; this reduces overall photosynthetic activity [62]. During water stress, a reduction in chlorophyll content usually results from chloroplast damage due to excessive overproduction of reactive oxygen species (ROS) [59]. In the present experiment, the foliar application of SA significantly increased CO_2 assimilation and stomatal conductance along with chlorophyll contents under water deficit treatments (Tables 1 and 2). Similar findings were reported in previous studies, where positive effects of SA on impaired photosynthetic processes under a limited supply of water were observed [23,24,60]. SA is a phytohormone that protects plants against chlorophyll degradation under stressful environments by decreasing the damage to the photosynthetic apparatus. Such an alteration is induced by an increase in compatible solutes' accumulation and the activity of the chlorophyll synthesizing enzymes, and a reduction in the concentration of ROS after the foliar application of SA [63]. In this study, intrinsic water use efficiency (WUE_i) significantly increased under both treatments (Table 2). Similar results were demonstrated in previous studies [23,25,38], where in enhancement in WUE_i was observed in plants under water deficit treatments. However, WUE_i enhanced significantly after foliar spray of SA, which was medicated by enhance in the CO_2 assimilation rate under water deficit treatment. Previous studies demonstrated that foliar application of SA promotes CO_2 assimilation and growth under stressful conditions by increasing the activity and efficiency of carboxylation by the rubisco enzyme under reduced stomatal conductance, due to stressful conditions [64].

4.3. The Osmolyte Accumulation

Proline content and soluble sugars are the most significant physiological parameters in plants for tolerance of a water deficit environment; they play an important role in defense mechanisms and maintaining cell turgor, gas exchange and growth parameters under a limited supply of water [30]. Various studies have reported that plants can tolerate water stress by increasing soluble sugar [24,30,63,65]. In the present study, proline and soluble sugars increased significantly under MS and HS, respectively (Table 2), which is consistent with previous studies [17,23,55,60,65,66]. However, the increase in proline concentration plays a key role in decreasing osmotic potential, enhancing growth, controlling water

absorption and cellular turgor pressure and protecting plants from ROS damage under water stress environments [1,67]. Previous studies have demonstrated that an increase in the concentration of soluble sugar in a water deficit environment linked to the breakdown of polysaccharides such as starch into glucose, promotes water potential and maintains cell volume to avoid wilting. Furthermore, it has been reported that enhancement of soluble sugar plays a significant role in sustaining the osmotic potential and cellular turgor pressure of a plant under a limited supply of water [22]. Phenolic compounds and soluble proteins are the most significant secondary metabolites that help in reducing the harmful effect of water deficits [67]. In this experiment, phenolic compounds and soluble proteins significantly increased under both water deficit (MS and HS) treatments, respectively (Table 2). Similar findings were reported in previous studies [23,55,57], where phenolic compounds and soluble proteins increased under a limited supply of water. Multiplies studies have demonstrated that plants upregulate the expression of phenolics synthesis enzymes; for example, phenylalanine ammonia-lyase, results in the increased production of phenolic compounds that are antioxidants enzymes in stressful conditions [68]. Furthermore, in the present study, the foliar application of SA further increased the concentration of proline, soluble sugars, phenolic compounds, and soluble proteins under MS and HS treatments, respectively (Table 2). These results are in line with previous studies which showed an increase in the phenolic compounds and soluble proteins [23,24,55]. Several other studies demonstrated an increase in proline contents after foliar application of SA because of the enhanced activity of the proline-synthesizing enzyme, gamma glutamyl kinase, and reduced activity of the proline-reducing enzyme proline oxidase [68]. Furthermore, the SA-mediated enhancement of phenolic compounds has been linked to the upregulation of phenolics-synthesizing enzymes such as phenylalanine ammonia-lyase PAL [68].

4.4. H_2O_2, O_2^-, along with MDA and EL% and Antioxidants

In plants, different environmental stresses, for example, water deficit, may lead to an over-production of reactive oxygen species ROS, for example, H_2O_2, O_2^-, $^1O_2$ and hydroxyl free radicals $^\bullet$OH, highly toxic molecules which create harmful effects to nucleic acids, proteins, lipids, and carbohydrates, thus causing cell death [4,55]. In this experiment, an increase in H_2O_2 and O_2^- was noticed in *M. alba* saplings under both soil water deficit treatments (Figure 2). Our results are consistent with previous research reporting increased production of H_2O_2 and O_2^- under water stress [23,24,55,57]. Multiple studies have demonstrated that an increase in ROS production alters the redox equilibrium, which ultimately affects plant development in a water-scarce environment [38]. The different types of abiotic stress have an extreme impact on the cell membrane which enhances the concentration of MDA contents and is related to the degree of lipid peroxidation due to oxidative stress and EL%, indicative of extent of cellular damage [57]. It has been reported that the enhancement in EL% is related to the lipid peroxidation of the cell membrane and osmotic imbalance [56]. In the current investigation, a significant increase in MDA content and EL% in *M. alba* saplings under both (MS and HS) treatments was observed (Figure 2). Similar observations with increased MDA content and EL% have been reported in previous studies [23,55,57,69]. Plants have effective antioxidants enzyme defense mechanisms that tolerance the different types of abiotic stresses. These antioxidants enzymes include SOD, POD, CAT, and APX which play an important role for scavenging the over production of ROS [67]. In the current study, the activity of antioxidant enzymes increased significantly under water deficit (MS and HS) and salicylic acid + water deficit treatments (Figure 3). However, the highest values were evidenced under the HS+1.0 treatment. Similar results with increased antioxidant enzyme activity have been reported in earlier investigations [23,55,57,69]. Among the antioxidants, SOD is considered the primary line of defense against excessive ROS production [70]. Other antioxidant enzymes such as CAT and APX subsequently scavenge the hydrogen peroxide produced by SOD to produce water and molecular oxygen. [17]. Catalase is mostly located in the peroxisome, where its main function is the detoxification of H_2O_2, while peroxidase converts hydrogen peroxide

to water in the plant cell [71]. In the present investigation, concentration of ROS such H_2O_2, O_2^-, along with MDA contents and EL%, was reduced significantly after foliar application of SA (0.5 and 1.0 mM) in *M. alba* saplings under MS and HS, respectively (Figure 3). Similar findings have been found in previous research where a reduction in H_2O_2, O_2^-, MDA content and EL% has been observed after foliar application of SA [23,24,60,72]. Moreover, in this study, the foliar application of SA significantly enhanced the activities of all antioxidant enzymes (SOD, POD, CAT and APX) under the water deficit and salicylic acid + water deficit treatments, respectively (Figure 3). Our results are in line with previous studies where in foliar application of SA increased antioxidant enzyme activity by reducing the ROS under different abiotic stresses such as water deficit [67]. In *Lippia citriodora* plants, the foliar application of SA has enhanced defense mechanisms through regulation of oxidative stress by increasing antioxidant enzyme production under water deficit treatments [72]. Through controlling oxidative stress by enhancing the synthesis of antioxidant enzymes under water deprivation, SA has been connected to plants' defense mechanisms [72]. Consequently, it can be concluded that SA applied topically significantly decreased ROS production, MDA, and EL%, and that this effect was mediated by an increased synthesis of antioxidant enzymes.

5. Conclusions

Water deficit had a negative impact on *M. alba* saplings, where a major reduction was found in different growth traits, dry biomass, and physiological parameters, respectively. However, the application of SA increases the growth parameters, dry biomass production, proline, soluble proteins, soluble sugar and total phenolic content. Moreover, a significant increase in the activities of SOD, POD, CAT and APX was found under both treatments (MS and HS). Therefore, it can be concluded that foliar spray of SA can help to alleviate the harmful effects of a water deficit on *M. alba* saplings, indicated by a reduction in MDA content, EL%, H_2O_2, and O_2^- and an increase in osmolyte concentration and antioxidants' enzyme activities under a soil water deficit. The results have demonstrated that under high stress, SA concentrations of 1.0 mM were most effective.

Author Contributions: Conceptualization, F.R. and O.G.; methodology, Z.Z. and F.R.; software, Z.R., M.U.K. and M.S.; validation, N.M., Z.Z. and M.A.I.; formal analysis, O.G., M.U.K. and M.M.; investigation, O.G. and F.R.; resources, Z.Z., F.R. and O.G.; data curation, Z.Z., F.R., N.M., M.M. and O.G.; writing—original draft preparation, Z.Z., Z.R., M.S. and M.A.I.; writing—review and editing, F.R. and O.G.; visualization, F.R., M.S. and M.U.K.; supervision, F.R.; project administration, Z.Z. and N.M.; funding acquisition, Z.Z. and O.G. All authors have read and agreed to the published version of the manuscript.

Funding: The Offices of Research, Innovation and Commercialization (ORIC) provided support for this research Grant no. A/C AO5213, University of Agriculture Faisalabad 38040, Pakistan.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Muhammad Atif (Associate Professor) for providing the laboratory for biochemical analysis and appreciate the support from the Open Access Publication Funds of the University of Göttingen.

Conflicts of Interest: The authors declare no conflict of interest.

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