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Foliar H_2O_2 Application Improve the Photochemical and Osmotic Adjustment of Tomato Plants Subjected to Drought

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Abstract: Water limits may have a disastrous impact on agricultural productivity, and the current climate change scenario presents additional problems for crops that rely on regular rainfall. Reactive oxygen species, such as hydrogen peroxide (H_2O_2), are a recognized stress-sensing mechanism in plants, and may be investigated as an approach for reducing stress impact via systemic acquired acclimation. Here, we looked at how H_2O_2 foliar application impacts tomato plants' photosynthetic activity, antioxidant system, sugar chemical profile, and osmotic adjustment during drought and recovery. The experiment was in randomized blocks, 3 × 2 factorial design, with no, one, or two foliar application of 1 mM H_2O_2, on plants that were either continually watered or subjected to drought. The plants were tested both during the drought period and after they had resumed irrigation (recovered). Leaf water potential, chlorophyll *a* fluorescence, gas exchange, lipid peroxidation, H_2O_2 concentrations, phenols, proline, antioxidant enzyme activity, and sugar chemical profile were all measured. Our findings showed that H_2O_2 application generated metabolic alterations in tomato plants independent of water status, and that two applications in drought plants resulted in a 30% decrease in oxidative stress during drought and faster recovery following irrigation return, with greater production of defence-related molecules such as the APX enzyme, phenols, arabinose, and mannose. Continually watered plants also benefited from H_2O_2 application, which increased carbon assimilation by 35%.

Keywords: hydrogen peroxide; *Solanum lycopersicum*; sugar profile; trehalose

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1. Introduction

Water deficiency is the most common abiotic stressor in agriculture [1], and a single incident can result in the loss or reduction of a crop's economic return. Climate change is exacerbating the situation, since extreme weather events, such as extended drought periods that are unpredictable, are anticipated to occur more frequently [2]. As a result, solutions for mitigating the impact of water scarcity are critical to the sustainability of agricultural practices.

A potentially useful method is based on acquired systemic acclimation, which happens because of long-distance communication between the plant's numerous organs and tissues [3]. A stressful stimulus, such as high light incidence, temperature, or osmotic stress, causes signals to be transmitted throughout the plant, which responds with gene expression, resulting in increased enzyme activity and antioxidant molecule action. Plants can develop a reaction and improve their defensive mechanism, resulting in higher performance even under harsh situations [3,4].

Reactive oxygen species (ROS) signalling is a well-known mechanism for plant stress detection, but the metabolic changes that follow this signalling have yet to be defined for

several plant species [5]. H_2O_2, for example, is engaged in the stomatal closure process, which is intimately related to plant and soil water levels. However, stomatal movement is merely the first visible response to a lack of water.

According to Ishibashi et al. [6], applying H_2O_2 to soybean plants prior to water stress boosted their tolerance to subsequent stress events, since soluble sugar concentrations rose in cells, resulting in better hydrological conditions. Other researchers found that exogenous H_2O_2 supply increased the performance of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) in wheat and corn plants subjected to water and saline stress, resulting in less cellular component lipid peroxidation [7,8]. Tomatoes resistant to drought produce more chemicals that detoxify ROS, including phenols and polyphenols [9], as well as enzymes including SOD, CAT, and APX [10]. Tomato mutant plants with low abscisic acid (ABA) production and drought tolerance accumulated more arabinose, glucose, fructose, and sucrose to compensate for increased stomatal conductance and to prevent dehydration [11].

Tomatoes are one of the most significant agricultural crops, with global cultivation distribution [12]. Although they are frequently grown in controlled and high-tech settings, most cultivation takes place in the field, where they are exposed to environmental conditions and changes. In this approach, we suggest that foliar application of H_2O_2 in tomatoes preceding water limitation induces changes in plant metabolism, resulting in a recovery owing to lesser oxidative damage. In this work, we explored how foliar H_2O_2 application affects tomatoes' photosynthetic activity, antioxidant system, sugar chemical profile, and osmotic adjustment during and after drought.

2. Materials and Methods

2.1. Plant Growth Environment, Experimental Design, and Treatments

The experiment was conducted between October 2020 and December 2020 in an experimental area of the Department of Biodiversity and Biostatistics at Universidade Estadual Paulista "Julio de Mesquita Filho", Botucatu, SP, Brazil, with geographic coordinates 22°53′09″ W, 48°26′42″ S and an average altitude of 800 m. The cultivation took place in a Van der Hoeven greenhouse with regulated temperature (25 ± 5 °C) and humidity (43 ± 10%). During the experiment, the photosynthetic photon flux density (836 ± 200 µmol m^2 s^{-1}) and ambient CO_2 content (410 ± 5 µmol mol^{-1}) were validated. We utilized tomato plants (*Solanum lycopersicum* L. cv. Micro-Tom wild subtype). After 12 days of sowing on a tray with vermiculite substrate, the plants were transplanted into individual 1 dm^3 pots with 130 g of vermiculite substrate. Fertilization was done every two days using nutrient solution nº 2 [13], commencing at 25% ionic strength and increasing to 50% after seven days. When most of the plants had bloomed, treatments began.

The experimental design employed was randomized blocks in a 3 × 2 factorial design, represented by three foliar treatments and two plant water condition treatments, with four blocks, each containing seven plants. The plants were treated with a foliar application of water (no H_2O_2), one foliar treatment of 1 mM H_2O_2 (one application of H_2O_2), or two foliar applications of 1 mM H_2O_2, separated by a 24 h interval (two applications of H_2O_2). After the second foliar treatment, 50% of the plants were watered every two days (watered plants) and 50% went without water for 12 days (drought plants) before being assessed. After this evaluation, all plants continued to receive nutrient solution (nº 2, 50% of ionic strength) for another 12 days, when a fresh evaluation was carried out. From then on, plants were continually watered and recovered from drought. Foliar spraying was done with a CO_2 backpack sprayer with a fan tip, using around 8 mL of the solution (deionized water + non-ionic adjuvant + H_2O_2 − BAKER™ 30% stabilized hydrogen peroxide − according to treatment) per plant.

2.2. Leaf Water Potential Measurement

The leaf water potential (LWP) was measured using a Decagon Device's WP4T dewpoint PotentiaMeter, Washington, DC, USA. Leaf samples from each replicate were finely

chopped (about 20 × 5 mm) and utilized for determination, as per the manufacturer's instructions, with water potential measurements represented in MPa.

2.3. Chlorophyll a Fluorescence and Gas Exchanges

The measurements were taken with an infrared radiation gas analyser (IRGA), model GSF 3000, Walz, GmbH, Effeltrich, Germany, at a saturating irradiance of 1200 µmol m^{-2} s^{-1}. The fluorescence variables were measured by a portable modulated light fluorometer (LED-ARRAY/PAM-Module 3055-FL, GmbH, Effeltrich, Germany) coupled to the GSF 3000, obtaining the effective quantum yield of the FSII (Φ_{PSII}), heat dissipation in the antenna complex ($D = 1 - Fv'/Fm'$), and energy not dissipated and not used in the photochemical phase ($Ex = Fv'/Fm' (1 - qP)$) [14,15]. The non-photochemical fluorescence quenching coefficient (qN) was determined after 30 min of acclimatizing the leaves to darkness. The gas exchange variables obtained were transpiration (*E*—mmol m^{-2} s^{-1}), stomatal conductance (*Gs*—mmol m^{-2} s^{-1}), and net carbon assimilation (*A*—µmol m^{-2} s^{-1}). The computed instantaneous water use efficiency (WUE_i—µmol CO_2 mmol H_2O^{-1}) was derived via the relationship between *A* and *E*. Measurements were taken between 9 and 11 a.m., on the second or third completely developed leaf.

2.4. Leaf Lipid Peroxidation Quantification and Hydrogen Peroxide Concentration

Rama Devi and Prasad [16] assessed lipid peroxidation using 100 mg of fresh frozen and milled material with reagents thiobarbituric acid and trichloroacetic acid in a water bath at 90 °C for one hour. The contents were centrifuged at 10,000× *g* for 15 min at room temperature, and the supernatant's absorbance was measured at 560 and 600 nm using a spectrophotometer. The results were represented in nmol of malondialdehyde g^{-1} of dry weight.

The hydrogen peroxide concentration of leaves was determined using 100 mg of fresh milled and frozen leaf sample, 0.1% trichloroacetic acid reagent, and centrifugation at 12,000× *g* for 15 min at 4° W, as described by Alexieva et al. [17]. The supernatant was collected and mixed with 0.1 M phosphate-buffered (pH 7) and 1 M potassium iodide, followed by a 1 h reaction in the dark and an absorbance reading at 390 nm using a spectrophotometer. The results were represented in µmol H_2O_2 g^{-1} of dry weight, based on the proportion of water in each sample and determined via relative water content.

2.5. Activity of Enzymes Superoxide Dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC: 1.11.1.6), Peroxidase (POD, EC 1.11.1.7) and Ascorbate Peroxidase (APX, EC: 1.11.1.1)

Enzymatic extraction was performed using the Kar and Mishra [18] (modified) technique using 100 mg of fresh, milled, and frozen leaf samples homogenized in 0.1 M potassium phosphate-buffered (pH 6.8) and PVPP added to prevent sample oxidation. The analysis of soluble proteins followed Bradford [19], with the enzymatic extract being homogenized with Bradford's reagent and absorbance reading on a spectrophotometer at 595 nm with a casein standard curve. SOD activity was measured according to Beauchamp and Fridovich [20]. The reaction system consisted of enzyme extract, 50 mM potassium phosphate-buffered (pH 7.8), 13 mM methionine, 75 µM NBT, 100 nM EDTA, and 2 µM riboflavin. The reaction occurred in the presence of light for 5 min at room temperature, with an absorbance measurement of 560 nm. Activity was measured in enzymatic units (U), with 1U representing the quantity of enzyme required to provide 50% inhibition in NBT reduction.

CAT activity was determined using the reaction method described by Peixoto et al. [21], which included enzymatic extract, 50 mM pH (7.0), and 12.5 mm hydrogen peroxide. Every 20 s, absorbance values at 240 nm were taken, and the molar extinction coefficient of hydrogen peroxide was calculated using the 39.4 mmol L^{-1} cm^{-1}. Enzyme activity was measured in nanomoles of H_2O_2 consumed min^{-1} mg $protein^{-1}$. The Teisseire and Guy [22] approach was used to conduct POD activity utilizing a reaction system that contained an enzymatic extract, 50 mm potassium phosphate-buffered (pH 6.5), 20 mm pyrogallol, and

5 mm hydrogen peroxide. The reaction was carried out at room temperature for 5 min, followed by an absorbance reading at 430 nm. The calculations were performed using the purpurogallin molar extinction coefficient of 2.5 mmol L^{-1} cm^{-1}. The enzyme activity has been expressed in µmol of purpurogallin min^{-1} mg $protein^{-1}$.

APX activity was determined using the reaction method described by Nakano and Asada [23], which included an enzymatic extract, 50 mM pH 7.0 potassium phosphate-buffered, 0.5 mM ascorbate, 10 mM hydrogen peroxide, and 0.1 mM EDTA. Every 20 s, absorbance values at 300 nm were taken, and the molar extinction coefficient of hydrogen peroxide was calculated using the 39.4 mmol L^{-1} cm^{-1} value. Enzyme activity was measured in nanomoles of H_2O_2 consumed min^{-1} mg $protein^{-1}$.

2.6. Total Phenols Leaf Concentration and Proline Quantification

Total phenols were extracted and measured in accordance with Bonoli et al. [24]. Extraction was performed on 100 mg of fresh, frozen, and milled leaves using a 50% (v/v) acetone solution, followed by 20 min in an ultrasonic bath. The reaction system included 1 mL of extract, Folin reagent (1:10), and a 20% Na_2CO_3 solution. The reaction was carried out in the dark for an hour, with subsequent absorbance measurements at 725 nm. Gallic acid was measured using a standard curve and represented as µg of total phenols of dry $weight^{-1}$.

Proline was measured using 250 mg of fresh, milled, and frozen leaf samples homogenized with 3% sulfosalicylic acid and centrifuged at 4000× g for 5 min, as described by Bates et al. [25]. Supernatant extract, glacial acetic acid, and acid ninhydrin were combined in a boiling water bath for an hour. The mixture was extracted with toluene, and absorbance was measured at 520 nm. The results were reported as µmol proline g of dry $weight^{-1}$ using a proline standard curve.

2.7. Soluble Sugars Chemical Profile Determination

The sugar profile was evaluated using high-performance ion chromatography (Dionex ICS-5000+, Thermo Fisher Scientific®, Sunnyvale, CA, USA). The extracts (100 mg of fresh, frozen, and milled leaf samples through triple extraction with 80% ethanol) and standards were filtered (0.22 µm) and analysed by chromatography using a quaternary pump, automatic sampler, DCS-5000 electrochemical detector (Thermo Fisher Scientific®, Sunnyvale, CA, USA), P-100 column (Carbopack, Thermo Fisher Scientific®, Sunnyvale, CA, USA), and gold working and Ag/AgCl reference electrodes. Eluent phases A, B, and C consisted of 640 mM sodium hydroxide, 0.5M sodium acetate, and ultrapure water, with a flow rate of 0.7 mL min^{-1} for 35 min. Soluble sugars were identified by comparing the retention time of the sample peak to the retention time of the standard mixes and injecting the standard solution together with the sample [26] (modified).

2.8. Statistical Analysis

Data were examined for variance homogeneity and normality, and differences between treatments and their interactions were then investigated for each assessment period. The data were compared using the Tukey test with a 5% probability. The heatmap was created with MetaboAnalyst 5.0 software (www.metaboanalyst.ca/; accessed on 22 May 2023).

3. Results

3.1. Leaf Water Potential

Drought plants were water-restricted until the twelfth day after foliar application treatments when the leaves had a potential water of −1.53 MPa, which was nearly 90% more negative than the watered plants (Figure 1a) and showed water stress. Twelve days after irrigation, there was no variation in the potential water of the leaves (Figure 1b). Foliar H_2O_2 application showed no influence on leaf water potential.

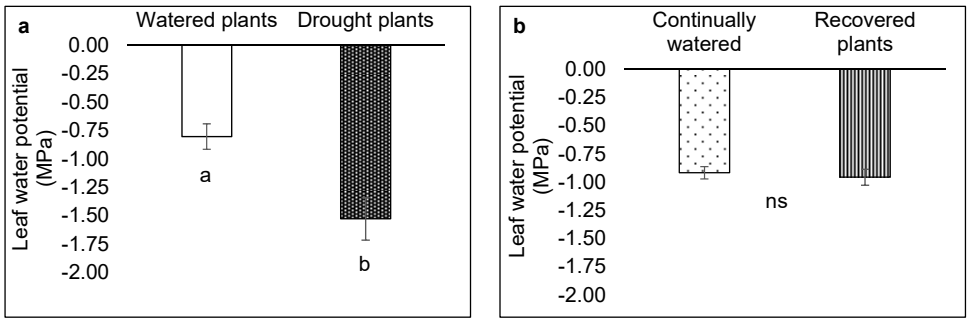

Figure 1. (**a**) Leaf water potential of tomato plants grown under optimal water supply (watered) or subjected to drought, and (**b**) after irrigation return, plants continually watered and recovered plants. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate means using the Tukey test (<0.05).

3.2. Chlorophyll a Fluorescence

Drought plants displayed lower Φ_{PSII} levels compared to those watered in the first evaluation (Figure 2a). A single application of H_2O_2 resulted in increased Φ_{PSII} and decreased qN, independent of plant water status (Figure 2b,c). Lower *D* and *Ex* values were observed in watered and drought plants, respectively, after a single H_2O_2 spray (Figure 2d,e). Irrigation resulted in full plant recovery and no change in Φ_{PSII} compared to continually watered plants (Figure 2f). However, the effect of a single H_2O_2 treatment may be seen in recovered plants with lower qN than those that did not receive the treatment (Figure 2g).

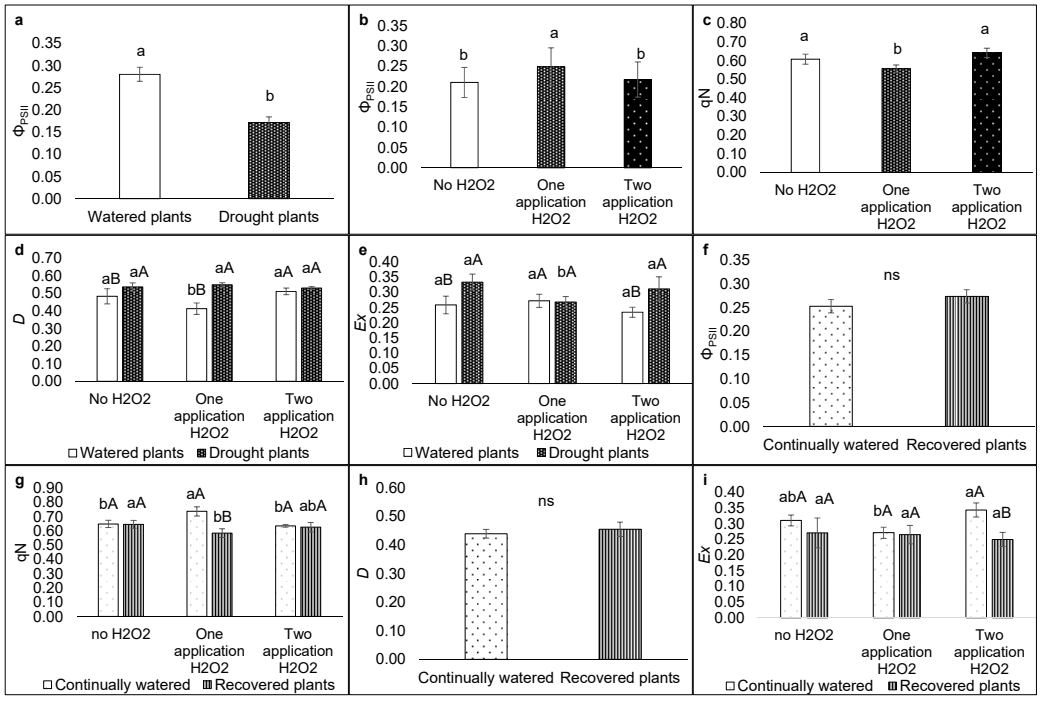

Figure 2. (**a**,**b**) Effective quantum yield of photosystem II (Φ_{PSII}), (**c**) non-photochemical fluorescence quenching coefficient (*qN*), (**d**) heat dissipation in the antenna complex (*D*) and (**e**) energy not dissipated and not used in the photochemical phase (*Ex*) in tomato plants grown under optimal water supply (watered) or subjected to drought and no, one, or two foliar applications of H_2O_2. After recovery due to irrigation return, (**f**) Φ_{PSII}, (**g**) qN, (**h**) *D* and (**i**) *Ex*. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition and uppercase letters compare water condition within H_2O_2 application.

3.3. Gas Exchanges

Watered plants treated with a single application of H_2O_2 performed better in the first evaluation, and those treated with two applications performed worse, with lower *E* and *Gs* (Figure 3a–c), while WUE_i was not influenced by the H_2O_2 application (Figure 3d) throughout the same time. Following irrigation, recovered plants showed lower *A* levels than continually watered plants (Figure 3g). However, among plants that did not receive H_2O_2 treatment, recovered plants exhibited the greatest WUE_i (Figure 3h).

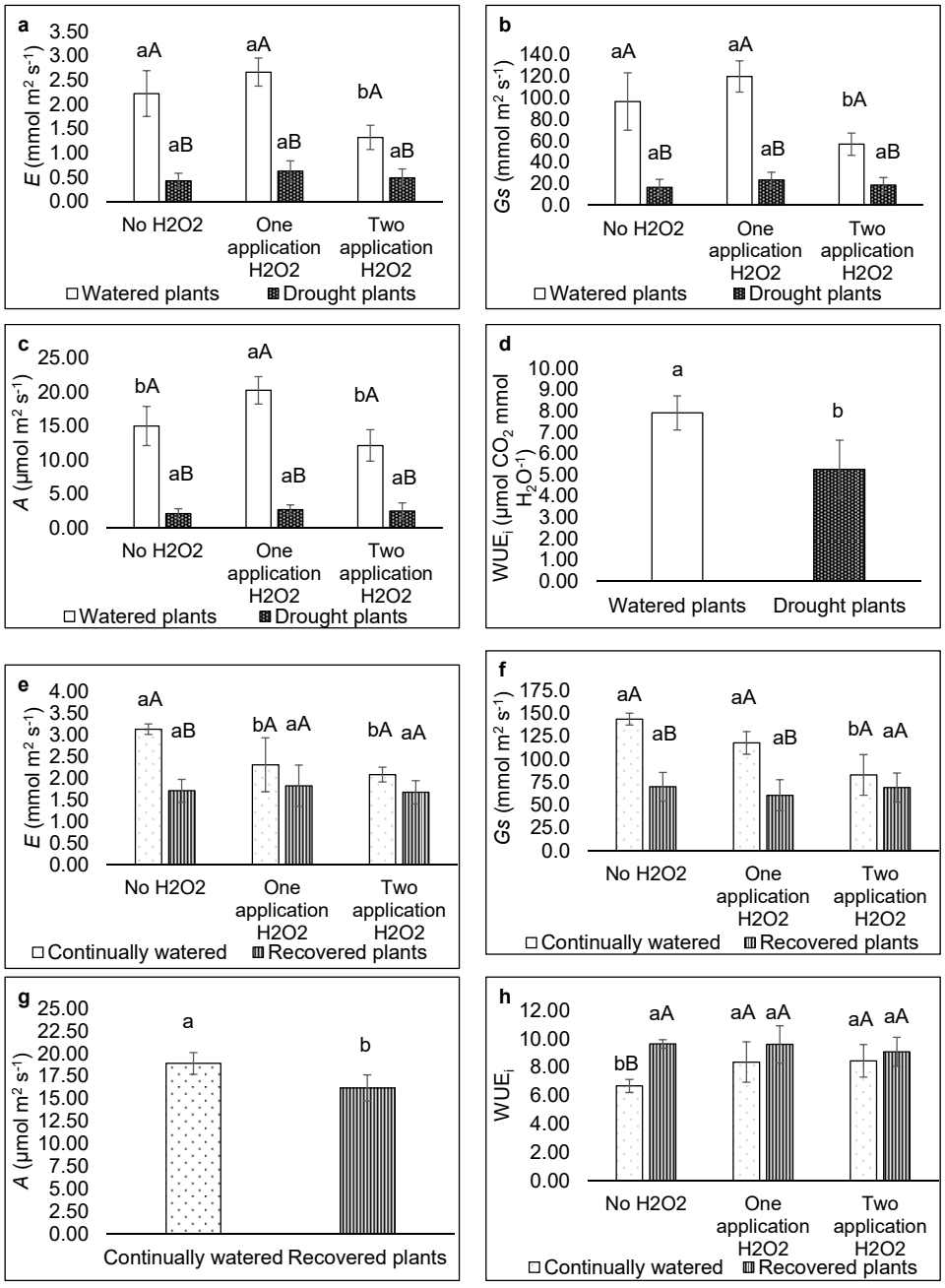

Figure 3. (**a**) Transpiration rate (*E*), (**b**) stomatal conductance (*Gs*), (**c**) net CO_2 assimilation (*A*) and (**d**) instantaneous water use efficiency (WUEi) in tomato plants grown under optimal water supply (watered) or subjected to drought and no, one or two foliar applications of H_2O_2. After recovery due irrigation return, (**e**) *E*, (**f**) *Gs*, (**g**) *A* and (**h**) WUEi. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition, and uppercase letters compare water condition within H_2O_2 application.

3.4. Leaf Lipid Peroxidation and H_2O_2 Concentration

Drought plants showed greater MDA and H_2O_2 concentrations than watered plants (Figure 4a,b). Drought plants that received H_2O_2 application had lower MDA than those that did not (Figure 4a), while H_2O_2 application reduced H_2O_2 leaf concentrations regardless of the water condition of plants (Figure 4c). MDA levels in all treatments were unchanged after irrigation (Figure 4d). Continually watered plants had a higher leaf concentration of H_2O_2 than recovered plants, and plants that had only one H_2O_2 treatment had a higher H_2O_2 concentration than those that did not receive any (Figure 4e,f).

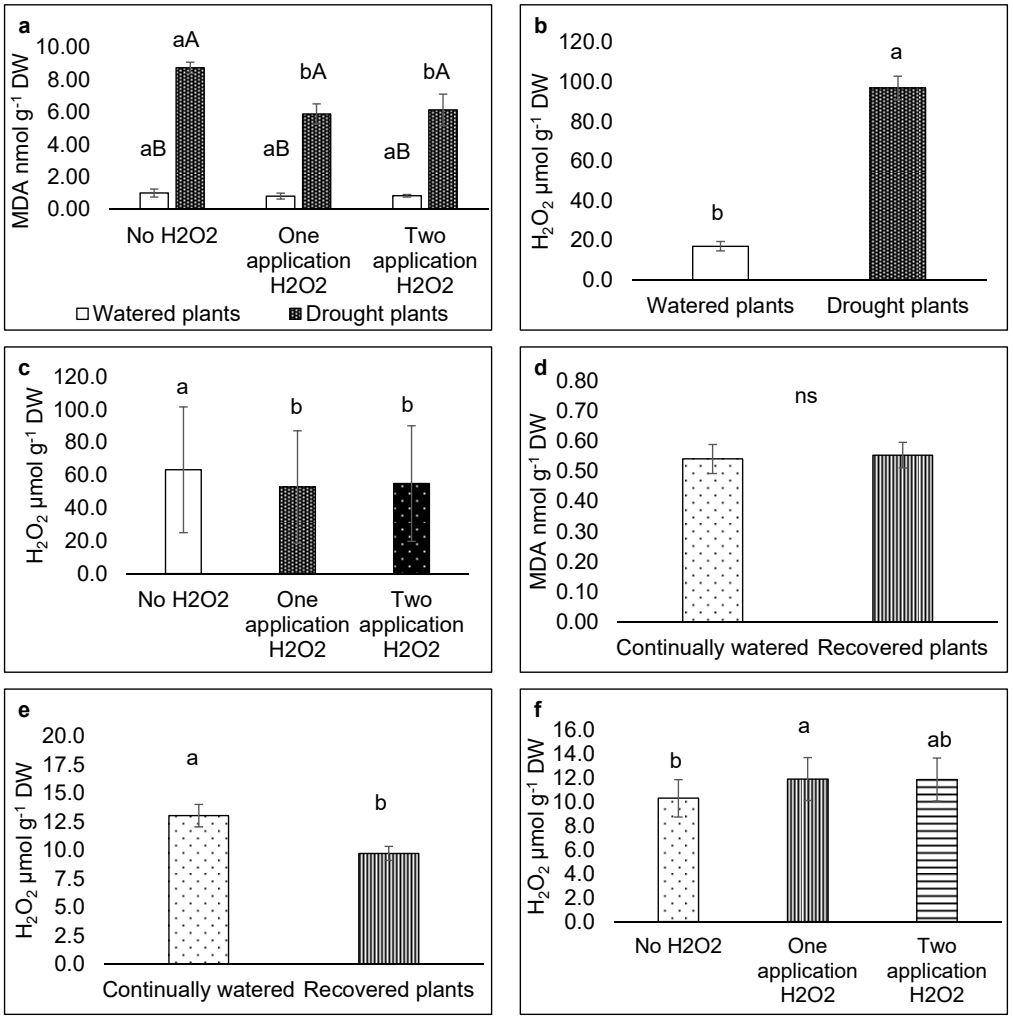

Figure 4. (**a**) Lipid peroxidation (MDA) and (**b**,**c**) concentration of hydrogen peroxide (H_2O_2) in leaf tomato plants grown under optimal water supply (watered) or subjected to drought and no, one, or two foliar applications of H_2O_2. After recovery due to irrigation return, (**d**) MDA, (**e**,**f**) H_2O_2. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition, and uppercase letters compare water condition within H_2O_2 application.

3.5. Antioxidant Enzyme Activity

During the water restriction period, drought plants displayed higher levels of SOD, CAT, and POD activity than watered plants (Figure 5a–c). At the same time, only drought plants that received a single H_2O_2 spray showed increased APX activity (Figure 5d). After irrigation, all plants that received a single H_2O_2 spray displayed increased CAT activity (Figure 5f). It was also shown that continually watered plants that received one H_2O_2 treatment had higher APX activity than those that did not, whereas recovered plants that had two H_2O_2 treatments had lower activity of this enzyme (Figure 5h). Among the

recovered plants, only those that did not receive H_2O_2 treatment exhibited higher APX activity than those that were continually watered.

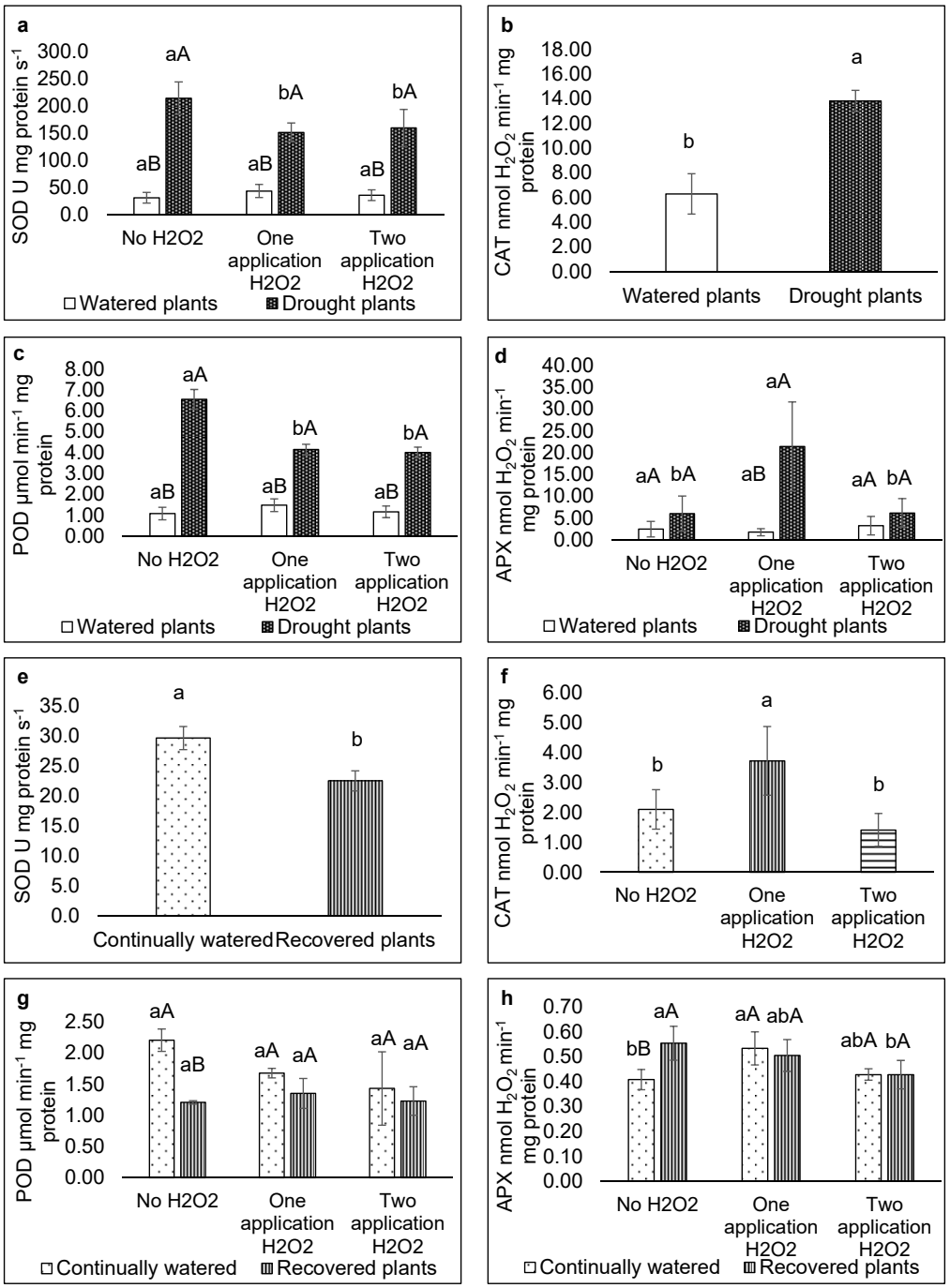

Figure 5. Antioxidant enzyme activity. (**a**) Superoxide dismutase (SOD), (**b**) catalase (CAT), (**c**) peroxidase (POD), and (**d**) ascorbate peroxidase (APX) in tomato plants grown under optimal water supply (watered) or subjected to drought and no, one, or two foliar applications of H_2O_2. After recovery due to irrigation return, (**e**) SOD, (**f**) CAT, (**g**) POD, and (**h**) APX. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition, and uppercase letters compare water condition within H_2O_2 application.

3.6. Phenolic Compounds and Proline

Drought plants had greater total phenol and proline contents in their leaves than watered plants (Figure 6a,b). During this time, drought plants treated with two H_2O_2 sprays had higher phenol and lower proline contents. After irrigation, only recovered plants that did not receive H_2O_2 treatment had reduced total phenol concentrations, although proline concentrations in recovered plants were higher than in continually watered plants (Figure 6c,d).

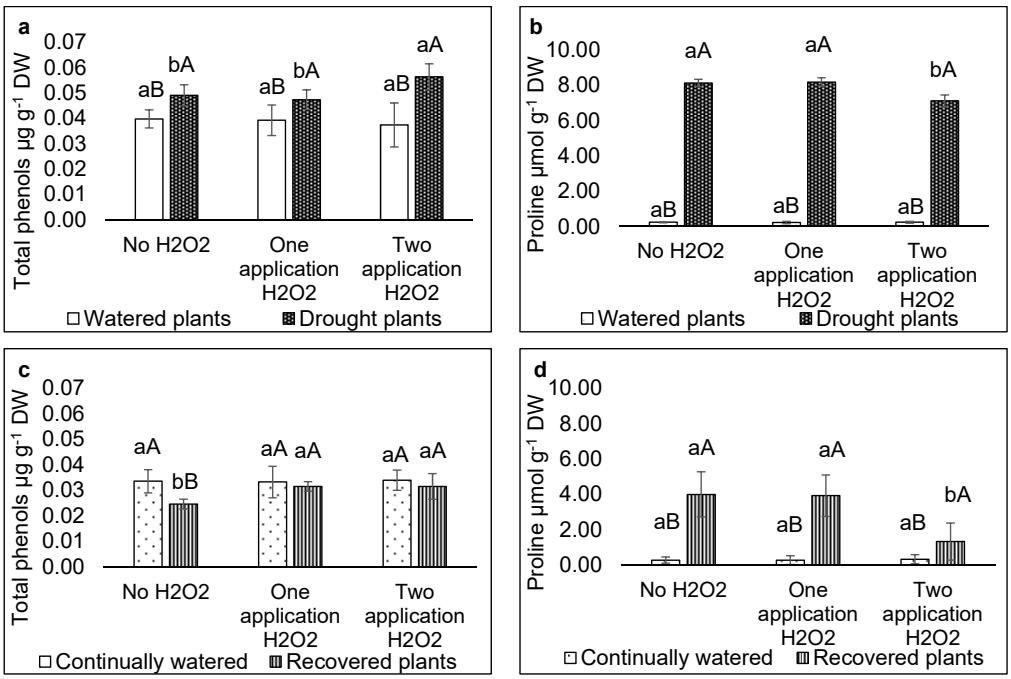

Figure 6. (**a**) Concentration of total phenols and (**b**) concentration of proline in leaf tomato plants grown under optimal water supply (watered) or subjected to drought and no, one, or two foliar applications of H_2O_2. After recovery due to irrigation return, (**c**) total phenols, (**d**) proline. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition, and uppercase letters compare water condition within H_2O_2 application.

3.7. Soluble Sugars Chemical Profile

In the first evaluation, drought plants had larger concentrations of the soluble sugars arabinose, glucose, fructose, and sucrose than watered plants (Figure 7b,d,f). Trehalose levels were not affected by treatments, but mannose levels were higher in watered plants that did not receive H_2O_2 application (Figure 7a,c). Plants that received one H_2O_2 application showed higher amounts of fructose regardless of water condition and higher glucose amounts only in drought plants. In drought plants, any H_2O_2 application resulted in the greatest mannose concentration, while higher concentrations of arabinose, glucose, and fructose were found in plants that received a single application of H_2O_2. Following irrigation, only recovered plants that did not receive H_2O_2 application had the greatest trehalose concentration (Figure 7g).

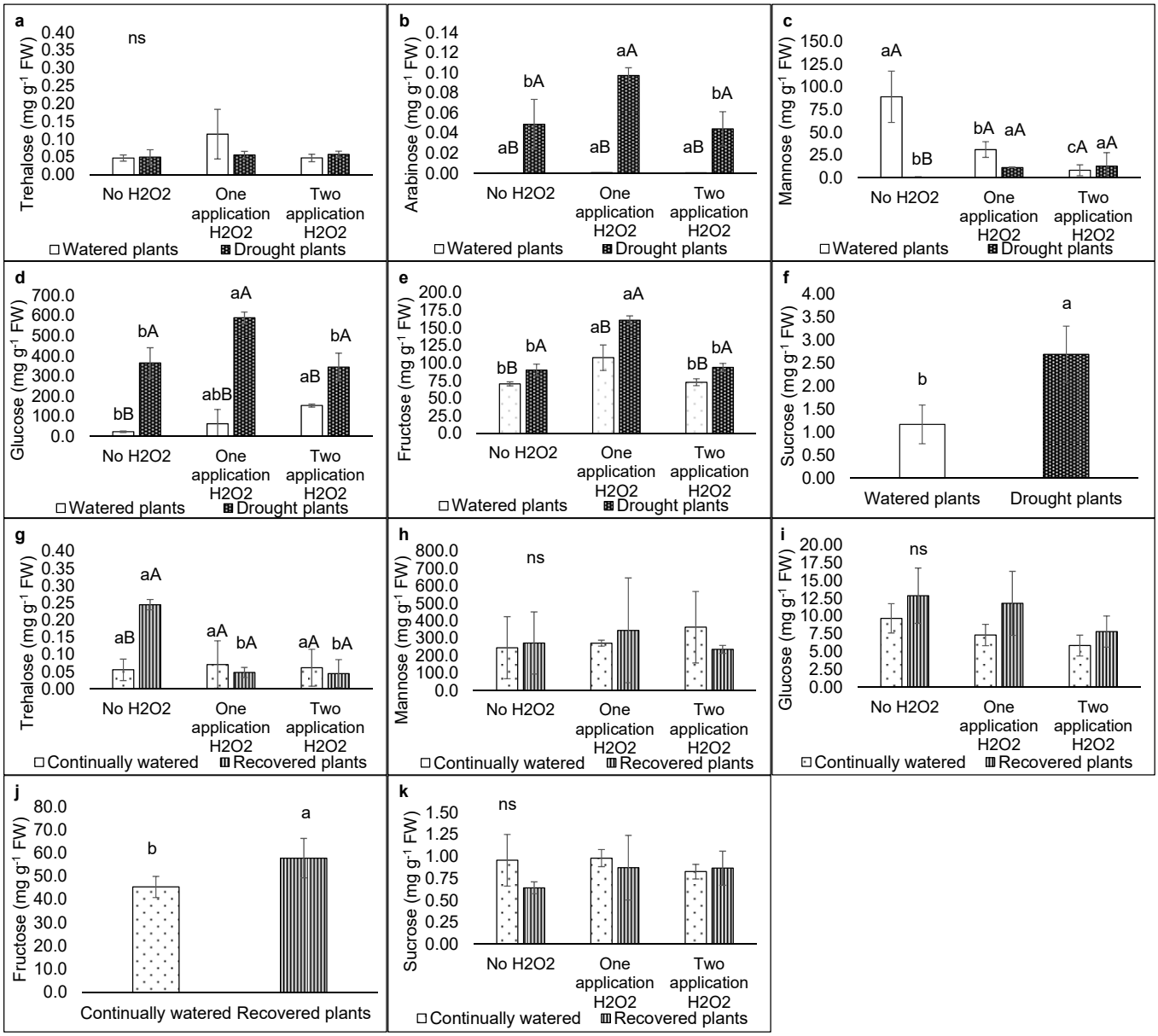

Figure 7. Soluble sugars leaf concentrations. (**a**) Trehalose, (**b**) arabinose, (**c**) mannose, (**d**) glucose, (**e**) fructose, and (**f**) sucrose in tomato plants grown under optimal water supply (watered) or subjected to drought and no, one, or two foliar applications of H_2O_2. After recovery due to irrigation return, (**g**) trehalose, (**h**) mannose, (**i**) glucose, (**j**) fructose, and (**k**) sucrose. Values correspond to the mean ± confidence interval (n = 4). Different letters differentiate the means using the Tukey test (<0.05), lowercase letters compare H_2O_2 application within each water condition, and uppercase letters compare water condition within H_2O_2 application.

3.8. Heatmap

Superior similarity was observed between plants that did not receive H_2O_2 application and plants that received two H_2O_2 applications during the water restriction period (Figure 8a). After the return of irrigation, the variables analysed showed greater similarity between recovered plants that received two applications of H_2O_2 and continually watered plants that did not receive or that received two applications of H_2O_2 (Figure 8b).

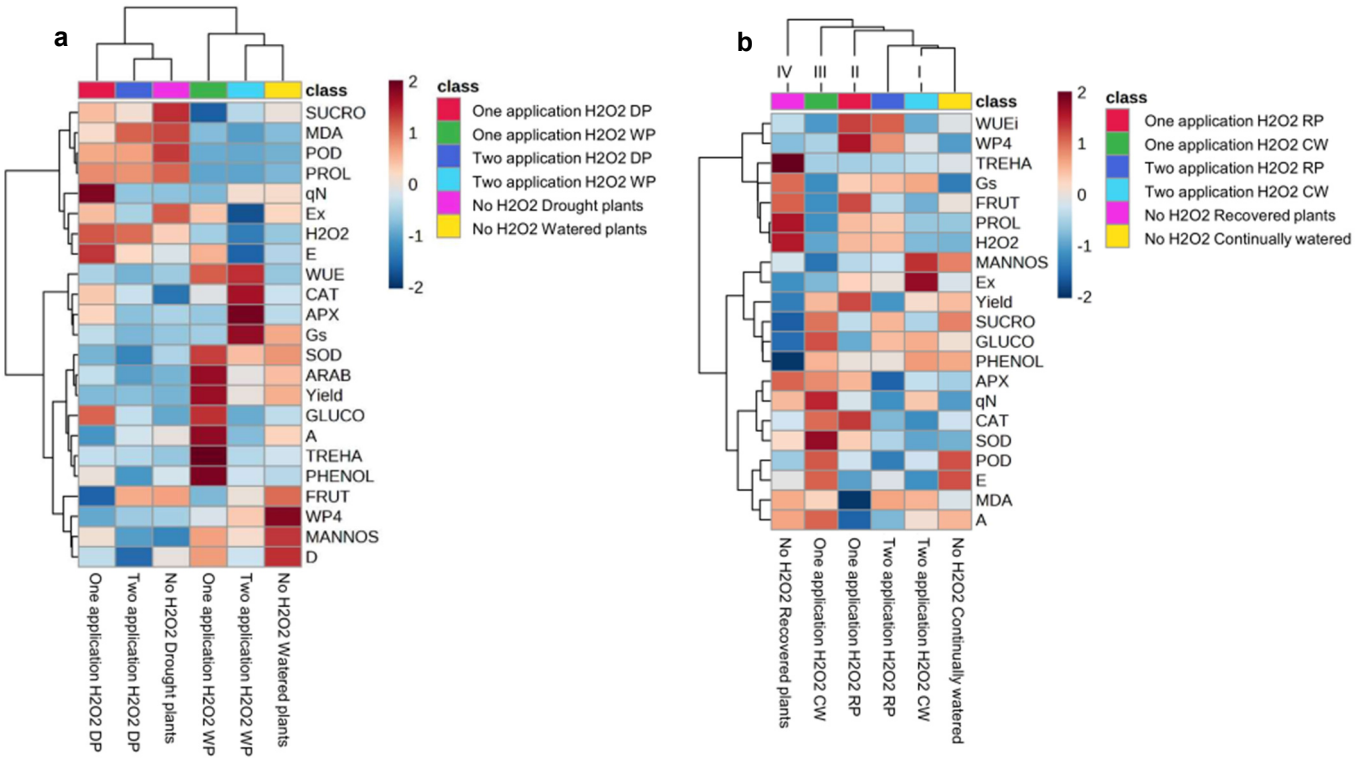

Figure 8. Heatmap. (**a**) During the drought period and (**b**) after recovery by the return of irrigation. Water potential of leaf (WP4), effective quantum yield of photosystem II (Yield), non-photochemical quenching coefficient of fluorescence (qN), heat dissipation in the antenna complex (*D*), energy not dissipated and not used in the photochemical phase (*Ex*), transpiration rate (*E*), stomatal conductance (*Gs*), net CO_2 assimilation (*A*), instantaneous water use efficiency (WUE), leaf lipid peroxidation (MDA), leaf concentration of hydrogen peroxide (H_2O_2), activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), leaf concentration of total phenols (PHENOL), proline (PROL), trehalose (TREHA), arabinose (ARAB), mannose (MANNOS), glucose (GLUCO), fructose (FRUT), and sucrose (SUCRO) in tomato plants grown under optimal water supply (watered—WP/continually watered—CW) or subjected to drought (DP)/recovery (RP) and no, one, or two foliar applications of H_2O_2.

4. Discussion

4.1. All the H_2O_2 Treatments Had No Effect on the Leaves' Potential Water Content

Plants with low water availability often respond to lower water potential in the leaves by accumulating solutes to maintain cell turgor and safeguard subcellular structures, as seen in this work (Figure 1a). Under drought conditions, plants synthesize soluble sugars and amino acids like proline, which are among the most osmotically active substances [27,28]. H_2O_2 treatment had no effect on the potential water of the leaves during water restriction or when irrigation was restored, but it did alter the accumulation of proline and the profile of soluble sugars in tomatoes subjected to drought (Figures 6b and 7).

4.2. One H_2O_2 Application Favoured the Photochemical Phase, Resulting in Higher ΦPSII and Decreased qN, D, and Ex

Drought decreased Φ_{PSII} levels in tomato plants (Figure 2a), supporting a prior study by Sperdouli and Moustakas [29]. Drought stress in plants changes the direction of energy harvested by photosystems, resulting in increased heat dissipation (*D*) to prevent excessive ROS production [30]. This occurs primarily because of the reduction in CO_2 availability caused by stomatal closure, which is required to reduce water loss through transpiration, increasing photorespiration and the requirement to extinguish excess energy that is not incorporated into organic molecules [31]. Plants treated with H_2O_2 demonstrated higher

Φ_{PSII} (Figure 2b), suggesting enhanced efficiency in converting light energy into reducing agents [32,33]. This is further corroborated by a drop in qN and *D* in watered plants and an increase in *Ex* in drought plants (Figure 2c–e), suggesting that a single application of H_2O_2 promotes photochemical processes at the expense of non-photochemical processes.

After irrigation return, recovered plants had similar Φ_{PSII} to those that were continually watered (Figure 2f), with no difference seen with H_2O_2 application. This indicates that the photoinhibition seen during water limitation was reversed. The decreased qN in recovered plants that received a single H_2O_2 application, as well as similar results in *D* and *Ex* in continually watered and recovered plants (Figure 2g–i), supports this hypothesis.

4.3. H_2O_2 Applications Increased the A and WUEi of Plants That Were Continually Watered

In watered plants, two H_2O_2 sprays lowered *Gs* and *E* levels. However, no decrease in *A* is seen (Figure 3a–c). Exogenous H_2O_2 can signal and modulate stomatal movements, as demonstrated by Jamaludin et al. [34]. According to Neill et al. [35], H_2O_2 plays a role in the pathway that promotes stomatal closure during abiotic stress by interacting predominantly with abscisic acid (ABA) and nitric oxide (NO). In response to water deprivation, ABA signalling drives the generation of H_2O_2 by NADPH oxidase, which leads to the production of NO by nitrate reductase and other enzymes like nitric oxide synthase, and the subsequent accumulation of NO in guard cells, signalling stomatal closure. As a result, two H_2O_2 applications may have caused a drop in *Gs* in the watered plants (Figure 3b).

Plants that received one H_2O_2 application showed greater *A* than those that received two H_2O_2 applications, which might be attributed to the latter's lower *Gs* (Figure 3b,c). Previous research has confirmed that exogenous H_2O_2 stimulates photosynthesis [34,36]. These findings can be explained by a set of evidence on the action of H_2O_2 in various stages of the photosynthetic process, such as in the increase in the initial activity of the RuBisCO enzyme, due to altering its activation state [37], by the upregulation of the enzyme RuBisCO in *Ficus deltoideae*, due to greater expression of the rbcL gene, of plastid DNA origin, which encodes RuBisCO subunits [34], or by increased activity of key enzymes in the Calvin–Benson cycle and sugar metabolism, such as sucrose phosphate synthase, which had its gene expression increased due to treatment with H_2O_2 in rice seedlings [36,38].

After irrigation, plants that were continually watered and had any H_2O_2 application exhibited lower *E*, whereas only those that received two H_2O_2 applications showed lower *Gs* (Figure 3e,f). Because there was no change in *A* rates, plants that received H_2O_2 showed increased WUE_i (Figure 3g,h). We note that among plants that did not receive H_2O_2 application, recovered plants had greater WUE_i than continually watered plants. Because WUEi values were equal between continually watered and recovered plants that received any H_2O_2 application, the current investigation showed the benefits of H_2O_2 application for better water resource utilization in plants grown under a regular water regime (continually watered plants).

4.4. Drought Plants with H_2O_2 Applications Showed Reduced Leaf Concentrations of MDA and H_2O_2

The increase in lipid peroxidation in drought-affected plants is demonstrated by the increase of MDA concentration, which was seen in our study (Figure 4a), and is a direct outcome of increased ROS formation, which causes oxidative damage to biological membranes. As previously noted, drought plants have surplus energy in their photosystems due to a reduction in CO_2 availability, which raises the need to employ alternate channels to extinguish energy, such as photorespiration. According to Foyer et al. [39], photorespiration produces a substantial quantity of H_2O_2 in photosynthetic tissues, and in addition other possibly more harmful ROS are generated spontaneously during electron flow in photosystems, such as singlet oxygen, superoxide, and hydroxyl radicals [40]. Although the drought enhanced ROS such as H_2O_2, it was shown that plants that received a single application of H_2O_2 had a smaller impact on membrane peroxidation, with less MDA concentration in leaf tissue (Figure 4a–c). Previous studies using exogenous H_2O_2 in drought-stressed

plants yielded similar results [8,41], demonstrating that it has signalling potential for plant acclimation and stress damage.

4.5. Drought Plants Showed Increased APX Activity after Receiving One Application of H_2O_2

The antioxidant enzymes SOD, CAT, and POD were more active in drought plants that did not receive H_2O_2, which, related to the greater accumulation of MDA observed in these plants, showed that the enzymes were not efficient in controlling oxidative damage (Figures 4a and 5a–c). Similarly, higher APX activity in drought plants that received one H_2O_2 spray may have cleared ROS, resulting in decreased MDA and H_2O_2 concentrations (Figures 4a,c and 5d). According to Miyake [40], APX is necessary for managing the alternate electron drain known as the water–water cycle, as well as the cyclic electron flow around photosystem I (FSI), which helps regulate electron transport and minimize photoinhibition. APX action occurs on SOD-produced H_2O_2, resulting in water and lowering ROS concentration in the photosystem [40]. The increased APX activity relative to CAT activity may indicate that the majority of H_2O_2 synthesis occurred in chloroplasts, where two of the five APX enzyme isoforms, located in the stroma and thylakoid membranes, act [42].

Following irrigation return, enhanced CAT activity was identified in continually watered or recovered plants that received one H_2O_2 application (Figure 5f), but only continually watered plants that had one H_2O_2 spray showed higher APX activity (Figure 5h). This might be linked to enhanced SOD activity reported in continually watered plants (Figure 5e), demanding a greater regulating effect of APX on H_2O_2. Plants that received H_2O_2 too showed higher mannose concentrations, which may have contributed to the synthesis of ascorbate, an electron donor for H_2O_2 reduction in water via APX action. Higher APX activity may suggest resilience to water stress, since increased tolerance to oxidative stress was seen in *Populus tomentosa* with high enzyme activity [43].

4.6. Two H_2O_2 Applications on Drought Plants Resulted in Higher Total Phenol Concentrations and Decreased Proline Levels

Despite the lowered activity of antioxidant enzymes in drought plants treated with two H_2O_2 sprays, total phenolic compound accumulation increased (Figure 6a), possibly acting in ROS removal and MDA regulation. Sánchez-Rodríguez et al. [9] found that stress, such as water restriction, promotes the production of phenolic chemicals. Due to their redox properties, these chemicals detoxify ROS while also acting as reducing agents and hydrogen donors. In their study of cherry tomato cultivars grown in drought conditions, researchers discovered an increase in phenolic compounds in the Zarina cultivar, which is resistant to water deprivation and accumulates less MDA and H_2O_2 under stress.

Exogenous H_2O_2 supply resulted in an increase in phenolic compounds in other species, including *F. Deltoideae* [34], suggesting that this ROS signal influences gene expression of phenolic compound precursor molecules. Previous research has shown that metabolism responds to environmental changes by signalling through sugar or ROS molecules, inducing the production of microRNAs such as miR858, which acts on transcript factors, MYB, which plays a critical regulatory role in phenylpropanoid biosynthesis, phenolic compound precursors, under stress conditions [44]. In the current investigation, only recovered plants that received H_2O_2 application had phenolic component concentrations comparable to continually watered plants (Figure 6c), indicating successful signalling for these plants.

Drought plants displayed higher proline accumulation compared to watered plants (Figure 6b). A significant rise in proline concentration in plants subjected to abiotic stressors is a predicted metabolic response, and it may account for more than 80% of free amino acids discovered in plant tissues, compared to 5% under normal conditions. According to Spormann et al. [45], higher proline accumulation in stressed plants is frequently reported as an indication of plant tolerance. However, this interpretation may be incorrect, since this buildup may be an indication or reaction generated by fight stress need, as under normal settings, the synthesis of this amino acid is controlled by negative feedback. Proline is

engaged in subcellular structure stabilization, ROS removal, buffering the redox potential of cells, and acting on water-deficit plants, particularly osmotic adjustment in combination with other suitable solutes such as soluble sugars [27,29]. In this study, we discovered that, while drought plants with H_2O_2 application had lower proline concentrations, a change in sugar profile could explain the maintenance of the leaves' water potential in comparison to plants that did not receive H_2O_2. After irrigation, only recovered plants that received two H_2O_2 sprays had lower proline concentrations (Figure 6d), suggesting that H_2O_2 signalling enables faster drought recovery. Proline catabolism occurs in mitochondria and is associated with oxidative respiration and power production. Thus, the product may be leveraged to increase growth return [27].

4.7. One Application of H_2O_2 to Drought Plants Resulted in Increased Quantities of Arabinose, Mannose, Glucose, and Fructose

H_2O_2 application changed the chemical composition of soluble sugars in drought plants and continually watered plants (Figure 7). Drought plants that received a single H_2O_2 application may have benefited from increased concentrations of glucose reductive sugars and fructose, which act in cellular osmotic balance, as well as arabinose and mannose, which are important cell wall components [11], as well as other signalling molecules such as flavonoids and peptides [46].

Živanović et al. [11] investigated how water shortage affects tomato plants with genotype savage subtype and mutant flacca, which do not release ABA and hence do not seal their stomata during drought circumstances. They observed that drought-tolerant mutant plants accumulate more soluble sugars in their leaves, particularly glucose, due to increased acid invertase enzyme activity and reduced sucrose transfer to sink regions. This state might have happened in plants that received a single H_2O_2 spray (Figure 7d). Two H_2O_2 sprays also raised mannose concentrations in drought plants (Figure 7c).

Other researchers discovered that the buildup of this sugar in mutant plants, which is increased by GMP gene mutation that catalyses mannose production, can be exploited as a technique to promote plant tolerance to osmotic stress [47]. Mannose in plants may be involved in the formation of ascorbic acid, a non-enzyme antioxidant capable of inhibiting ROS detoxification [48]. As seen in this study, drought plants that given one H_2O_2 application had decreased MDA and H_2O_2 accumulation (Figure 4a,c). It implies a contribution to mannose buildup in defensive chemical production.

Watered plants that did not receive H_2O_2 application had greater mannose concentrations than drought plants, which might be attributed to reduced reducing sugar content (Figure 7c–e). Watered plants treated with H_2O_2 had a lower mannose content than drought plants. This might be attributed to decreased H_2O_2 leaf concentrations, which may have reduced signalling to the buildup of this sugar, even boosting its integration into cell walls forming hemicellulose polymers, as emphasized by Gilbert et al. [49].

Trehalose content increased in recovered plants that did not receive H_2O_2 application after irrigation return (Figure 7g), as did fructose concentration in recovered plants compared to continually watered plants (Figure 7j). Our drought-stressed tomato plants did not accumulate trehalose during the stress period, unlike wild subtype tomato plants [11]. This occurred after irrigation return on recovered plants that did not receive H_2O_2. According to the researchers, trehalose modulates starch breakdown through trehalose-6-phosphate, allowing for more soluble sugar buildup. Trehalose's other documented activities in water-deficit plants include high water retention capacity, power supply for metabolic processes, and molecular signalling for defensive chemicals [48]. Thus, increased concentrations of trehalose detection in recovered plants that did not receive H_2O_2 application may indicate that these plants were still encouraging metabolic changes to overcome stress overall, even if MDA levels were comparable to continually watered plants (Figure 4d).

4.8. Plants That Did Not Receive H_2O_2 Application Exhibited More Similarity with Plants That Received Two H_2O_2 Applications throughout the Water Limitation Period: Irrigation-Return-Recovered Plants That Received Two H_2O_2 Applications and Revealed Greater Similarity with Continually Watered Plants That Did Not Receive or Received Two H_2O_2 Applications

During the water restriction phase, the heat map separated into two different clusters (Figure 8a), demonstrating a high degree of similarity in responses between plants watered as opposed to plants exposed to drought independently of H_2O_2 application. These two primary clusters were differentiated by combining plants that did not receive or received two H_2O_2 applications, allowing us to conclude that a single H_2O_2 application changes tomato metabolism in a more pronounced way. It is worth noting that, among watered plants, a negative trend (blue squares) was seen for sucrose in plants that received H_2O_2, particularly one H_2O_2 application, indicating a large export of this sugar for sink-tissue in development, as indicated by Xu et al. [50]. The positive trend (red squares) in *A* and Φ_{PSII} supports this idea. Although the plants that received two H_2O_2 applications did not exhibit significance for *A* and Φ_{PSII}, it showed for WUE_i, suggesting greater water resource use. Watered plants that did not receive H_2O_2 application, on the other hand, showed a positive trend for *D*, indicating a requirement for photoprotection, with dissipation of a percentage energy collected by antenna complexes [30]. Among drought plants, those that did not receive H_2O_2 application showed a positive tendency for sucrose. According to Živanović et al. [11], this contributes to osmotic adjustment but implies sugar retention at the sources and less exportation to sinks. *Ex* also revealed a positive trend in these plants, which might have contributed to additional energy accumulating in photosystems, potentially causing damage [51], which was verified by a positive trend in MDA.

After irrigation return (Figure 8b), the continually watered plants that did not receive or received two H_2O_2 applications, as well as the recovered plants that received two H_2O_2 applications, exhibited strong similarities and formed a cluster (I). The most distinguishable cluster was formed by recovered plants that had not received H_2O_2 application (IV). The remaining two clusters (II and III) lie situated between clusters I and IV. Cluster II was approximated to cluster I based on the positive trend in WUE_i, LWP, *Gs*, proline, and H_2O_2 seen in the H_2O_2-applicated recovered plants. Cluster IV, which did not receive H_2O_2 application, showed a positive trend for trehalose and proline, which are generally observed in plants under stress, for osmotic adjustment and signalling for defence compounds [45,48], and H_2O_2, which may have signalled these substances. Cluster III, with continually watered plants that received a single H_2O_2 application, showed a positive trend in *A*, *E*, Φ_{PSII}, antioxidant enzymes, and sucrose. The consistency of the findings obtained with two H_2O_2 applications in recovered and continually watered plants implies that ROS-induced signalling resulted in acquired systemic acclimation.

5. Conclusions

We anticipate that H_2O_2 foliar application can aid in the development of options, such as acquired systemic acclimation, to deal with unexpected dry spells, including new challenges induced by climate change. This is because drought plants that received H_2O_2 application showed less oxidative damage under water restriction, with metabolic changes indicating the production of plant defence molecules such as the APX enzyme, phenols, arabinose, and mannose. Plants that were not subjected to drought and received H_2O_2 application exhibited no losses, including better photochemical performance and water consumption efficiency, allowing them to make the most of available light and water. We hope that our findings will help to advance and spark further research on ROS signalling activation in plant metabolism, resulting in greater output and adaptation to severe environments.

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