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Hydrogen Peroxide Improves the Antioxidant Defence System in Salt Stressed-*Allium cepa* Plants

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Abstract: As one of the active oxygen species that is widely generated in many biological systems and mediates various physiological and biochemical processes in plants, exogenous hydrogen peroxide (H_2O_2) in very low concentrations improves salt-tolerance in some plant species. Therefore, two field experiments were conducted in 2013/14 and 2014/15 to study the effect of foliar sprays at concentrations of 1 and 2 mM H_2O_2 on growth, yield, plant water relations, osmoprotectants and the activity of antioxidant system in two onion varieties grown under saline soil condition (EC_e = 7.94 - 8.81 dS/m). Exogenous H_2O_2 enhanced salt stress tolerance in onion plants by reducing the endogenous H_2O_2 and lipid peroxidation, and increasing enzymatic (i.e., superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) and non-enzymatic antioxidant (i.e., ascorbic acid and glutathione) activity. Moreover, H_2O_2 application significantly affected photosynthetic efficiency and plant water status as evaluated by relative water content and membrane stability index. These results were positively reflected by the increase in plant growth, productivity and water use efficiency under salt stress conditions. H_2O_2 may participate in enzymatic and non-enzymatic antioxidant activity, inducing salt-tolerance in onion plants.

Keywords: Antioxidants, Productivity; H_2O_2, Onion, Photosynthetic Performance, Salinity

1. Introduction

Salt stress is considered one of the major limiting factors to plant growth and crop production in many areas, particularly in arid and semi-arid regions. Salt stress negatively affects plant morphology and physiology through osmotic and ionic stress, and changes biochemical responses in plants [1]. It causes osmotic stress (a physiological drought problem), adversely affecting water relations and ion homeostasis in plants, leading to toxic-ion effects on metabolic processes [2]. This, in turn, leads to an excessive generation of reactive oxygen species (ROS), which causes damage to lipids, proteins and DNA [3]. Negative effects generated under salt stress are usually caused by elevated Na^+ and Cl^- concentrations in soil or irrigation water. Salt stress decreases photosynthetic attributes [4], plant growth and development [5], and stimulates the activity of antioxidant system [6, 7]. To overcome these salt stress effects, plants develop several mechanisms to induce their tolerance such as ion homeostasis, osmotic adjustments, stress damage control and repair, and growth regulation [8].

Onion (*Allium cepa* L.) is one of the most commercially valuable vegetables grown worldwide. The average annual production in the last five years in Egypt is put at 2,113,749 tons [9]. Onions are rated as a salt sensitive crop, bulb yield severely declines for every unit increase in soil salinity (ECe) [10].

In recent years, a growing interest has been observed with some ROS in very low concentrations to support plant growth in stress conditions, including hydrogen peroxide (H_2O_2). H_2O_2 is a signaling molecule in plants [11] and acts as a second messenger in response to various stresses, including salt stress in plants [12, 13]. It acts as a key regulator in a wide range of physiological processes such as photosynthesis, growth, and development [14, 15]. Recent works show that exogenous application of H_2O_2, at low concentrations, significantly improved the antioxidant defense system in salt-stressed plants [13, 16]. It plays a dual role in plants under both normal and stress conditions, and at high concentration it initiates programmed cell death [17]. However, at low concentrations H_2O_2 acts as a signal molecule and is involved in acclamatory signaling triggering tolerance against salt stress [13, 18].

The objective of this study was to evaluate the potential of foliar H_2O_2 application to alleviation of salt stress in onion plants and study their growth, yield, photosynthetic pigments, osmoprotectants, water relations, membrane stability and antioxidant system in the presence of both salinity and H_2O_2 applications. The hypothesis tested is that H_2O_2 will enhance the activity of the antioxidant system that will protect the stress generated by soil salinity. In addition, H_2O_2 will help in enhancing onion growth and production under the adverse effects of soil salinity.

2. Materials and Methods

2.1. Plant Material, Experimental Design and Treatments

Two field experiments were conducted in 2013/14 and 2014/15 at a private farm in Sennoris District, Fayoum, Southwest Cairo, Egypt between latitudes 29° 02′ and 29° 35′ N and longitudes 30° 23′ and 31° 05′ E. The climatic data of studied area indicate that the total rainfalls does not exceed 7.5 mm/year and the mean minimum and maximum annual temperatures are 14.5 and 31.0°C in January and June, respectively. The evaporation rates coincide with temperatures where the lowest evaporation rate (1.9 mm/day) was recorded in January while the highest value (7.3 mm/day^{-}) was recorded in June. According to the aridity index [19]; the area is located under hyper-arid climatic condition. These landforms are characterized by less than 3.5% surface slopes with an elevation vary from 49 m below sea level to 26 m above sea level.

Healthy seeds of two varieties [i.e., Giza 20 and Giza Red] of onion (*Allium cepa* L.) were sown on 30 and 25 September 2013 and 2014 respectively. The produced transplants were transported and replanted on 7 December and harvested on 6 May for both seasons. Total surface area used for the experiment was 550 m^2 divided into 20 experimental plots of 16.5 m^2 each (1.1 m wide × 15 m long). These plots included eight planting rows placed 10 cm apart with a distance of 15 cm between plants. These plant densities are a typical of onion production in Egypt. The soil was fertilized with NPK fertilizer according to the recommendations of the Ministry of Agriculture and Land Reclamation (450 kg ha^{-1} ammonium nitrate (33.5% N), 400 kg ha^{-1} calcium super-phosphate (15.5% P_2O_5), and 150 kg ha^{-1} potassium sulphate (48% K_2O)).

Onion plants were irrigated in 2-d interval using the amounts of applied water as 100% in a drip irrigation method. The daily ETo was calculated from weather data according to the following equation of FAO-PM [20]:

$$ETo = \frac{0.408\ \Delta\ (R_n - G) + \gamma \frac{900}{T_{mean} + 273} u_2\ (e_s - e_a)}{\Delta + \gamma (1 + 0.34\ u_2)}$$

Where: *ETo* is the reference evapotranspiration (mm day^{-1}), Δ is the slope of the saturation vapor pressure curve at air temperature (kPa C^{-1}), R_n is the net radiation at the crop surface (MJm^{-2} d^{-1}), G is the soil heat flux density (MJm^{-2} d^{-1}), γ is the psychometric constant = (0.665 × 10^{-3} × P), kPa C^{-1} [20], P is the atmospheric pressure (kPa), U_2 is the wind speed at 2 m height (m s^{-1}), e_s is the saturation vapor pressure (kPa), e_a actual vapor pressure (kPa) ($e_s - e_a$) is the saturation vapor pressure deficit (kPa), and T_{mean} is the mean daily air temperature at 2 m height (°C).

Average daily *ETo* in Fayoum region was estimated using the monthly mean weather data for a 15-year period (January 1998 – December 2012) of Etsa station. The average daily *ETo* was 2.17, 1.64, 2.29, 3.35 and 5.02 ETo mm/day in December, January, February, March and April, respectively.

Crop water requirements (*ETc*) were estimated using the crop coefficient according to equation:

$$\mathrm{ETc} = \mathrm{ETo} \times \mathrm{Kc}$$

Where: *ETc* is the crop water requirement (mm day^{-1}) and *Kc* is the crop coefficient.

The lengths of the different crop growth stages were 15, 25, 70, and 40 days for initial, crop development, mid-season and late season stages, respectively. The crop coefficients (*Kc*) of initial, mid and end stages were 0.7, 1.05 and 0.75, respectively [20].

The amount of irrigation water applied was calculated according to the following equation:

$$\mathrm{IWA} = \frac{\mathrm{A} \times \mathrm{ETc} \times \mathrm{Ii} \times \mathrm{Kr}}{\mathrm{Ea} \times 1000 \times (1 - \mathrm{LR})}$$

Where IWA is the irrigation water applied (m^3), A is the plot area (m^2), ETc is the crop water requirements (mm day^{-1}), Ii is the irrigation intervals (day), Kr is the covering factor, Ea is the application efficiency (%) (Ea = 85), and LR is the leaching requirements.

All other recommended agricultural, disease and pest management practices were followed as recommended by the Egyptian Ministry of Agriculture and Land Reclamation.

Water and soil analyses were carried out according to [21] and [22] and the data are shown in Tables (1 – 3). Based on the EC values shown in Table (3), the soil is classed as being strongly saline according to [23]. In addition, according to [24], scale the used irrigation water lies within the second

categories for salinity and sodicity levels (C_2S_1, ECiw = 0.75 – 3.00 dS/m and SAR < 6.0). The experiments were arranged in a randomized complete block design, with 3 levels of H_2O_2 (0, 1.0 and 2.0 mM), and three replicate plots.

Twenty days after transplanting (DAS), onion seedlings in each plot were sprayed to run-off with 0 (tap water as a control), 1.0 and 2.0 mM H_2O_2, and then the sprays were repeated at 40 and 60 DAS. The concentrations of H_2O_2, and the number and timing of sprays were based on results from a preliminary pot trial (data not shown). To ensure optimal penetration into leaf tissues, 0.1% (v/v) Tween-20 was added to the foliar sprays as a surfactant.

Table 1. *Chemical composition of irrigation water.*

Ionic concentration (Meq/l)								EC[a] (dS/m)	pH	SAR[b]
CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}	Ca^{++}	Mg^{2+}	Na^+	K^+			
2013/14										
0.00	4.35	16.73	6.82	7.34	6.84	12.4	1.32	2.67	7.46	5.38
2014/15										
0.00	4.21	15.32	6.41	6.42	5.39	12.71	1.42	2.53	7.41	7.40

[a]EC means the average electrical conductivity, [b]SAR means sodium adsorption ratio.

Table 2. *Some initial physical properties of the experimental soil.*

Layer (m)	Particle size distribution				Bulk density	K_{sat}	FC	WP	AW
	Sand %	Silt %	Clay %	Texture class	g/cm^3	m/h	(%)	(%)	(%)
2013/14									
0 – 0.3	79.2	10. 0	10.8	LS	1.60	0.02	25.3	9.7	15.6
0.3 – 0.6	77.2	10.1	10.7	LS	1.55	0.015	22.2	12.1	10.1
2014/15									
0 – 0.3	78.3	9.8	10.6	LS	1.67	0.025	24.5	9.7	15.6
0.3 – 0.6	77.8	11.7	11.9	LS	1.61	0.015	22.1	11.2	13.3

F.C=Field capacity, W.P = wilting point, A.W= Available water, LS= loamy sand, and K_{sat}=Hydraulic conductivity.

Table 3. *Some initial chemical properties of the experimental soil.*

Properties	value	
	2013/14	2014/15
pH [at a soil: water(w/v) ratio of 1:2.5]	7.75	7.86
ECe (dS/m; soil – paste extract)	7.94	8.81
CEC($cmol_c$ /kg)	11.15	11.10
$CaCO_3$ (%)	4.83	4.81
Organic matter (%)	1.20	1.10
ESP(exchangeable sodium percentage)	13.46	14.62
Soluble ions:		
Ca^{2+} (meq/l)	21.31	25.32
Mg^{2+} (meq/l)	20.14	22.14
Na^+ (meq/l)	37.12	40.32
K^+ (meq/l)	1.23	1.34
CO_3^{2-} (meq/l)	0.00	0.00
HCO_3^- (meq/l)	3.40	4.38
Cl^- (meq/l)	39.21	44.25
$SO4^{2-}$ (meq/l)	37.19	40.49
Exchangeable cations:		
Ca^{2+} (meq/100g soil)	5.16	5.11
Mg^{2+} (meq/100g soil)	2.83	2.78
Na^+ (meq/100g soil)	1.64	1.61
K^+ (meq/100g soil)	1.56	1.51
Available nutrients:		
N (%)	0.005	0.004
P (mg/kg soil)	530.20	523.80
K (mg/kg soil)	71.20	69.90
Fe (mg/kg soil)	3.60	3.40
Mn (mg/kg soil)	10.64	10.60
Zn (mg/kg soil)	0.72	0.70
Cu (mg/kg soil)	0.53	0.50

2.2. Measurements of Growth, Yield and Water Use Efficiency (WUE)

Ninety-day-old onion plants were carefully removed from each experimental plot, and the shoots of plants were weighed to record their fresh weights, and then placed in an oven at 70ºC till the constant weight to record their dry weights. At the end of experiments, all onion plants in each experimental plot were removed to estimate the total bulb yield.

WUE values as kg yield per m^3 of applied water were calculated for different treatments after harvest according to the following equation [25]:

$$\text{WUE} = \frac{\text{Bulb yield (Kg ha}^{-1})}{\text{water applied (m}^3\text{ ha}^{-1})}$$

2.3. Assessment of Chlorophyll Fluorescence

On two different sunny days, chlorophyll fluorescence was measured using a portable fluorometer (Handy PEA, Hansatech Instruments Ltd, Kings Lynn, UK). One fully expanded mature leaf was chosen per plant to conduct the fluorescence measurements. Maximum quantum yield of PS II F_v/F_m was calculated using the formulae; $F_v/F_m = (F_m - F_0) / F_m$ [26]. F_v/F_0 reflects the efficiency of electron donation to the PSII RCs and the rate of photosynthetic quantum conversion at PSII RCs. F_v/F_0 was calculated using the formulae; $F_v/F_0 = (F_m - F_0) / F_0$ [27]. Performance index of photosynthesis based on equal absorption (PI_{ABS}) was calculated as reported by [28].

2.4. Measurement of Free Proline and Total Soluble Sugars (TSS)

Proline was extracted and determined (in mg per g of leaf DW) using the method described by [29]. A dried leaf sample

(0.5 g) of leaf tissue was ground in 10 ml of 3% (v/v) sulphosalicylic acid, and the mixture was then centrifuged at 10,000 × *g* for 10 min. Two-ml of freshly prepared acid-ninhydrin solution was added to 2 ml of the supernatant in a test-tube, and the tube was incubated in a water bath at 90ºC for 30 min. The reaction was terminated in an ice-bath, and the reaction mixture was then extracted with 5 ml of toluene and vortex-mixed for 15 s. The tube was allowed to stand for at least 20 min in the dark at room temperature to separate the toluene and aqueous phases, and the toluene phase was then collected carefully into a test tube. The absorbance of the toluene phase was read at 520 nm, and the concentration of proline was determined from a standard curve prepared using analytical grade proline and expressed as mg per g of leaf DW.

TSS were extracted and determined according to [30]. A dried leaf sample (0.2 g) was homogenized in 5 ml of 96% (v/v) ethanol, and then washed with 5 ml 70% (v/v) ethanol. The extract was centrifuged at 3500 × g for 10 min, and the supernatant was stored at 4°C prior to determination. The reaction mixture of 0.1 ml of the ethanolic extract and 3 ml of freshly-prepared anthrone reagent [150 mg anthrone plus 100 ml of 72% (v/v) sulphuric acid] was placed in a boiling water bath for 10 min, and was then cooled. The absorbance of the mixture was recorded at 625 nm using a Bauschand Lomb-2000 Spectronic Spectrophotometer.

2.5. Determination of Membrane Stability Index (MSI) and Relative Water Content (RWC)

The MSI was determined using duplicate 0.2 g samples of fully-expanded leaf tissue [6]. The leaf sample was placed in a test-tube containing 10 ml of double-distilled water. The content of the test-tube was heated at 40°C in a water bath for 30 min, and the electrical conductivity (C_1) of the solution was recorded using a conductivity bridge. A second sample was boiled at 100°C for 10 min, and the conductivity was measured (C_2). The MSI was calculated using the formula:

$$\mathrm{MSI}(\%) = \left[1 - \left(\frac{\mathrm{C1}}{\mathrm{C2}}\right)\right] \times 100$$

RWC was estimated using 2 cm-diameter fully-expanded leaf discs [31]. The discs were weighed (fresh mass; FM) and immediately floated on double-distilled water in Petri dishes for 24 h, in the dark, to saturate them with water. Any adhering water was blotted dry and the turgid mass (TM) was measured. The dry mass (DM) was recorded after dehydrating the discs at 70°C until the constant weight. The RWC was then calculated using the following formula:

$$\mathrm{RWC}(\%) = \left[\frac{(\mathrm{FM} - \mathrm{DM})}{(\mathrm{TM} - \mathrm{DM})}\right] \times 100$$

2.6. Determination of Total Ascorbic Acid (AsA) and Glutathione (GSH)

Extraction of AsA from leaf samples and its determination were conducted according to the method of [32]. Fresh leaf sample (1 g) was homogenized in liquid N_2 and extracted with 5% (w/v) TCA. At 4°C, the homogenate was centrifuged for 5 min at 15,600 × *g*. The supernatant was then transferred to a new reaction vessel and immediately assayed for the ascorbate concentration in a reaction mixture containing supernatant, 10 mM DTT, 0.2 M phosphate buffer (pH 7.4), 0.5% NEM, 10% TCA, 42% H_3PO_4, 4% 2,2′-dipyridyl and 3% $FeCl_3$.

The level of GSH was determined as described by the method of [33]. GSH was extracted from filtered FW in 2 volumes of extracting buffer (2% sulfosalicylic acid, 1 mM Na_2EDTA and 0.15% AsA) and homogenized. The homogenate was centrifuged at 12,000 × *g* for 5 min. An aliquot of supernatant was then used for the measurement of GSH in leaf samples by GSH assay kit (Sigma Chemical Co., USA).

2.7. Determination of Hydrogen Peroxide (H_2O_2)

H_2O_2 level was determined as the described method of [34] with a slight modification. The mixture of 2 ml of cold acetone extract of the sample, titanium reagent (0.2 ml of 20% titanic tetrachloride in concentrated HCl, v/v), 0.4 ml of NH_4OH to form hydroperoxide-titanium complex, was centrifuged at 12,000 ×g for 10 min. The precipitate was dissolved in 2 ml of 2 M H_2SO_4 and absorbance of the solution was measured at 415 nm against a reagent blank. Concentration of H_2O_2 (in µmol per g of fresh leaf) was determined using the standard curve plotted with known concentration of H_2O_2.

2.8. Determination of Lipid Peroxidation

By measuring the level of thiobarbituric acid reactive substance (TBARS), the level of lipid peroxidation was determined according to [35]. Fresh leaf samples were mixed with 1 ml of 10% TCA and 1 ml of 0.67% thiobarbituric acid (TBA), and heated in a boiling water bath for 15 min. TBARS was determined spectophotometrically by absorbance at 535 nm and expressed as nmol of MDA per g fresh leaf samples.

2.9. Determination of the Antioxidant Enzyme Activities

Antioxidant enzymes were extracted from 1 g of fresh onion leaves. The biomass was filtered and homogenized in liquid N_2 with 0.05 M phosphate buffer (pH 7.0) containing 0.1 M EDTA and 1% PVP at 4 °C. The leaf biomass: extraction buffer (w/v) proportion was 1:2, respectively. At 4°C, the homogenate was centrifuged for 10 min at 15,000 ×*g*. The supernatant was then dialyzed overnight in phosphate buffer. The activity determination of the selected enzymes was performed. In the homogenate, protein concentration was also determined according to [36].

The activity of superoxide dismutase (E.C. 1.15.1.1) was assayed according to the method of [37]. The ability of enzyme to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) and the change in absorbance was measured at 560 nm. The reaction mixture consisted of 25 mM phosphate buffer (pH 7.8), 65 µM NBT, 2 µM

riboflavin, enzyme extract, and TEMED and the reaction mixture was exposed to light of 350 µmol m^{-2} s^{-1} for 15 min. The activity of enzyme was expressed as nmol of H_2O_2 per mg of soluble protein per min.

The activity of catalase (EC 1.11.1.6) was determined as outlined in the method of [38]. The rate of H_2O_2 decomposition at 240 nm was measured spectrophotometrically and calculated using a molar extension coefficient ε = 45.2 mM^{-1} cm^{-1}. The reaction mixture consisted of phosphate buffer, 0.1 mM H_2O_2 and enzyme extract. One unit of catalase activity was presumed as the amount of enzyme that decomposed 1 nmol of H_2O_2 per mg of soluble protein per min at 30 °C.

The activity of total ascorbate peroxidase (APX; EC 1.11.1.11) was determined using the method described by [39]. The reaction mixture consisted of phosphate buffer, 5 mM sodium ascorbate, 0.1 mM H_2O_2 and enzyme extract. The activity of APX was determined as a reduction in the absorbance of ascorbate at 290 nm and calculated using a molar extension coefficient ε = 2.8 mM^{-1} cm^{-1}. The activity of enzyme was calculated as the amount of the enzyme that oxidized 1 nmol of ascorbate consumed per mg of soluble protein per min at 30°C. The activity of enzyme was expressed as nmol ascorbate oxidized per mg of soluble protein per min.

The activity of glutathione reductase (EC 1.6.4.2) was determined according to [40]. The reaction mixture consisted of 10 mM GSSG, 1 mM Na_2EDTA, 200 mM phosphate buffer and enzyme extract was pre-incubated at 25°C for 5 min. The reaction was initiated by the addition of 1 mM NADPH and the rate of oxidation of NADPH was measured spectrophotometrically at 340 nm. The activity of enzyme was expressed as nmol NADPH oxidized per mg of soluble protein per min.

2.10. Statistical Analysis

The statistical analysis of the experimental data was carried out using ANOVA procedures in GenStat statistical package (version 11) (VSN International Ltd, Oxford, UK). Combined analysis of data of the two seasons was conducted and difference between means was compared using least significant difference test (LSD) at 5% level ($p \leq 0.05$).

3. Results

3.1. Growth of Onion Plants as Affected by H_2O_2 and Saline Soil Conditions

Significant reduction (P < 0.05) was observed in growth in terms of shoot fresh weight (FW) and shoot dry weight (DW)] of the two varieties of onion plants under salt stress condition. However, exogenous H_2O_2 remarkably promoted onion plants growth under salt stress (Table 4). The level of 1 mM H_2O_2 was found to be more effective, increasing shoot FW and shoot DW by 44.6 and 65.2%, and 46.7 and 68.7% in the Giza Red and Giza 20 varieties, respectively compared to their controls. The data also indicate that the Giza 20 variety was more sensitive to soil salinity, while it was more responsive to the foliar application with 1 mM H_2O_2.

Table 4. *Effects of spray treatments with H_2O_2 (mM) on growth (fresh and dry weights) of two varieties of onion plants grown on a saline soil.*

Treatment		Shoot fresh weight/plant (g)	Shoot dry weight/ plant (g)
Variety	H_2O_2		
Giza Red	0	72.6 ± 4.3c	7.5 ± 0.4b
	1	105.0 ± 5.8ab	11.0 ± 0.6a
	2	100.7 ± 5.5b	10.6 ± 0.5a
Giza 20	0	64.3 ± 1.5d	6.7 ± 0.2c
	1	106.2 ± 5.7ab	11.3 ± 0.7a
	2	114.3 ± 6.4a	10.6 ± 0.6a

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

3.2. Total Bulb Yield and Water Use Efficiency (WUE) of Onion Plants as Affected by H_2O_2 and Saline Soil Conditions

Table 5 shows the weight total bulb yield and WUE of the two varieties under saline soil conditions (EC_e = 7.94 - 8.81). Total bulb yield and WUE of the two varieties of onion plants were significantly increased by the foliar application of H_2O_2 compared to untreated control plants. The applied level of 1 mM H_2O_2 was noticed to be more effective, significantly increasing total yield and WUE by 74.9 and 30.7%, and 75.2 and 30.7% in the Giza Red and Giza 20 varieties, respectively compared to their controls. The data also show that the Giza Red variety had more yield and WUE than the Giza 20 variety which was more sensitive to soil salinity.

Table 5. *Effects of spray treatments with H_2O_2 (mM) on total yield and water use efficiency (WUE) of two varieties of onion plants grown on a saline soil.*

Treatments		Total yield (ton/ha)	WUE (kg bulbs/m^3 of water)
Variety	H_2O_2		
Giza Red	0	30.7 ± 0.9e	3.51 ± 0.12e
	1	53.7 ± 1.5a	6.15 ± 0.20a
	2	48.8 ± 1.2b	5.56 ± 0.18b
Giza 20	0	36.8 ± 1.0d	4.20 ± 0.14d
	1	48.1 ± 1.2b	5.49 ± 0.17b
	2	41.1 ± 1.1c	4.69 ± 0.15c

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

3.3. Chlorophyll Fluorescence of Onion Plants as Affected by H_2O_2 and Saline Soil Conditions

Changes in the photosynthetic efficiency were evaluated 90 days after transplanting (Table 6). Foliar application of H_2O_2 significantly increased *Fv/Fm*, Fv/F_0 and PI of the two varieties of onion plants compared to those of the control plants that received no exogenous H_2O_2. The applied level of 1 mM H_2O_2 was more effective, increasing the above attributes by 9.5 and 11.0%, 93.9 and 85.3%, and 151.2 and 134.0% in the Giza Red and Giza 20 varieties, respectively compared to their controls.

Table 6. *Effects of spray treatments with H_2O_2 (mM) on chlorophyll fluorescence of two varieties of onion plants grown on a saline soil.*

Treatments		F_v/F_m	F_v/F_0	PI
Variety	H_2O_2			
Giza Red	0	0.74 ± 0.02b	2.29 ± 0.11c	2.09 ± 0.60d
	1	0.81 ± 0.03a	4.44 ± 0.23a	5.25 ± 0.83a
	2	0.80 ± 0.03a	4.18 ± 0.21a	5.22 ± 0.69a
Giza 20	0	0.73 ± 0.02b	2.31 ± 0.11c	2.03 ± 1.01d
	1	0.81 ± 0.03a	4.28 ± 0.21a	4.75 ± 0.53b
	2	0.80 ± 0.03a	3.60 ± 0.17b	3.96 ± 0.44c

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

3.4. Osmoprotectants, Membrane Stability Index (MSI) and Relative Water Content (RWC) as Affected by H_2O_2 and Saline Soil Conditions

Data introduced in Tables 7 showed that the application of H_2O_2 significantly reduced the concentrations of free proline and total soluble sugars (TSS), and significantly increased MSI and RWC in the two varieties of onion plants compared to untreated control plants. The applied level of 1 mM H_2O_2 was found to be more effective, reducing the concentrations of free proline and TSS by 21.4 and 29.4%, and 37.0 and 23.8%, and increasing MSI and RWC by 20.9 and 19.4%, and 10.7 and 11.2% in the Giza Red and Giza 20 varieties, respectively compared to their controls.

Table 7. *Effects of spray treatments with H_2O_2 (mM) on leaf free proline, total soluble sugars (TSS), membrane stability index (MSI) and relative water content (RWC) of two varieties of onion plants grown on a saline soil.*

Treatments		Free proline (mg/g DW)	TSS (mg/g DW)	MSI (%)	RWC (%)
Variety	H_2O_2				
Giza Red	0	0.14 ± 0.01b	3.65 ± 0.12a	62.68 ± 1.93c	70.45 ± 1.20b
	1	0.11 ± 0.01d	2.30 ± 0.08d	75.78 ± 2.26a	77.96 ± 1.58a
	2	0.12 ± 0.01c	2.61 ± 0.09c	70.51 ± 2.08b	77.45 ± 3.91a
Giza 20	0	0.17 ± 0.01a	3.87 ± 0.13a	60.41 ± 1.81b	71.91 ± 0.55b
	1	0.12 ± 0.01c	2.95 ± 0.10b	72.11 ± 2.18ab	79.93 ± 2.57a
	2	0.14 ± 0.01b	3.09 ± 0.10b	70.29 ± 2.09b	78.85 ± 1.10a

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

3.5. Non-enzymatic Antioxidants, Hydrogen Peroxide (H_2O_2) and Lipid Peroxidation (MDA) as Affected by H_2O_2 and Saline Soil Conditions

Table 8 shows that, under saline soil conditions (EC_e = 7.94 - 8.81), the application of H_2O_2 significantly increased the concentrations of ascorbic acid (AsA) and glutathione (GSH), but significantly decreased the concentrations of H_2O_2 and MDA in the two varieties of onion plants compared to untreated control plants. The applied level of 1 mM H_2O_2 was found to be more effective, increasing the concentrations of AsA and GSH by 55.8 and 55.8%, and 24.6 and 25.0%, and reducing H_2O_2 and MDA by 67.6 and 73.4%, and 51.7 and 54.8% in the Giza Red and Giza 20 varieties, respectively compared to their controls.

Table 8. *Effects of spray treatments with H_2O_2 (mM) on the activity of leaf antioxidants; ascorbic acid (AsA), glutathione (GSH), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) of two varieties of onion plants grown on a saline soil*

Treatments		AsA (ng ascorbate/mg protein)	GSH (nmol GSH /mg protein)	H_2O_2 (µmol/g FW)	MDA (nmol of MDA/g FW)
Variety	H_2O_2				
Giza Red	0	515.4 ± 6.2c	28.1 ± 0.12c	8.84 ± 0.03b	1.49 ± 0.03a
	1	802.8 ± 9.8a	35.0 ± 0.18a	2.86 ± 0.01d	0.72 ± 0.01c
	2	716.2 ± 8.8b	32.8 ± 0.15b	3.28 ± 0.01c	1.00 ± 0.02b
Giza 20	0	527.4 ± 6.6c	28.4 ± 0.13c	9.90 ± 0.04a	1.55 ± 0.03a
	1	821.8 ± 9.9a	35.5 ± 0.20a	2.63 ± 0.01d	0.70 ± 0.01c
	2	692.3 ± 8.5b	32.4 ± 0.15b	3.40 ± 0.02c	0.94 ± 0.02b

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

3.6. Antioxidant Enzyme Activities as Affected by H_2O_2 and Saline soil Conditions

The activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are shown in Table 9. Under saline soil conditions (EC_e = 7.94 - 8.81), the application of H_2O_2 significantly increased the activities of SOD, CAT, APX and GR in the two varieties of onion plants compared to untreated control plants. The applied level of 1 mM H_2O_2 was more effective, increasing the activities the above enzymes by 22.4 and 28.2%, 51.8 and 51.0%, 28.2 and 35.4%, and 51.4 and 38.7% in the Giza Red and Giza 20 varieties, respectively compared to their controls.

Table 9. *Effects of spray treatments with H_2O_2 (mM) on the activity of leaf antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) of two varieties of onion plants grown on a saline soil*

Treatments		SOD	CAT	APX	GR
Variety	H_2O_2	(nmol NO_2 /mg protein/ min)	(nmol H_2O_2 /mg protein/min)	(nmol ascorbate oxidized/mg protein/min)	(nmol NADPH oxidized /mg protein min)
	0	78.2 ± 0.4c	19.5 ± 0.1c	50.7 ± 0.3c	20.8 ± 0.1c
Giza Red	1	95.7 ± 0.7a	29.6 ± 0.2a	65.0 ± 0.5a	31.5 ± 0.3a
	2	89.3 ± 0.6b	24.4 ± 0.2b	58.0 ± 0.4b	26.7 ± 0.2b
	0	77.2 ± 0.4c	19.4 ± 0.1c	49.4 ± 0.3c	21.2 ± 0.1c
Giza 20	1	99.0 ± 0.8a	29.3 ± 0.2a	66.9 ± 0.5a	29.4 ± 0.2a
	2	86.9 ± 0.6b	23.5 ± 0.2b	56.8 ± 0.4b	26.0 ± 0.2b

[#]Values are means ± SE (n = 6). Mean values in each column followed by a different lower-case-letter are significantly different by least-significant difference test (LSD) at $P \leq 0.05$.

4. Discussion

By stimulating the overproduction of reactive oxygen species (ROS) through various organelles and enzymes, salt stress negatively affects different processes during seed germination, growth and flowering that negatively reflects in plant productivity [5]. To avoid effects of salt stress, plants adopt several strategies such as ion homeostasis, osmotic adjustment, and improvement of antioxidant defense system [41]. As recently reported, exogenous application of H_2O_2 increases tolerance to many environmental stresses including salt stress [13, 15, 16]. In these works, the role of H_2O_2 as an acclimation response has been well reported to improve the activities of antioxidants and to reduce the peroxidation of membrane lipids, which was consistent with our results (Tables 8 and 9). H_2O_2 has also been reported to promote plant defense system that improves growth and photosynthetic ability of plants under salt stress [15], which also agreed with our findings (Tables 4 and 6). The work in [42] reported a dual role of H_2O_2 during biotic and abiotic stresses. As an element of oxidative stress, it deleteriously affects cell components on the excess accumulation, and simultaneously it induces protective mechanisms, particularly at the early stage of plant stress response. H_2O_2 can act as a signaling molecule for stress adaptation and plant application with H_2O_2 can lead to programmed cell death and regulate plant development [42].

It has been observed in [13] that exogenous H_2O_2 treatment reduced the harmful effects of salt stress on growth of wheat, and suggested in [43] the multiple positive effects of H_2O_2 on root system, leaf and coleoptiles growth of wheat seedlings. In our study, salt stress caused a significant reduction in all growth parameters of Giza Red and Giza 20 varieties (Table 4), but the spray applications of H_2O_2, particularly the lower level (1 mM) significantly increased the growth of these varieties, which reflected in the final yields (Table 5).

Although H_2O_2 is known as the central signaling molecule in stress responses, little information explains how it affects the photosynthetic machinery [44]. Salt stress partially inhibited photosynthesis by the reduction in photosynthetic pigments and chlorophyll fluorescence (*Fv/Fm*, *Fv/F0* and PI; Table 6), but H_2O_2 application may be associated with the H_2O_2-mediated increase in ascorbic acid (AsA) and glutathione (GSH) concentrations (Table 8), which acted as an antioxidants and protected photosynthetic machinery from salt-induced ROS. It has been shown that pre-treatment of seeds with H_2O_2 increase the net photosynthetic rate in wheat seedlings [15, 45]. In the present study, chlorophyll fluorescence decreased under salt stress through the decrease in intermediates of chlorophyll biosynthesis [46], leading to a decreased absorption of light by the chloroplast, indirectly impairing photosynthesis [15]. The F_v/F_m, F_v/F_0 and PI were used as a noninvasive method to determine the functional state of the photosynthetic machinery. The F_v/F_m, F_v/F_0 and PI were significantly reduced by salt stress, but H_2O_2 application significantly improved these components in leaves of salt-stressed plants (Table 6). The highest F_v/F_m, F_v/F_0 and PI were observed in the leaves of salt-stressed plants sprayed with 1 mM H_2O_2. However, H_2O_2 application was a remedy for plants under salt stress effects. The reduction in the F_v/F_m, F_v/F_0 and PI provides an indicator of photo-inhibitory damage caused by the incident photon flux density when plants are subjected to a wide range of environmental stresses [47].

The reduction in growth and yield of onion plants (Giza Red and Giza 20) grown under salt stress was associated with a reduction in water potential, decreasing the water use efficiency (WUE; Tables 4 and 5), but the application with H_2O_2 reversed these effects and increased WUE, which may be related to the increase in relative water content (RWC; Table 7). H_2O_2 treatments enabled the leaf to maintain a high level of RWC by regulating the osmolality in the leaf, alleviating the effects of salt stress. The increase in water potential and osmotic potential might help stabilization of proteins and increase photosynthesis [15]. Under salt stress, osmotic stress is triggered by an excess of salt in the soil, and ionic stress is caused by the over-accumulation of salt in plant cells. These stresses individually affect the physiological status of plant [48, 49]. Exogenous application of H_2O_2 showed amelioration of the salt effects and increased membrane stability index (MSI) and RWC, maintaining turgid cells for healthy metabolic processes and membrane integrity.

It has been suggested in [50] that H_2O_2 application induces the accumulation of compatible-solutes (i.e., polyols, sugars,

and amino acids including proline) in stressed plants to allow the maintenance of water uptake and cell turgor under drought induced by salt stress. The reduction in free proline and soluble sugars by H_2O_2 application in this study (Table 7) may be attributed to the crucial role of H_2O_2 in mitigating the negative salt effects. Exogenous H_2O_2 treatment has been shown to prevent the increase of oxidative stress and endogenous H_2O_2 concentration in plants (Table 8) and enhance tolerance of plants to salt stress by enhancing the production of enzymatic and non-enzymatic antioxidants (Tables 8 and 9), which can quench the ROS and decrease lipid peroxidation (MDA; Table 8).

To avoid water imbalance within plants during stress, CO_2 fixation reduces after stomatal closure by abscisic acid, limiting the oxidation of NADP, the prime acceptor of electrons during photosynthesis. Thus, when ferrodoxine is reduced in the photosystem, free oxygen radicals are generated by Mehler reaction [51]. This transfer of one, two, or three electrons generates the formation of $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$, respectively, with grave consequences for DNA, lipids, and proteins [52]. This affects the integrity of cell membranes as well as the activity of numerous enzymes and the functioning of the photosynthetic machinery [53]. In our study, the increased contents of H_2O_2 in the salt-stressed plants were significantly reduced when plants applied with H_2O_2 (Table 8). This is accompanied by the increased contents of antioxidants such as AsA and GSH (Table 7), and antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Table 6), which enable onion plants to overcome salt stress by lowering the ROS damage. Lipid peroxidation, in this study, was measured as malondialdehyde (MDA) content. Membrane destabilization is generally attributed to lipid peroxidation [54], which ultimately gives rise to the compound MDA [55]. Therefore, MDA levels are related to the extent of damage to the ultrastructure of cell membranes [56]. MDA is a biochemical indicator of stress, where it inhibits the production of biomass and reduces the possibilities of plant adaptation to stress [57]. MDA content under salt stress, like the content of H_2O_2 (Table 8), was higher than its content when H_2O_2 was applied to onion plants. This could indicate a positive impact on the biomass of salt stressed-onions treated with H_2O_2. Several studies have indicated that the oxidative damage generated during salt stress is due to the overproduction of ROS such as $O_2^{\bullet-}$ and H_2O_2 and antioxidant activity alterations [58]. To avoid the damage caused by the oxidative stress, plants have developed many antioxidant systems, including SOD that constitutes the first line of defence against ROS [59] by reducing the $O_2^{\bullet-}$ radical to H_2O_2. H_2O_2 can serve as a substrate for numerous enzymes such as CAT. This enzyme is located in the peroxysomes where the H_2O_2 concentration is very high, and thus H_2O_2 is eliminated by peroxidases. Also, APX is considered one of the most important enzymes in the reduction of this reactive molecule [60]. The regenerating enzymes DHAR and GR as a fundamental part of the Halliwell–Asada cycle form a part of the regeneration of AsA from DHA using GSH as a reducing power [61]. In turn, the GSH consumed can be regenerated from its oxidized form (GSSG) by the reaction of GR [62]. Our results show that H_2O_2 treatments supported the activities of SOD, CAT, APX and GR compared to the salt stressed-control plants (Table 9). The substrates of the Halliwell–Asada cycle, AsA and GSH, also act as antioxidants in an isolated way by being involved in the direct reduction of ROS during different types of stress [63]. This is reflected in the total concentrations of AsA and GSH in this study, which are increased with the H_2O_2 application under salt stress, to overcome the accumulation of $O_2^{\bullet-}$, where the AsA can directly eliminate $O_2^{\bullet-}$ and H_2O_2 in a non-enzymatic way [62]. Here, salt stress tolerance in onion plants, was improved by the application of H_2O_2 which alleviated salt stress as indicated by improved chlorophyll, enzymatic and non-enzymatic antioxidants, and plant growth and productivity when compared to the controls. This might be due to cytokinin mediated green effect. Findings of this study suggest that exogenous application of H_2O_2, particularly at the lower level (1 mM) promotes the expression of stress–response genes and increases salt stress tolerance. In addition, inducing the expression of ROS-related stress–response genes by H_2O_2 application is an effective means of enhancing resistance to subsequent stress, and we recommend performing additional studies of the potential application of H_2O_2 to plant production under stress.

5. Conclusion

This study suggests that spraying onion plants (either Giza Red or Giza 20 variety) with 1 mM H_2O_2 improves the response of onion plants to salt stress (7.94 - 8.81 dS/m) through the increased activity of the antioxidant system, including enzymatic and non-enzymatic antioxidants, increased plant water relations and reduced endogenous H_2O_2 and MDA concentrations. Therefore, H_2O_2 may be considered beneficial in onion production to help plants to overcome the harmful effects of salt stress conditions.

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