EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

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PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Changes in the gene expression level of salicylic acid, morphological, biochemical parameters and antioxidant enzyme activity in three pretreated Egyptian citrus rootstocks by copper sulphate or mannitol to enhance their salt tolerance

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Climate change is the main concern in the current global situation. Salinity is one of the most crucial side effects of climate change. The citrus genus is the most popular fruit crop in the world. In Egypt, citrus is considered the first fruit crop and is planted in several areas and in newly reclaimed areas. Salinity affects several genes and parameters of citrus yield. The objective of this study was to study changes in the gene expression level of salicylic acid (SA) and antioxidant enzyme activity in three salted Egyptian citrus rootstocks (Troyer citrange, Sour orange and Volkamin lemon), and to study the effect of salt stress on some morphological and other biochemical parameters, in addition to evaluate the alleviation effect of presoaking with 20mM mannitol and 5mM $CuSO_4$. Under salinity conditions, the results proved a significant increase in SA gene expression level. Similarly, trehalose, MDA and H_2O_2 contents and the activity of five antioxidant enzymes were significantly increased. Conversely, shoot length, number of leaves and photosynthetic pigments were significantly decreased under salt stress. However, root length increased as a type of adaptation. Presoaking the rootstocks with 5mM $CuSO_4$ and 20mM mannitol significantly changed the expression level of the SA gene, recovered the harmful effect of salt stress, and weakened the oxidative stress caused by salinity and mannitol was more effective than $CuSO_4$. Thus, the present findings displayed the changes in SA gene expression and may recommend presoaking with mannitol and $CuSO_4$ to improve the salt sensitivity of citrus rootstock for use in salted areas.

Keywords: Citrus rootstocks, Salt stress, Presoaking, SA gene expression, Antioxidant enzymes

ARTICLE HISTORY
Submitted: December 21, 2023
Accepted: September 01, 2024

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DOI: 10.21608/ejbo.2024.257058.2623

EDITED BY: H. Zahran

INTRODUCTION

The citrus genus is the most widespread fruit crop worldwide and has a sizable global market (Abobatta, 2018). It belongs to the Rutaceae family, subfamily Aurantoideae. Citrus is a flowering tree that includes many species. Major citrus varieties are Sour oranges, Mandarin, Grapefruits, Limes, and sweet oranges. Egyptian citrus is the most well-known and significant fruit produced in Egypt with a total area under cultivation of 520,000 acres. According to the Egyptian Ministry of Agriculture and Land Reclamation, orange exports are estimated to reach 1.7 MMT in 2022–2023. According to the Citrus Annual Reports Cairo, Egypt, EG2022-0033, orange exports made up 83.2% of all citrus exports in the period 2021-22022. approximately 69% of all Egyptian citrus is produced as oranges. In Egypt, Navel oranges are the most widely grown fruit. Khalily orange, Baladi orange, Blood orange, and sweet orange are also very important varieties of orange (Abobatta, 2018).

Salinity is one of the most crucial and oldest abiotic stresses affecting large areas of the earth's irrigation land caused by NaCl and climate changes. There are many factors affecting the salinity yield relationship such as the physical and chemical conditions of the soil, climate, and farming practices (Shrivastava & Kumar, 2015). Recently, rising soil salinity has been a significant issue in Egyptian soils. In the Nile Delta, between 30 and 40 percent of the soil is classified as salt-affected soil. It is believed that seawater intrusion, particularly in the coastal region of the Nile Delta, salt buildup in the upper soil layers because of improper irrigation management, and poor drainage conditions are the main causes of salinity in Egyptian soils (Elshinnawy & Almaliki, 2021). The major irrigation water source in Egypt is the Nile River. However, a lack of irrigation water in reclaimed lands and low-quality groundwater cause soil salinization in reclaimed lands. Therefore, sustainable solutions are needed for solving salinity issues such as improving drainage systems and the development of salt-tolerant varieties of fruits and vegetables (Mohamed, 2016).

The main abiotic stress threatening citrus plants is salinity, which reduces plant growth and yield due to water shortages and ionic toxicity resulting in massive losses in yield (El-Habashy, 2018). Therefore, developing citrus rootstocks that tolerate salinity stresses is one of the foremost objectives of breeding programs to produce citrus plants suitable for salinity problems (Pathania & Singh, 2021). Plants respond to abiotic stress such as salinity in many ways, including accumulating several osmolytes such as trehalose, sugar alcohol, proline, and glycine

(Türkan & Demiral, 2009; Khatab et al., 2021; Latif et al., 2021; Sadak et al., 2024) or producing compounds with hormonal action such as salicylic acid (SA) (Fragnière et al., 2011). The accumulation of osmolytes (trehalose and mannitol) can compensate for the high salt stress in the soil and reduce the high concentrations of salt in the vacuole (Türkan & Demiral, 2009). Mannitol is found in several organisms and has critical functions in biotic and abiotic stress responses. Mannitol-producing transgenic plants exhibited enhanced tolerance to salt stresses (Rathor et al., 2020). Trehalose is a nonreducing disaccharide that can diminish stress. Trehalose is less reactive than other types of sugars; therefore, its accumulation cannot destroy cellular structures (Tekadal, 2021).

According to numerous studies, under salt stress, the endogenous level of SA increases. Endogenous levels of SA help plant growth stimulate resistance to diseases as well as salt stress by lowering DNA injury and reducing oxidative stress. Exogenous treatment with SA increased tomato production (*Solanum lycopersicum*) under salinity stress (Naeem et al. 2020) increased seed germination of *Leymus chinensis* under salt-alkali stress (Hongna et al., 2021) and enhanced the root system in *Cucumis sativus* under salt stress (Miao et al., 2020). Jayakannan et al. (2013) proved that SA restored the membrane permeability of *Arabidopsis* by improving salinity tolerance. Nazar et al. (2011) claimed that SA relieves photosynthesis decline by enhancing antioxidant metabolism in *Vigna radiata* cvs.

Additionally, plant growth requires eight heavy metals as micronutrients, including copper (Cu), which is related to various physiological and biochemical processes in plant cells. Copper is a cofactor for many important enzymes and plays a key role under stress conditions. However, the exposure of plants to high levels of copper can also negatively influence plant metabolism and development (Chen et al., 2022).

Plants under salinity and other abiotic stresses can produce reactive oxygen species (ROS). These ROS can cause cellular damage by interacting with essential macromolecules and metabolites such as proteins and DNA. Additionally, ROS can induce lipid peroxidation and DNA damage, causing cell death (Mesquita et al., 2019). To protect cells and tissue from oxidative damage, plants must produce several antioxidant enzymes (Sahu et al., 2022; Budran et al., 2023; Taha et al., 2023).

Thus, this work aimed to evaluate the expression level of the endogenous SA gene and study some morphological and biochemical parameters and the oxidative state of rootstocks during the development of three citrus rootstocks that can tolerate salinity stress using mannitol and copper sulphate.

MATERIALS AND METHODS

Materials and treatment groups

Three Egyptian citrus rootstock Troyer citrange (*Citrus sinensis Osb.,* L *x Poncirus trifoliate* L. Raf.*)*, Sour orange (*Citrus aurantium* L.), and Volkamin lemon (*Citrus Volkameriana* Ten. & Pasq.) were supplied by the Citrus Department, Horticulture Research Institute, Egypt. The planting experiment was performed in a greenhouse at the Horticulture Research Institute, Egypt. Seeds were grown in pots with diameters of 35 cm and 40 cm in depth with 7 kg of sandy loam soil. Seeds of each rootstock (Troyer citrange, Sour orange, and Volkamin lemon) were divided into 9 treatment groups (50 seeds per treatment). They were rinsed with running tap water for 1 h, and then the seeds were disinfected with 10% NaOCl for 30 min and washed three times in sterilized distilled water.

The 9 treatment groups (50 seeds per treatment) were as follows:

1. In the control group, seeds were soaked in distilled water for 12 hours before planting, and the soil was watered twice per week to its maximum capacity for 3 months.
2. After one month of salt treatment with 25 mM NaCl, seedlings reached 10 cm and were then irrigated twice per week by 40 ml 25 mM NaCl for 3 months.
3. After one month of salt treatment with 50 mM NaCl, the seedlings reached 10 cm, and then they were irrigated twice per week with 40 ml of 50 mM NaCl for 3 months.
4. Seeds were presoaked in 5 mM $CuSO_4$ for 12 h before planting. They were water irrigated twice per week for 3 months.
5. Seeds were presoaked in 5 mM $CuSo_4$ for 12 h before planting. After one month, the seedlings reached 10 cm, and they were irrigated twice per week with 40 ml of 25 mM NaCl for 3 months.
6. Seeds were presoaked in 5 mM $CuSo_4$ for 24 hours before planting. After one month, the seedlings reached 10 cm, and they were irrigated twice per week with 40 ml of 50 mM NaCl for 3 months.

7. Seeds were presoaked in 20 mM mannitol for 12 h before planting. They were water irrigated twice per week for 3 months.
8. Seeds were presoaked in 20 mM mannitol for 12 h before planting. After one month, the seedlings reached 10 cm, and they were irrigated twice per week with 40 ml of 25 mM NaCl for 3 months.
9. Seeds were presoaked in 20 mM mannitol for 12 h before planting. After one month, the seedlings reached 10 cm, and they were irrigated twice per week with 40 ml of 50 mM NaCl for 3 months. Only 20 seedlings were collected from each treatment group after 3 months for the following analyses.

Morphological parameters

Three morphological parameters of each citrus rootstock seedling (shoot length, root length, and leaf numbers) for the 9 groups were measured.

Biochemical analysis

Chlorophyll content (chlorophyll a, b and total) and carotenoids were determined as described by Arnon (1949) and concentrations of pigments for each citrus rootstock seedling leaf were determined (Lichtenthaler & Buschmann, 2001). The trehalose content in each citrus rootstock seedlings leaf was determined using the method of Li et al. (2014).

Quantitative Real-time PCR analysis of salicylic acid (*SA*) gene expression

A total of 200 mg of fresh leaves of each citrus rootstock treatment group was ground using liquid nitrogen and total RNA was extracted using the TRIzol reagent (Invitrogen). RNA quantity and quality were assessed. First-strand cDNA was synthesized with 1 µg of total RNA and oligo (dT) 20 primers using Super Script III RNase H Reverse Transcriptase (Promega). The expression level of endogenous salicylic genes was analyzed by qRT-PCR (MyGo mini, UK) using *SA* gene-specific primers (SA F 5`-TTCTTCCACTTCGTCGGGTG-3` (Tm 54°C), SA R 5`-TGGACGCTAAGTTGTGTCTCT-3` (Tm 54°C). PCR cycle was used to amplify *SA* gene products from cDNA. The initial denaturation at 94°C for 3 minutes, denature (94°C) for 45 seconds, anneal (57°C) for 45 seconds, extend (72°C) for 1 min for 40 cycles, and final extension 72°C for 6 min. The qRT-PCR products were analyzed using *ACT1* (actin) as a housekeeping gene (*Actin* gene primer F 5'-CCAAAGGCCAACAGAGAGAAGAT-3' (Tm 57°C, T_a 52°C), R: 5'-TGAGACACACCATCACCAGAAT-3' (Tm 57°C, T_a 52°C). The Applied Biosystems 7900 HT Fast Real-Time PCR System software was used to calculate CT values and relative abundance (Livak & Schmittgen, 2001).

Oxidative stress

Hydrogen peroxide (H_2O_2) levels and the amount of malondialdehyde (MDA) were studied as two indicators to evaluate the oxidative damage. One hundred mg of fresh leaves from each treatment group of each citrus rootstock seedling were dissolved in 2 ml of 0.1% trichloroacetic acid (TCA) (Heath & Packer, 1968). After centrifugation for 15 min at 4000 xg, they were filtered and used to determine H_2O_2 levels and lipid peroxidation by determining the amount of MDA, using the thiobarbituric acid (TBA) assay method (Heath & Packer, 1968). Two ml of 0.5% TBA in 20% TCA was added to 0.5 ml of supernatant and then heated for 30 min in water bath at 95°C. The mixture was quickly poured into ice. All tubes were centrifuged at 10000 xg for 10 min and the absorbance was assessed at 532 nm. MDA content was calculated through an extinction coefficient of 155 mM^{-1} cm^{-1}. H_2O_2 was measured after reaction with potassium iodide spectrophotometrically at 390 nm (Sergiev et al., 1997), and the amount of H_2O_2 was given as mmole g^{-1} FW.

Antioxidant enzyme activity
Preparation of enzyme extract

A weight of 200 mg from fresh leaves of each citrus rootstock treatment group was ground using liquid nitrogen, and then crude enzyme extract was extracted by ice extraction buffer containing 50 mM phosphate buffer pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% (v/v) Triton X-100, and polyvinyl polypyrrolidone, the homogenates were centrifuged at 10,000 xg for 20 min at 4°C. The supernatants were considered as enzymes crude extract and immediately used for determination of the enzyme activity of peroxidase (POX), polyphenol oxidase (PPO), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Davenport et al., 2003). To estimate the enzyme activity of POX (EC 1.11.1.7), the synthesis of the oxidized product (tetraguaiacol) was measured (Tatiana et al., 1999). The enzyme activity was calculated using an extension coefficient of tetraguaiacol (25.5 mM^{-1} cm^{-1}). Enzyme activity of PPO activity (EC 1.14.18.1) was calculated using an extension coefficient of pyrogallol (2.47 mM^{-1} cm^{-1}) as reported by Raymond et al. (1993). The APX (EC

1.11.1.11) activity, was determined as reported by Nakano & Asada (1981). The unit of activity is expressed as mmole of ascorbic acid oxidized min^{-1} at 25°C. The activity of GR (EC 1.6.4.2) was measured according to Jiang & Zhang (2001).

Glutathione reductase follows the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by monitoring the oxidation of NADPH as visualized by a decrease in absorbance at 340 nm at 25°C. GR activity was calculated using a molar extinction coefficient of 6.22 × 103 M^{-1} cm^{-1} for NADPH at 340 nm for 1 min, expressed as mmol NADPH $min^{-1}g^{-1}FW$. Catalase activity (EC 1.11.1.6) was assayed spectrophotometrically at 240 using the Aebi (1984) method which measures the rate of H_2O_2 decomposition at room temperature. Catalase activity was calculated using an extinction coefficient of 0.0436 mM^{-1} cm^{-1}, and one unit (U) was defined as the quantity of enzyme that decomposes 1 µmol of H_2O_2 per minute at 25°C, pH 7.0.

Statistical analysis

All the results were represented as means ± SD. Results were represented statistically analyzed using one-way analysis of variance (ANOVA) to find out the difference in mean values among the accessions for variability assessment when the F- test was significant ($p \leq 0.05$) using SPSS 26.0 software (IBM, 2019) (Identical letters indicate non-significant differences).

RESULTS

Morphological parameters

Salt treatment significantly reduced the growth of the three citrus rootstocks. The salt treatment reduced shoot length and the number of leaves. This reduction was highly significant after treatment with 50 mM NaCl. Presoaking rootstocks with both $CuSO_4$ and mannitol alleviates salinity stress in the three rootstocks. The effect of mannitol was more significant than that of $CuSO_4$. Troyer citrange rootstock was the most resistant type and responded positively to mannitol and $CuSO_4$. The shoot length of Troyer citrange decreased by more than 50% after treatment with 50 mM NaCl reached 12 cm. $CuSO_4$ caused the increment to 13 cm, while mannitol significantly increased the shoot length to 15 cm. The number of leaves significantly decreased from 51 to 13 leaves after treatment with 50 mM NaCl. Mannitol alleviates the depression effect of salinity. The leaf number increased to 15 leaves (Table 1). The Sour orange *rootstock was the most sensitive type to salinity stress.* The shot length of Sour orange decreased to 6 cm after treatment with 50 mM NaCl. $CuSO_4$ caused the increment to reach 8 cm, while mannitol significantly increased the shoot length to 9 cm. The number of leaves significantly decreased from 6 leaves after treatment with 50mM NaCl, while increased to 9 leaves by presoaking with mannitol (Table 1). The root length data in Table 1 revealed a significant increase in root length after all treatments compared with their respective controls. The maximum root length in each *citrus* rootstock was achieved after treatment with 5m $MCuSO_4$ and 50 mM NaCl.

Biochemical analysis

Chlorophyll a, b and total in the three rootstocks showed significant decreases after treatment with 25 and 50 mM NaCl (Table 2). Both $CuSO_4$ and mannitol could not alleviate the depression effect of NaCl on chlorophyll content. The carotenoid content showed a fluctuating pattern. Presoaking Sour orange and Volkamin lemon with 5 mM $CuSO_4$ alone caused a significant increase in carotenoid content while presoaking with mannitol caused a significant decrease in carotenoid content in the three rootstocks. Copper sulfate could reduce the salinity stress effect in salt-treated plants by 25 mM NaCl by increasing carotenoid content (Table 2). The osmolyte trehalose significantly accumulated after all treatments compared with the control groups and was highly significant after treatment with 50 mM NaCl (Table 2). Seed soaking with 5 mM $CuSO_4$ and 20 mM mannitol recovered the toxic effect of NaCl and caused a decrease in the accumulation of trehalose contents compared with the corresponding salt-treated plants.

Quantitative Real-time PCR analysis of SA gene expression

The gene expression level of *SA* was quantified to investigate the effect of salt stress on the endogenous SA content in the three citrus rootstocks. The results showed that *SA* gene expression was significantly upregulated under salt stress. Treatment with 50 mM NaCl upregulated *SA* gene expression by about 5-fold in the Troyer citrange rootstock and about 2-fold in *Sour orange and* Volkamin lemon *rootstocks.* All plants treated rootstocks with $CuSO_4$, and mannitol showed significant downregulation in *SA* gene expression as compared with salt-treated rootstocks (Figure 1)

Table 1. Shoot height and root length and number of leaves of control and treated rootstocks.

Type of rootstock	Treatment Parameter	Control	NaCl (25mM)	NaCl (50mM)	$CuSO_4$ (5mM)	$CuSO_4$ (5mM) + NaCl(25mM)	$CuSO_4$ (5mM) + NaCl(50mM)	Mannitol 20mM	Mannitol (20mM) + NaCl(25mM)	Mannitol (20mM) + NaCl(50mM)	F value
Troyer citrange	Shoot length (cm)	29 a ±0.17	16 c,d,e ±0.31	12 e ±0.04	21 b ±0.28	17 b,c,d ±0.61	13 d,e ±0.14	26 a ±0.42	19 b,c ±0.07	15 c,d,e ±0.35	15.16***
	Number of leaves	51 a ±0.02	26 c,d,e ±0.42	13 e ±0.01	35 b ±0.26	22 b,c,d ±0.61	12 d,e ±0.03	37 a ±0.001	25 b,c ±0.28	15 c,d,e ±0.18	139.50***
	Root length (cm)	23 c,d ±0.31	21 d ±0.07	28 b,c,d ±0.59	33 a,b ±0.26	31 b,c ±0.01	39 a ±0.14	25 b,c ±0.39	28 b,c,d ±0.24	26 b,c,d ±0.08	4.82**
Sour orange	Shoot length (cm)	14 a ±0.001	8 d,e ±0.20	6 e ±0.08	11 b,c ±0.62	9 b,c,d ±0.25	8 c,d,e ±0.73	12 b ±0.89	10 b,c,d ±0.005	9 b,c,d ±0.03	7.99**
	Number of leaves	28 a ±0.21	12 e,f ±0.52	6 g ±0.001	19 d,e ±0.052	16 c,d ±0.64	8 f,g ±0.01	22 b ±0.08	17 c ±0.29	13 d,e ±0.43	31.01***
	Root length (cm)	14 e ±0.52	17 d,e ±0.08	22 c,d ±0.21	25 b,c ±0.79	22 c,d ±0.08	30 a ±0.52	19 c,d,e ±0.01	29 a,b ±0.07	21 c,d ±0.49	9.56***
Volkamin lemon	Shoot length (cm)	21 a ±0.32	16 a,b,c ±0.29	8 e ±0.05	19 a,b ±0.16	13 c,d,e ±0.53	9 d,e ±0.64	20 a,b ±0.27	15 b,c,d ±0.42	12 c,d,e ±0.21	6.72***
	Number of leaves	28 a ±0.51	15 b ±0.20	7 d ±0.49	26 a ±0.02	19 b ±0.08	11 c,d ±0.11	26 a ±0.09	24 a ±0.39	14 c ±0.06	24.38***
	Root length (cm)	16 c ±0.04	25 b ±0.63	28 b ±0.22	25 b ±0.23	29 b ±0.37	37 a ±0.01	23 b ±0.48	26 b ±0.02	28 b ±0.17	8.03***

Identical letters indicate non-significant differences

Table 2. Chlorophyll, carotenoid, and trehalose contents (mg g^{-1}FW) of control and treated rootstocks.

Type of rootstock	Treatment Parameter	Control	NaCl (25mM)	NaCl (50mM)	$CuSO_4$ (5mM)	$CuSO_4$ (5mM) + NaCl(25mM)	$CuSO_4$ (5mM) + NaCl(50mM)	Mannitol 20mM	Mannitol (20mM) + NaCl(25mM)	Mannitol (20mM) + NaCl(50mM)	F value
Troyer citrange	Chl a	45.65 a ±0.03	32.29 c ±0.02	20.41 f ±0.005	32.28 c ±0.05	26.90 d ±0.02	19.09 f ±0.002	37.28 b ±0.02	33.65 c ±0.001	22.98 e ±0.030	98.62***
	Chl b	10.42 a ±0.006	6.76 b ±0.002	6.48 b ±0.004	9.22 a,b ±0.004	9.94 a,b ±0.010	8.17 a,b ±0.010	10.25 a ±0.006	6.95 b ±0.003	8.01 a,b ±0.007	3.18**
	Total Chl	56.07 a ±0.003	39.05 c,d ±0.005	26.89 f ±0.025	41.50 c ±0.001	36.84 d ±0.002	27.26 c ±0.025	47.53 f ±0.080	40.60 f ±0.001	30.99 c ±0.005	67.87***
	Carotenoid	4.62 a ±0.001	5.26 a ±0.029	4.23 a,b ±0.031	3.50 a,b,c ±0.004	2.61 b,c ±0.001	2.58 b,c ±0.003	3.47 a,b,c ±0.006	3.52 a,b,c ±0.078	2.10 e ±0.009	3.16*
	Trehalose	0.016 d ±0.013	0.026 b ±0.002	0.038 a ±0.071	0.018 d ±0.003	0.024 c ±0.006	0.027 b ±0.001	0.017 d ±0.005	0.022 c ±0.007	0.026 b ±0.001	132.19***
Sour orange	Chl a	23.96 a ±0.002	22.27 a,b ±0.025	20.83 b ±0.003	22.64 a,b ±0.014	22.28 a,b ±0.003	20.44 b ±0.004	22.62 a,b ±0.009	21.43 b ±0.001	20.73 b ±0.030	2.39**
	Chl b	12.64 ±0.009	12.18 ±0.005	11.57 ±0.046	12.54 ±0.002	12.43 ±0.004	12.64 ±0.002	12.78 ±0.020	12.36 ±0.002	12.19 ±0.007	0.46
	Total Chl	36.60 a ±0.002	34.44 a,b ±0.008	32.40 b ±0.002	35.19 a,b ±0.004	34.71 a,b ±0.001	33.09 b ±0.003	35.40 a,b ±0.064	33.80 a,b ±0.001	32.92 b ±0.001	2.00*
	Carotenoids	3.14 a ±0.001	3.26 a ±0.001	3.07 a ±0.022	3.53 a ±0.030	3.18 a ±0.001	2.62 a,b ±0.002	2.84 a ±0.007	1.71 b ±0.042	1.69 b ±0.009	3.66**
	Trehalose	0.008 i ±0.067	0.023 c ±0.005	0.029 a ±0.001	0.012 g ±0.002	0.020 e ±0.030	0.028 b ±0.005	0.009 h ±0.040	0.018 f ±0.049	0.021 d ±0.006	49.5***
Volkamin lemon	Chl a	25.17 a ±0.009	24.50 a,b ±0.003	21.7 b,c,d ±0.002	23.4 a,b,c ±0.005	22.86 a,b,c,d ±0.012	20.39 d ±0.071	23.60 a,b,c ±0.017	23.49 a,b,c ±0.002	21.19 c,d ±0.002	3.23**
	Chl b	13.80 ±0.002	14.90 ±0.036	13.033 ±0.001	14.37 ±0.001	14.02 ±0.004	13.84 ±0.020	13.80 ±0.001	13.63 ±0.001	12.74 ±0.057	0.56
	Total Chl	38.97 a ±0.006	39.41 a ±0.022	34.76 b ±0.001	37.78 a,b ±0.060	36.88 a,b ±0.002	34.23 b ±0.004	37.40 a,b ±0.001	37.12 a,b ±0.009	33.93 b ±0.001	2.98**
	Carotenoids	2.78 ±0.044	2.45 ±0.001	2.98 ±0.001	2.96 ±0.002	2.79 ±0.003	2.24 ±0.047	2.59 ±0.002	2.58 ±0.003	2.92 ±0.007	0.192
	Trehalose	0.009 i ±0.001	0.016 d ±0.001	0.021 a ±0.068	0.013 g ±0.020	0.015 e ±0.009	0.018 b ±0.072	0.010 h ±0.014	0.014 f ±0.003	0.017 c ±0.006	74.25***

Identical letters indicate non-significant differences

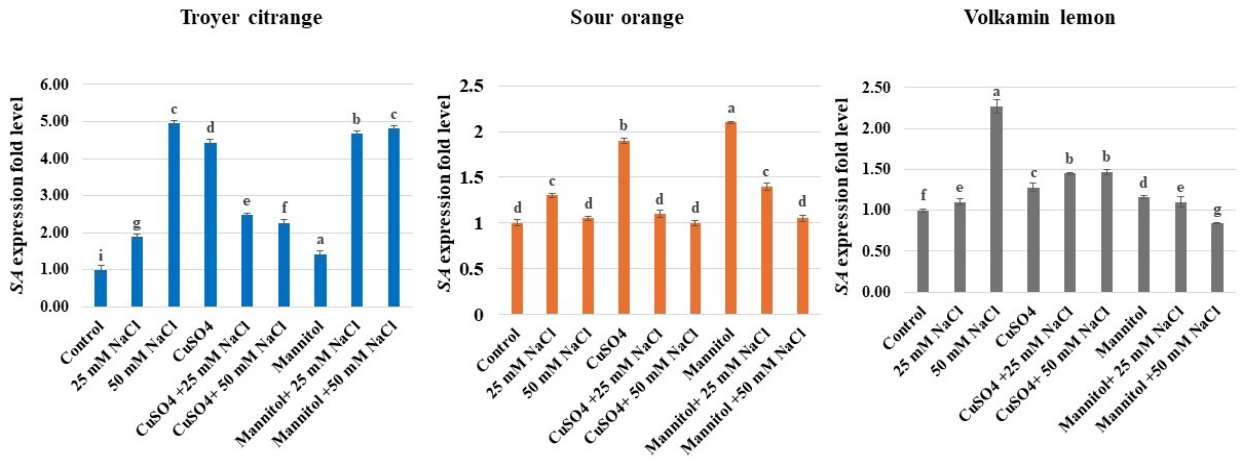

Figure. 1 Salicylic gene expression level of control and treated rootstocks treated with NaCl, $CuSO_4$ and mannitol.

except for treatment with 20 mM mannitol and 50 mM NaCl in the Troyer citrange *rootstock*. $CuSO_4$ was more efficient than mannitol. There was approximately a 4-fold increase in *SA* gene expression after treatment with 20 mM mannitol and 50 mM NaCl in the Troyer citrange *rootstock. The minimum upregulation level* of *SA* gene was achieved after treatment with 20 mM mannitol and 50 mM NaCl in Sour orange rootstock (Figure 1).

Oxidative stress

Salt treatment caused a significant accumulation of MDA and 50 mM NaCl caused the highest accumulation (Figure 2). Volkamin lemon was the most salt-tolerant rootstock, representing the lowest accumulation of MDA, while Troyer citrange *was* the most salt-sensitive rootstock, showing the highest accumulation of MDA. Presoaking of the three rootstocks with mannitol alleviated the toxic effect of saline stress more than $CuSO_4$, exhibiting the lowest accumulation of MDA (Figure 2). The accumulation of H_2O_2 was highly observed after salt treatment by 50 mM NaCl. Volkamin lemon was the most nonoxidative stressed rootstock, representing the lowest accumulation of H_2O_2, while Troyer citrange *was* the most oxidative stressed rootstock, showing the highest accumulation of H_2O_2. Mannitol treatment showed a more potent effect than $CuSO_4$ reducing H_2O_2 accumulation (Figure 3).

Antioxidant enzyme activity

The results showed that the activities of the antioxidant enzymes POX, PPO, APX, GR and CAT in the three citrus rootstocks exposed to salinity stress were significantly increased, compared with those of the control plants (Figures 4-8). All enzyme activities were markedly increased under salt stress concentrations compared with the corresponding controls. Treatment with 50 mM NaCl caused the maximum enzyme activities for all enzymes in the three citrus rootstocks. The highest POX, PPO, APX, GR and CAT activities were detected in the Troyer citrange. Presoaking the rootstocks with $CuSO_4$ caused a moderate effect and reduced the enzyme activities compared with salt-treated plants, while presoaking with mannitol caused a significant reduction in the activity of most enzymes (Figures 4-8).

DISCUSSION

Salinity stress affects plant division, growth, yield, morphology, genetics and biochemical characteristics (Shahid et al., 2020). To avoid the negative effects of salinity, plants use several strategies, including improving the antioxidant system inside the cell, osmotic regulation, and ion homeostasis (Mohamed et al., 2021; Taha et al., 2023). This work revealed a reduction in shoot length and number of leaves after treatment with salt stress. Seedling growth, shoot height, and root length were the most sensitive parameters to salt stress. These results agree with Zahra et al. (2020), who found that salinity affected the growth, morphological, and biochemical characteristics of maize species. The amazing data was the increment in root length in all treatments compared with the control. This increase in root length may be a type of adaptation to survive in a salt-stress environment. The increase in the root/shoot ratio represents an adaptive advantage to increase the ability of the root to provide the shoot system by the requested elements, as reported by Lu and Fricke (2023). Root growth usually tolerates salt stress more than shoot growth, so there is an increase in root/shoot percentage when plants are under saline stress (Bernstein, 2013). Roots can detect environmental stresses and respond against stresses (Muthert et al., 2020) and vigorously change in response to stresses, by altering some parameters, such as root length, growth and branching style, and modifying root cell wall structures (Zou et al., 2022).

The reduction in photosynthesis pigment content (chlorophyll a and b) observed in this study is concurrent with Singh et al. (2014), who reported that, under saline stress, the observed reduction in chlorophyll in citrus rootstocks may be due to a decline in the activity of enzymes and/or chlorophyll degradation (Mansoor et al., 2022). Additionally, Hameed et al. (2023) previously stated that under saline conditions, the membranes of cell organelles are destroyed, so the chlorophyll content rarely remains regular because chlorophyll is produced by plastids, which are membrane-bound pigments, and their integrity depends on membrane stability.

Although Cu is an important microelement that is necessary for regular plant development and plays a key role under stress conditions, copper sulphate did not decrease the reduction impact of salt stress on photosynthesis pigments. Hossain et al. (2020) observed a significant decrease in the chlorophyll a and b contents, and carotenoids, after treatment of *Lens culinaris* (lentils) with 3 mM copper sulphate.

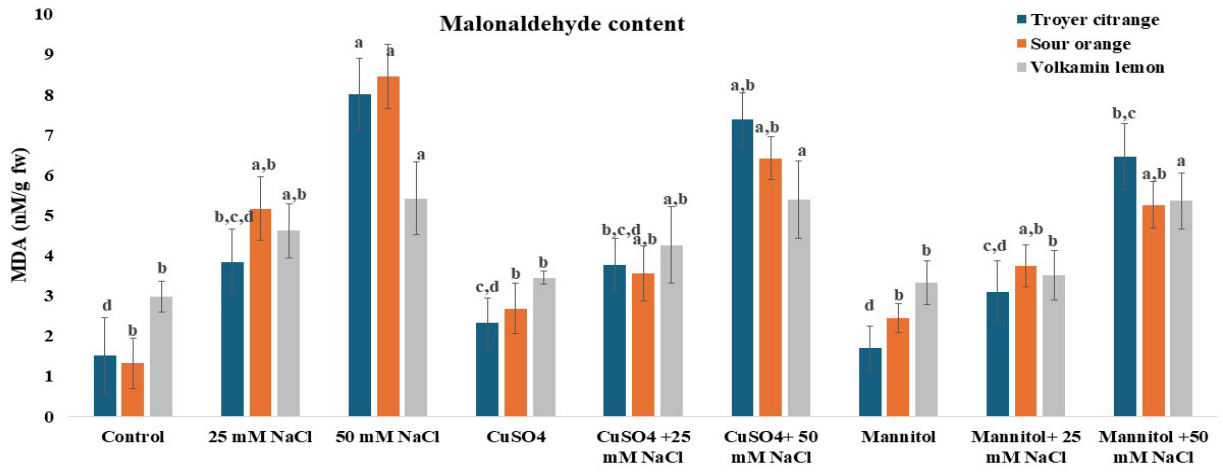

Figure 2. Malonaldehyde content of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

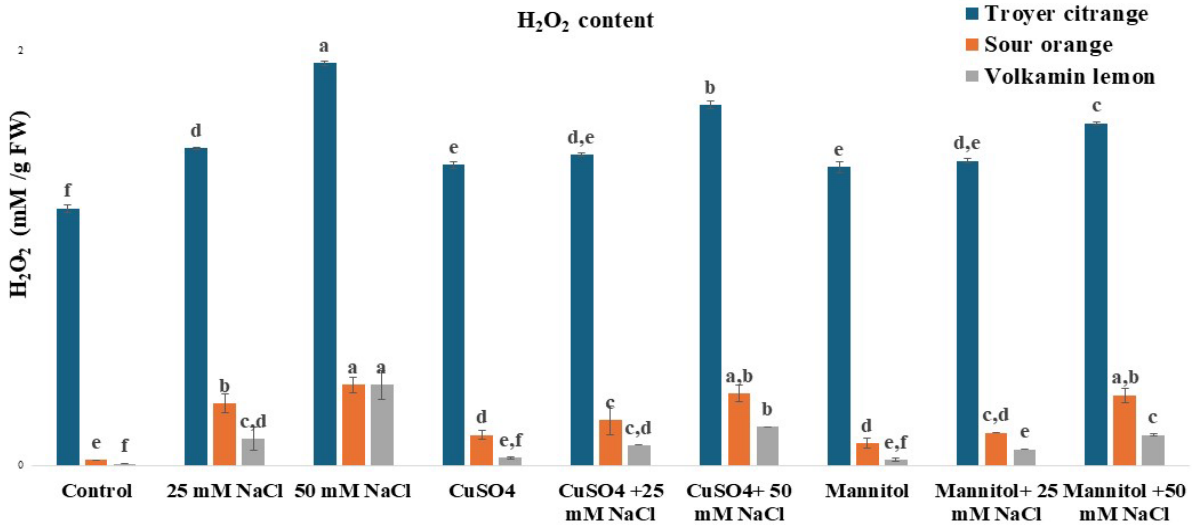

Figure 3. Hydrogen peroxide contents of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

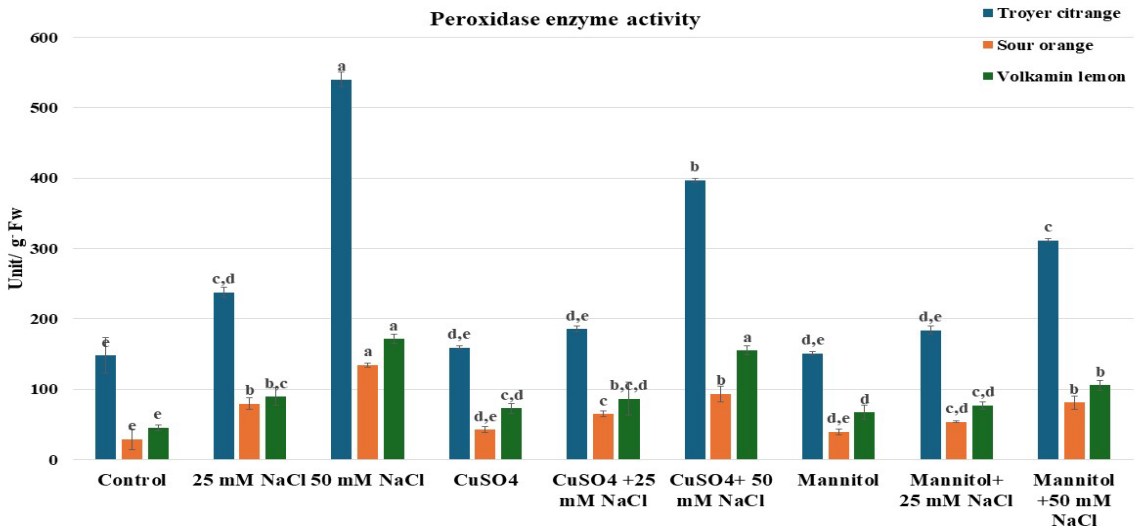

Figure 4. Peroxidase enzyme activity of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

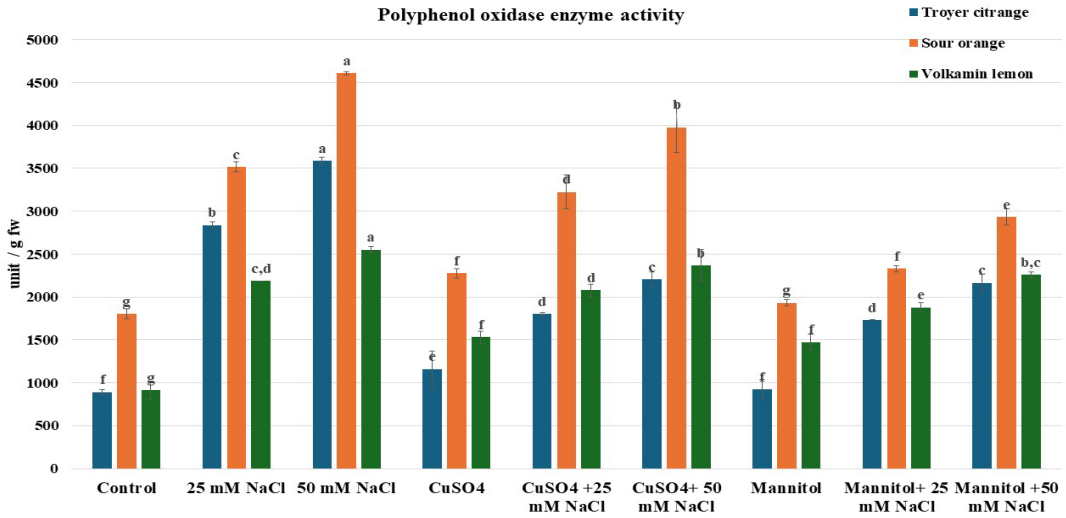

Figure 5. Polyphenol oxidase enzyme activity of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

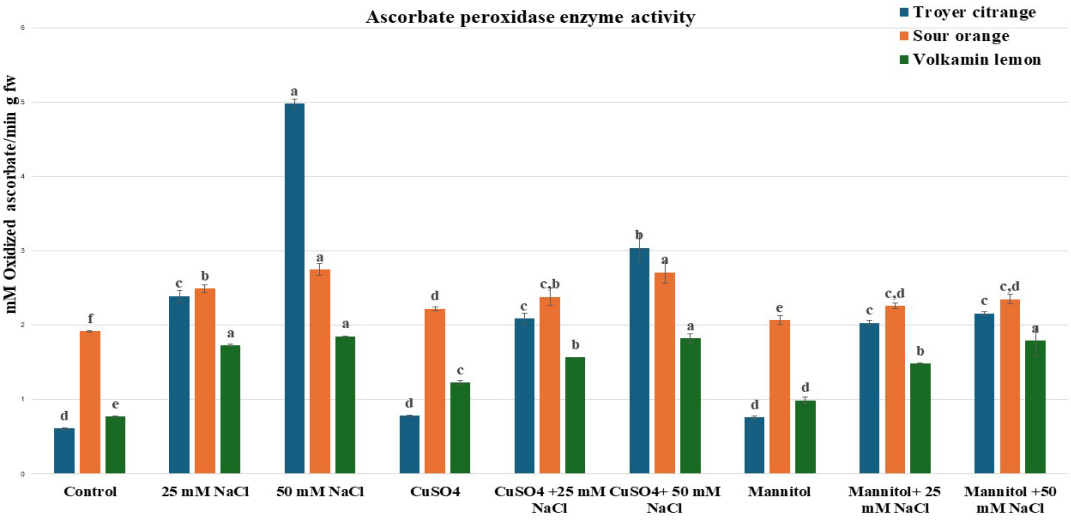

Figure 6. Ascorbate peroxidase enzyme activity of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

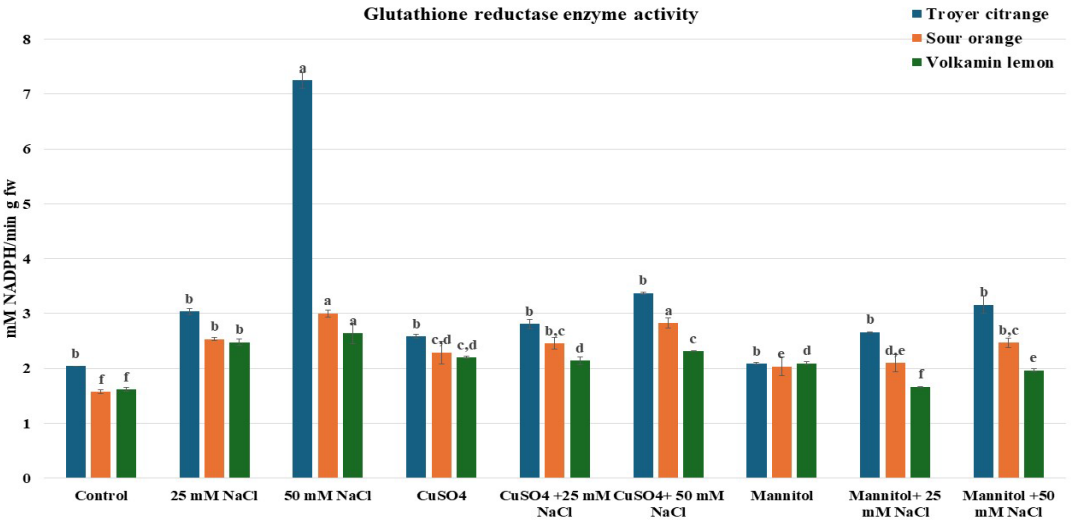

Figure 7. Glutathione reductase enzyme activity of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

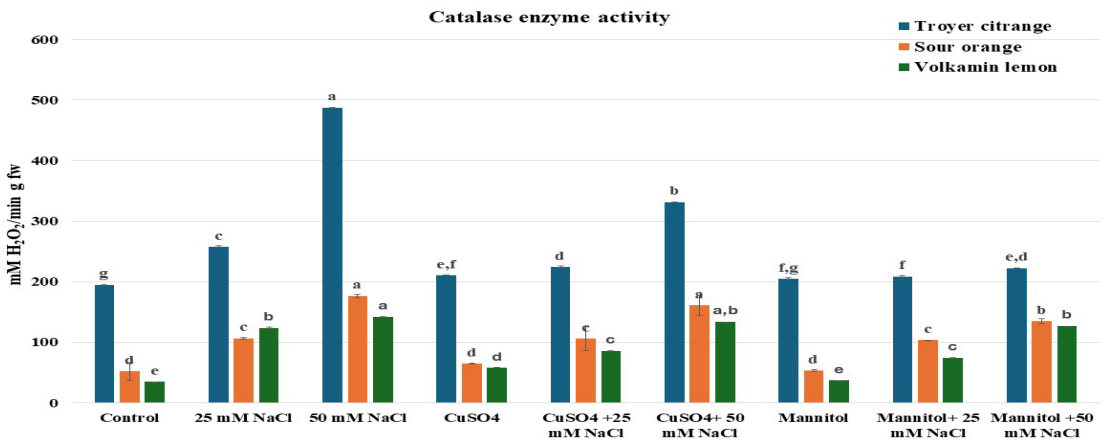

Figure 8. Catalase enzyme activity of control and treated rootstocks with NaCl, $CuSO_4$ and mannitol.

The present results detected a significant accumulation of trehalose in plants under salinity levels. Trehalose is a compatible solute that accumulates in plants under abiotic stress. Therefore, it may act as an osmo-protective agent, facilitating the ability of salted plants to tolerate the harmful influences of salt stress. Trehalose was previously used to stimulate the resistance of rice seedlings to salt stress and decrease the expression levels of some antioxidant enzymes (Abdallah, 2016). Salinity promotes the overproduction of reactive oxygen species (ROS) (Taha et al., 2023). Previous studies proved that *SA* gene expression is upregulated under abiotic stresses that trigger ROS (Yuan & Lin, 2008). Salicylic acid is an endogenous signaling molecule used to tolerate abiotic stresses. It regulates cellular activities, including antioxidant defense, nitrogen metabolism, and photosynthesis, to prevent plant cell death. Several plant genes linked to *SA-dependent activation* are known to be upregulated by salt stress (Ogunsiji et al., 2023). Upregulation of *SA* gene expression was observed to control the defense and resistance processes (Abdallah, 2016). In the present study, the *SA* expression level was upregulated under salt stress to protect citrus rootstocks from the adverse effects of salt stress. Guo et al. (2019) proved that SA protects barley seedlings against Cd stress by affecting several mechanisms of cadmium detoxification. Presoaking with $CuSO_4$ and mannitol caused significant downregulation of *SA* expression levels compared with *SA* expression levels in salt-stressed plants, indicating that $CuSO_4$ and mannitol may have the ability to protect citrus rootstocks from salt stress. The presence of ROS damages cellular proteins, DNA, and lipids, causing membrane lipid peroxidation (malondialdehyde accumulation) (Taha et al., 2023). The MDA content increased in the salt-sensitive Troyer citrange in response to salt stress; however, the Sour orange and Volkamin lemon rootstocks showed less accumulation of MDA. This increase in MDA content indicated an increase in lipid peroxidation and weakness of the cell membrane (Mahmoud et al., 2020). Volkamin lemon seedlings displayed significant MDA accumulation under salinity stress (Khalid et al., 2020). Salinity increased H_2O_2 content in the plant tissues, which greatly induced DNA damage. Pre-soaking of the rootstocks with $CuSO_4$ and mannitol reduced the MDA content and H_2O_2, indicating their ability to improve the oxidative stress in rootstocks and enhance the defense and salt tolerance of rootstocks (Hu et al., 2020). Additionally, André and Villain (2017) proved that mannitol functions as a scavenger of ROS, thereby preventing peroxidation of lipids and resulting in cell damage.

The preset data revealed a significant increase in all estimated antioxidant enzymes (POX, PPO, APX, GR and CAT) under salt stress. This increase could be considered a defensive way to decrease oxidative damage caused by salt stress (Nounjan et al., 2012). Antioxidant enzymes play a catalytic role in converting ROS into stable, harmless compounds, making them the most effective defence against cell damage by ROS. Various antioxidant enzymes are involved in the scavenging of ROS in citrus rootstocks (Singh et al., 2014). The antioxidant enzymes POX, APX and CAT toxify H_2O_2, converting it to H_2O and O_2 to increase the stress tolerance of plants (Nounjan et al., 2012). Peroxide enzymes are important antioxidant enzymes involved in the scavenging of H_2O_2 and are also important for plant growth, lignification, and suberization processes (Jbir et al. 2001). Exogenous pretreatment with $CuSO_4$ and

mannitol reduced antioxidant enzyme activities compared with plants stressed by NaCl. André & Villain (2017) proved that mannitol plays a major role as a free radical scavenger, therefore removing excess H_2O_2 produced by salt stress conditions. As previously reported by Toscano et al. (2023), the increase in APX and CAT activities in plants treated with mannitol with NaCl reduced the level of lipid peroxidation because of the ability of APX and CAT to diminish H_2O_2 and minimize membrane injury under salt stress. Glutathione reductase (GR) is an important protective enzyme for many plants against oxidative stress. It catalyzes the NADPH-dependent reduction of oxidized glutathione. Salt stress and pretreatment with $CuSO_4$ and mannitol rootstocks increased GR activity, which may be used to reduce the activity of the ASC-GSH cycle. Also, APX and POX were increased by salt stress, and they reported the importance of those two enzymes for citrus rootstock to tolerate salt stress (Toscano et al., 2023).

CONCLUSIONS

In conclusion, salt stress by NaCl affects and causes oxidative stress on the three citrus rootstocks (Troyer citrange (*Citrus sinensis Osb.,*L *x Poncirus trifoliate* L. Raf.*)*, Sour orange (*Citrus aurantium* L.), and Volkamin lemon (*Citrus Volkameriana* Ten. &Pasq), causing changes in some SA gene expression, morphological and biological parameters and increasing lipid peroxidation and the antioxidant activity of POX, PPO, APX, GR and CAT. Pre-soaking the rootstocks with 5 mM $CuSO_4$ and 20 mM mannitol significantly changed the expression level of the SA gene, recovered the harmful effect of salt stress, and weakened the oxidative stress caused by salinity. Mannitol was more effective than $CuSO_4$. Thus, the present findings displayed the changes in SA gene expression and may recommend pre-soaking with 20 mM mannitol and 5 mM $CuSO_4$ to alleviate rootstock from the negative effects of salt stress, making them suitable for planting in salted soil.

ETHICS APPROVAL

No animals or humans were involved.

AUTHOR CONTRIBUTIONS

Data collection, methodology and analysis were performed by W.M. Experimental design, data collection and methodology were achieved by E.M.S. Revising the first draft of the manuscript was done by Th. R. Conceptualization, experimental design, supervision, data collection, data collection, interpretation of the results, and writing of the original manuscript were produced by Sh.S.S. All the authors have read and approved the final manuscript.

DATA AVAILABILITY

All data generated or analyzed in this study are included in this manuscript.

FUNDING

No funds were received

ACKNOWLEDGEMENTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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