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Influence of Seed Priming with Trehalase Inhibitors on Inducing Salinity Tolerance in Sour orange and Troyer citrange Rootstocks

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ABSTRACT

Citrus fruits are among the largest fruit crops in the world. These crops are exposed to several challenges that cause severe yield losses due to abiotic stress each year. Where water stress is a major environmental factor, breeding approaches need to develop tolerant rootstocks that can mitigate risks from climate and other key abiotic factors. In this work, the impact of two Trehalase enzyme inhibitors, Validamycin and Zn^{2+}, on increasing Trehalose accumulation and inducing salinity tolerance in two citrus rootstocks, sour orange and Troyer citrange, was studied. Our findings suggest that application of pre-soaking of seeds with 30 μM of Validamycin A and 20 mM ZnSO4 alleviated the adverse effects of salinity by stimulating growth, increasing total chlorophyll content, and reducing antioxidant enzyme activity under severe salt stress conditions (50 mMNaCl) compared with the untreated control. An additional point of interest was to detect the change in the expression of the Trehalose gene in response to salt stress with or without Val/Zn treatment.

Key words: Citrus, rootstock, Trehalose, Salinity, Tolerance.

INTRODUCTION

Citrus fruits are the most significant fruit crop in Egypt and one of the largest fruit crops worldwide. Citrus is a genus of the *Rutaceae* family that includes many of the most significant fruit crops including different species of grapefruit, orange, lemon, and tangerine, among others (Mabberley 2008). With a planted area of 172,200 hectares in 2022–2023 (USDA 2022), Egypt will be one of the world's top exporters of fresh oranges and the sixth-largest producer of oranges overall (Omar & Tate 2018). Abiotic challenges like salt, drought, and cold frequently affect citrus crop productivity, and these challenges have a big global impact. Therefore, in the event of climate change, the selection and development of novel rootstocks that are resistant to abiotic stress are essential. One of the most serious challenges today is the salinization of agricultural soils around the world, which severely restricts the amount of cultivated land as well as crop yield and quality. NaCl is the component in salty soils that causes the most damage. In plants exposed to saline environments, K^+ and Mn^{2+} ions drop while Na^+ and Cl^- ions rise. Major osmotic inhibition of water availability and toxic effects of salt ions that result in salt damage and nutritional imbalances are two of salinity's major inhibitory effects on plants. To endure a variety of intricate abiotic challenges, plants have evolved complex adaptation systems. Several viable alternative control strategies are based on the application of chemicals that increase the resistance of plants by inducing defense mechanisms (Pel and Pieterse, 2013; Pushpalatha et al., 2013). Understanding these intricate features in higher plants has significantly advanced with the development of new technologies like genomics and genetic engineering. Different physiological and molecular adjustments are made by fruit crops to deal with excessive salts in the soil and irrigation water. Different defense systems used to stop salt from entering plants may be active at the molecular, cellular, or entire plant levels. These reactions, however, are generally genotype-specific, suggesting that some mechanisms that activate in one genotype might not function in others. Complex physiological features, metabolic processes, and molecular or gene networks have a role in plant adaptation or tolerance to salt stress (Almeida et al., 2007). Plants employ a variety of defense mechanisms in response to osmotic stress to deal with unfavorable conditions. Their detection and subsequent signal transmission are the early events that start metabolic reactions by activating numerous stress-responsive genes (Bray, 1997). Trehaose, a non-reducing disaccharide, is one of them and is essential for maintaining metabolic homeostasis and abiotic stress tolerance in a variety of organisms. Trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) are the enzymes that catalyze it. According to Blazquez et al. (1998), this osmoprotectantst abilizes proteins and membrane structures under pressure. Many plants have reportedly shown improved abiotic stress tolerance when exogenous and endogenous TPS-encoding genes are overexpressed (Almeida et al., 2007). Because the enzyme Trehalase hydrolyzes Trehalose to glucose, Trehalose levels are typically low in plants. Therefore, by decreasing plant Trehalase activity or by expressing the Trehalose biosynthetic genes under stress-specific regulation, it should be possible to induce enhanced Trehalose accumulation (Lunn et al., 2014).This enzyme is crucial to the metabolism of Trehalose because it either participates directly in the assimilation of exogenous Trehalose or regulates the level of Trehalose in the cell. Various chemicals, such as 20 mM Na^{+}, K^{+}, Li^{+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, or Fe^{3+}, as well as Validamycin A, were used to inactivate the enzyme. The non-systemic antibiotic Validamycin A (C20H35NO13) also functions as a fungicide. Streptomyces hygroscopicus, lemon-variety bacteria, is fermented to create it. Growing data in recent years suggests that Trehalose and its derivatives may function as signaling molecules that provide plant resilience to a range of stressors (Mostofa et al., 2015). In this study, two Trehalase inhibitors, Validamycin A and ZnSO4, were used to increase Trehalose accumulation in both sour orange and Troyer citrange rootstocks under salt stress conditions. Furthermore, we measured the effect of this stimulus on resistance to salt stress and assessed the expression level of the Trelaose gene.

MATERIALS AND METHODS

The experiment was conducted in a greenhouse and biotechnology lab at the Horticulture Research Institute, ARC, Egypt, in 2021–2022. In order to study the influence of Validamycin A and ZnSO4 as soaking agents on the response of two citrus rootstocks, sour orange (*Citrus aurantium L.*) and Troyer citrange(*Citrus sinensisOsb.*, *(L) x Poncirus trifoliate (L) Raf.)*, to saline solutions (25 and 50 mMNaCl). The seeds of the investigated citrus rootstock were obtained from the Citrus department of the Horticulture Research Institute, ARC. The trial was designed as three groups: the first is soaking the seeds of investigated rootstocks in distilled water for 12 hours before planting as a control group; the second is soaking the seeds in 30µ Validamycin A for 12 hours before planting; and the third is soaking the seeds in 20 mMZnSO4 for 12 hours. After 30 days, seedlings were transferred to pots (diameter 35 x 40 cm, containing 7 kg of sandy loam soil) and watered for one month under greenhouse conditions. After one month of growth in pots, each group was irrigated twice a week with 0, 25, and 50 mMNaCl for three months. Pots were then arranged in a randomized complete block design in a greenhouse; the experiment was replicated for two years.

Biochemical Analysis

The total quantities of chlorophyll a, b, and carotenes in fresh leaves were calculated using the method described by Lichtenthaler and Buschmann (2001). The method given by Shimada and associates (Shimada et al., 1992) was used to evaluate the stable radical DPPH's antioxidant potential. The enzymes activities of catalase (CAT) polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) were estimated according to Aebi (1984), Kumar and Khan (1982), Tatiana et al. (1999), Nakano and Asada (1981), and Chung and Hurlbert's (1975), respectively.

RNA extraction and quantification of gene expression with RT-qPCR

Using a gene-specific primer, quantitative real-time PCR (RT-qPCR) was used to examine the trehalose gene's expression patterns. RNA was extracted from untreated and treated plants using the Trizol reagent (Invitrogen) for stress-induced expression experiments. Using Super Script III RNase H Reverse Transcriptase from Promega, 1 µ of total RNA and oligo (dT) 20 primers were used to create first-strand cDNA. The actin gene F: CCAAAGGCCAACAGAGAGAAGAT, R: TGAGACACACCATCACCAGAAT and the Trehalose gene primers *Tre* F: 5'-CAACTGCTGAATCGGGATGG- 3', *Tre* R: 5'-TTCTGAGGTCACACTGTCCC- 3' were used in the qRT-PCR(MyGo mini, UK). Initial denaturation at 94°C for 3 minutes, denature (94°C) for 45 seconds, anneal (58°C) for 60 seconds, extend (72°C) for 2 minutes per cycle for 35 cycles, and final extension (72°C) for 8 minutes. This procedure is used to amplify gene-specific products from cDNA. The PCR efficiency for the reference and target genes were identical in the treatment and control samples. CT values and relative abundance were determined using the Applied Biosystems 7900 HT Fast Real-Time PCR System software.

Statistical Analysis

SPSS 16 (SPSS Inc., Chicago, IL, USA) was used to analyze the data and determine the F values. The F- test was used to calculate the variance's significance at the 5% level of significance.

RESULTSAND DISCUSSIONS

Growth parameters

Changes in plant growth of sour orange and Troyer citrange rootstocks as affected by NaCl salinity are shown in Fig (1). The present study confirmed the reduction in plant height, leaf number, and root length at high salinity levels. The reduction in these parameters was greater at 50 mMNaCl, while the highest values of these parameters were attained for plants treated with Validamycin, followed by plants treated with $ZnSO_4$ under normal and stress conditions in both of the investigated rootstocks. Plants treated with Valid and Zn^{2+} under normal or salt stress conditions (25 mMNaCl) showed a significant increase in plant length and number of leaves compared to control plants. Meanwhile, the longest root length was observed with plants treated with Validamycin with 25 mMNaCl (Fig. 1).

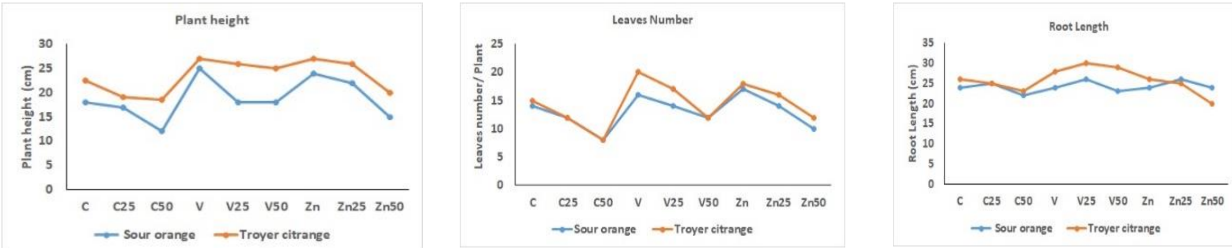


Fig. (1). Growth characteristics of sourorange and Troyer citrange rootstock seedlings under NaCl stress, C: control plant;C25: Plants treated with 25 mMNaCl; C50: Plants treated with 50 mMNaCl; V: Val-treated plants; V25: Val- treated plants +25 mMNaCl; V50: Val- treated plants +50 mMNaCl;Zn: Zn- treated plants; Zn25: Zn- treated plants + 25 mMNaCl, Zn50; Zn- treated plants + 50 mMNaCl.

Osmotic adjustment, a key process of salinity tolerance, is brought about by the buildup of compatible solutes in stressed plants (Shawky and Talaat, 2014). To maintain the water-relationship, stabilize the enzyme, protein complex, and membranes under saline conditions, many plant species produce compatible solutes like sucrose (Sami et al., 2016), mannitol (Patel and Williamson, 2016), glycine-betaine (Kurepin et al., 2017), proline (Slama et al., 2015), and Trehalose (Kosar et al., 2018). Trehalose, a glucose non-reducing disaccharide, has been found to stabilize biological structures and is one of the compatible solutes. One of the primary ways that plants combat osmotic stress is by accumulating Trehalose. It produces proteins with anti-oxidant effects and compatibility qualities (Ashraf and Harris, 2004) as well as an increase in the activity of hydrolytic enzymes such SOD, APX, CAT, POX, and others (Sorkheh et al., 2012). In this work, salinity treatments significantly affected plant growth in terms of height, number of leaves, and length of roots in citrus sour orange and Troyer citrange, and these effects increased with increasing salinity (Fig. 1). However, pretreatment of these seedlings with Val/Zn showed better growth under salinity conditions compared to control plants. These results are consistent with Nasto et al. (1999) who showed that growth of citrus varieties was significantly reduced at NaCl concentrations above 15 mM. Cooper and Peynado (1953) noted that citrus trees, both young and old, grew less quickly after being exposed to salt. According to Peynado and Young (1969), in saline environments, trees grew less and showed greater signs of salt harm. The reduction in relative growth rate brought on by NaCl treatment was shown to be more scion cultivar dependent when a relationship between the two variables was established (Banuls et al., 1990).

Influence of rootstocks plant treatments on biochemical analysis.

Photosynthesis pigment

The analysis of pigment contents, chlorophyll a, b, and carotenoids in the investigated rootstocks revealed a significantly varied response under all treatments. We observed a reduction in chlorophyll pigment in response to salt stress (Table 1). There was a significant increase in the level of chlorophyll a in Val-treated plants of the investigated rootstocks compared to control plants. There were reductions observed in chlorophyll a, b, and carotenoids under severe salinity (50 mMNaCl) in both rootstocks compared to control plants. Whereas in sour orange, there was a significant increase in Chl a and Chl b in Val-treated plants, followed by Zn^{2+} under severe salinity stress (50 mMNacl) compared with control. Meanwhile, no significant differences with Troyer citrangewere observed.

Table (1): Photosynthetic pigments of treated and untreated two citrus rootstocks under NaCl stress.

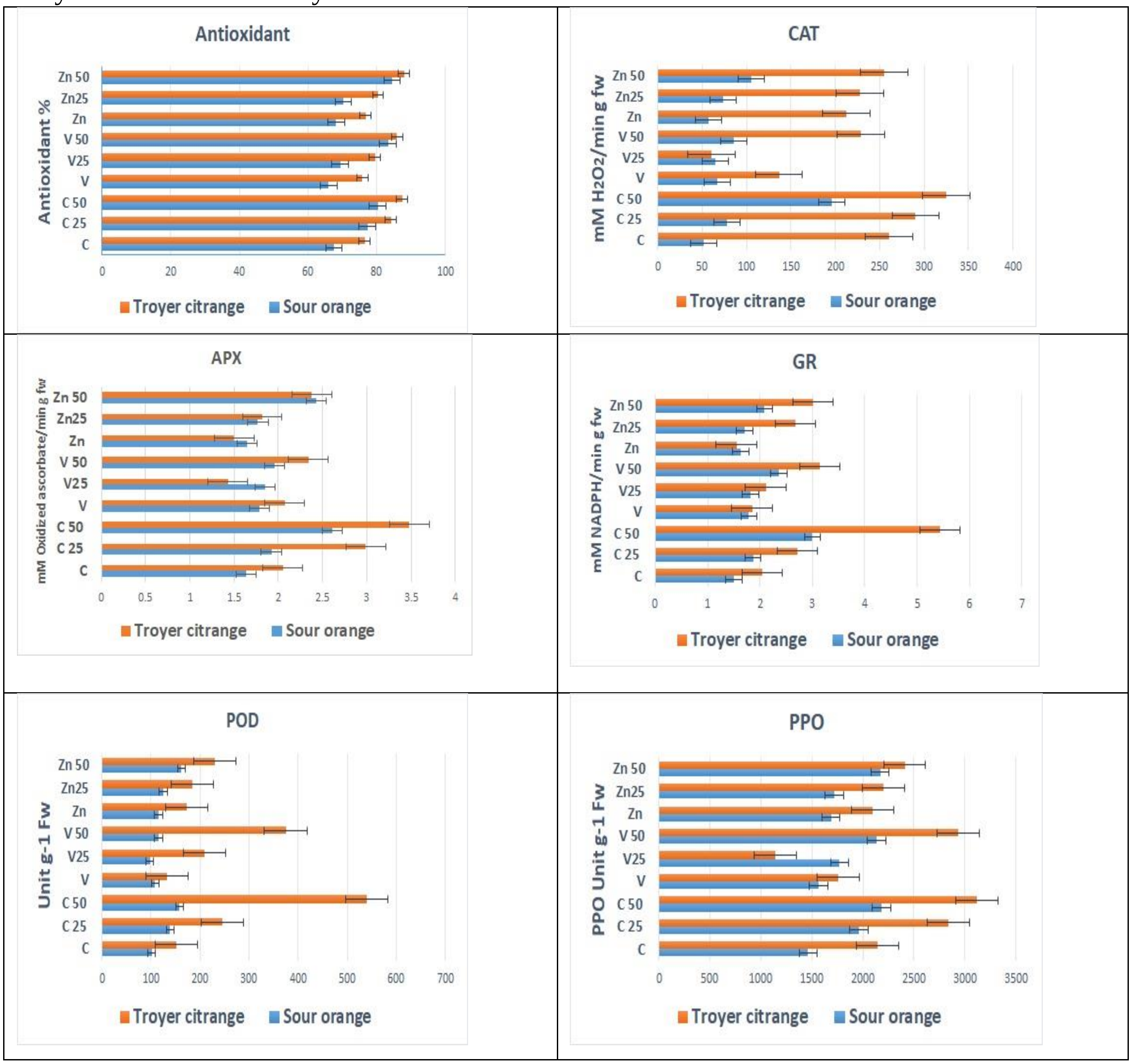
Treatment	Chlorophyll a (mg/g)		chlorophyll b (mg/g)		Carotenoids (mg/g)	
	Sour orange	Troyer citrange	Sour orange	Troyer citrange	Sour orange	Troyer citrange
C+ 0 NaCl	1.36c	2.06ab	0.48bc	0.47a	0.77abc	0.90b
C+ 25mM NaCl	1.35c	1.61b	0.46bcd	0.33bc	0.74abc	1.08b
C+ 50mM NaCl	0.41e	1.42b	0.10e	0.27c	0.26c	0.83b
Val+ 0 NaCl	2.07a	2.25a	0.63a	0.43ab	0.36bc	1.03b
Val + 25mM NaCl	1.64b	1.54b	0.59ab	0.27c	0.92ab	0.76b
Val + 50 mMNaCl	1.51bc	1.42b	0.34d	0.25c	1.02a	0.81b
Zn +0 NaCl	1.35c	1.60b	0.50abc	0.44ab	0.20c	1.84a
Zn + 25mM NaCl	1.10d	1.48b	0.53abc	0.45ab	0.21c	1.87a
Zn + 50 mMNaCl	0.90d	1.43b	0.45cd	0.26c	0.19c	1.01b
F-Value	110.11***	2.51*	13.58***	5.52***	3.02*	4.78**

*.**,***significant difference at P<0.05, P<0.01,0.001respectively.
Means in the same column with the same letter have no significant difference.

The studied rootstocks had different responses to salt stress, according to the examination of their pigment concentrations, chlorophyll a, b, and carotenoids. Chlorophyll a, b, and carotenoids were found to be lower in both rootstocks when exposed to severe salinity (50 mMNaCl) than in control plants. Our findings supported Salim et al. (2019), who claimed that salt stress hada negative impact on the amount of chlorophyll. Under various stresses, Hamani et al. (2020) detected a decrease in Chl a, Chl b, and total chlorophyll concentration. Under severe salinity stress (50 mMNaCl), there was a considerable rise in Chla and Chl b in Val-treated plants and Zn-treated plantsin sour orange compared to control. Meanwhile, no significant differences with Troyer citrange were observed. Our results are consistent with those described by Said (2017), who found that Validamycin A treatment was successful in lessening the inhibitory effect of salt stress on photosynthetic pigments. Stress conditions cause the chloroplast ultra structure to be destroyed and the amount of chlorophyll to decrease, both of which lower photosynthetic activity (Sidhu et al., 2017). Zeid (2009) suggested that the accumulation of Trehalose in plants has been hypothesized to preserve the osmotic potential and stability of the chloroplast envelope. Higher chlorophyll levels under soaking treatment may be related to their ability to repair salt-dependent damage.

Antioxidant and the enzymes activities

The results in (Fig.2) revealed a significant increase in the antioxidant activity of the DPPH radical in both rootstocks under salinity conditions in all treatments. There was a significant decrease in antioxidant activity in Val/Zn- treated plants in both rootstocks under medium salinity stresses (25 mMNaCl) compared to control plants. Plants produce several antioxidant enzymes, such as CAT, APX, GR, POD, and PPO, to treat cell damage caused by stress at the oxidative level. In Fig. (2), the results showed that antioxidant enzymes CAT, APX, GR, POD, and PPO increased significantly with increasing as well salt stress in all treatments as compared to the control. The treatment of plants with Val/Zn had reduced antioxidant content under stress conditions compared to control plants without treatment on both investigated rootstocks. The CAT enzyme activity recorded the lowest value in plants pretreated with Val/Zn at low and high levels of salinity compared to untreated plants on both rootstocks (Fig. 2). Although salinity significantly restricted the growth on both sour orange and Troyer citrange seedlings caused by NaCl treatment, the pretreatment seedlings with Val and Zn^{2+} were more tolerant to salt stress than untreated ones, which can be attributed to the higher capacity of the treated seedlings to control ROS formation and to activate enzymatic and non-enzymatic antioxidant defenses.



Fig(2):Influence of Validamycin A and ZnSO4 treatments on Antioxidant activity, CAT, APX, GR, POD, and PPO enzymes content of Troyer citrange and sour orange rootstocks under salt conditions (0, 25, 50 mMNaCl).

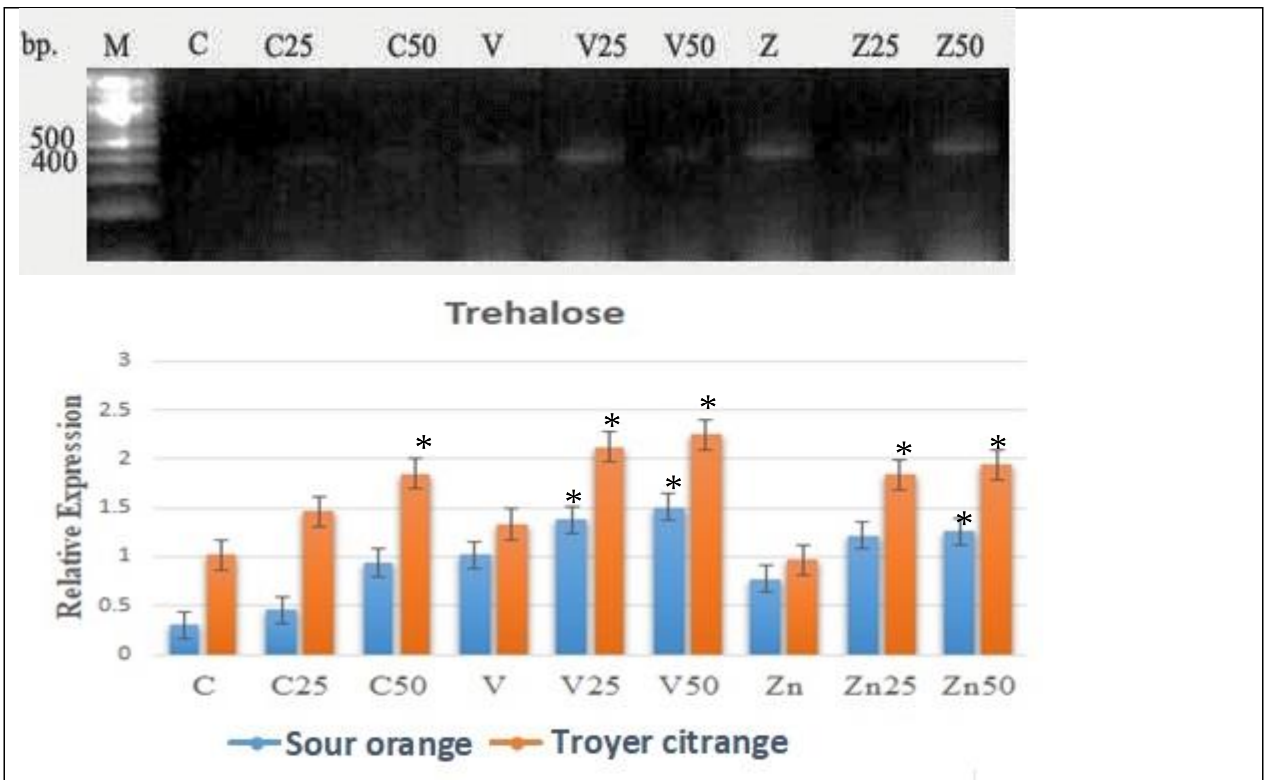
The antioxidant defense system protects the plant from salt-induced oxidative damage by detoxifying the ROS and maintaining the balance of ROS generation during salt stress (Hasanuzzaman et al., 2021). According to studies by Misra et al. (2006), and Tunc-Ozdemir et al. (2009), oxidative stress is one of the main factors that harm plants when they are exposed to salinity. Our research showed that plants under salinity stress enhanced antioxidant pathways compared to control plants (Fig. 2). The CAT enzyme activity recorded the lowest value in plants pretreated with Val/Zn at low and high levels of salt compared to untreated plants on both rootstocks (Fig. 2). Our results demonstrated that increasing salinity enhanced CAT, APX, GR, POD, and

PPO activity up to 50 mMNaCl. To address oxidative cell damage brought on by stress, plants produce a number of antioxidant enzymes, including CAT, APX, GR, POD, and PPO (Hatami et al., 2018). Since the CAT enzyme separates H_2O_2 into oxygen and water, it is crucial for plants to be able to withstand salinity. Many plants have been found to have a substantial association between their ability to tolerate salt and their enzymatic antioxidant defense system (Misra et al., 2006; Tunc-Ozdemir et al., 2009). The maintenance of cellular redox equilibrium, which is primarily provided by some non-enzymatic and enzymatic antioxidants like APX, CAT, POD, and GR, is necessary for plant defense against increased ROS under stressful conditions (Yan et al., 2013). These enzymes coordinated actions make it easier to maintain equilibrium between the production and detoxification of ROS (Hanin et al., 2016). The priming of the seeds with both Val/Zn before planting of investigated rootstocks alleviated the adverse effects of salinity by stimulating the growth and reducing antioxidant activity under severe salt stress conditions (50 mMNaCl) compared with the untreated control (Fig. 2).

Expression analysis of *Tre*gene

The relative expression of the *Tre* gene in sour orange and Troyer citrange rootstocks pretreated with Valdamycin A and $ZnSO_4$ is shown in Fig (3). The results indicated that pretreatment of both rootstocks with Val and Zn^{2+} induce several up regulations of *Tre*-encoding gene. A significant upregulation of *Tre*gene expression was observed under the two levels of salinity (25 and 50 mMNaCl) in treated and untreated plants (Fig. 3). Up regulation of *Tre*gene expression was recorded and resulted in a 2.25- and 1.95-fold increase in Val-treated and Zn^{2+} treated plants, respectively, of Troyer citrangeunder severe salinity (50 mMNaCl) compared with control plants. Moreover, Validamycin and ZnSO4 also significantly induced 1.51 and 1.26- fold increase in Val- and Zn^{2+} treated plants, respectively, of sour orange under severe salinity (50 mMNaCl) compared with control plants (Fig 3).

Plants can adapt and lessen the effects of stress by altering their genes and gene expression (Huang et al. 2019). Citrus is affected by salinity stress, which is managed through agronomic, breeding, and molecular methods. Plants employ a variety of defense mechanisms in response to osmotic stress to deal with unfavorable circumstances. Their detection and subsequent signal transmission are the early events that start metabolic reactions by activating numerous stress-responsive genes (Bray, 1997). The expression patterns of the *Tre* gene in response to salt stress (25, 50 mMNaCl) in the two rootstocks are shown in Fig. (3), to ascertain the effect of Val/Zn pretreatment on the expression of the *Trehalose*gene. In both treated and untreated plants, the results of the qRT-PCR experiments indicated up regulation at the two salinity levels (25 and 50 mMNaCl).In sour orange and Troyer citrange rootstocks treated with Valdamycin A and ZnSO4, the relative expression of the *Tre* gene results in numerous up regulations of *Tre*-encoding genes. These findings are consistent with those of Renard et al. (2007) and Smeekens (2017), who demonstrated that spraying Trehalose on wheat leaves increases their resistance to water stress and powdery mildew. The stimulation of genes involved in the manufacture of different osmolytes, such as proline, glycine betaine, and trehalose, as well as low molecular weight proteins, is a characteristic alteration in expression during a period of water scarcity (Ramanjulu and Bartels, 2002). Trehaose is essential for maintaining metabolic homeostasis and abiotic stress tolerance in a variety of organisms. Trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) are the enzymes that catalyze it. According to Blazquez et al. (1998), this osmo protectant stabilizes proteins and membrane structures under stress. Many plants have reportedly shown improved abiotic stress tolerance when exogenous and endogenous TPS-encoding genes are overexpressed (Almeida et al., 2007). Because the enzyme Trehalase hydrolyzes Trehalose to glucose, Trehalose levels are typically low in plants. It is a particularly competitive Trehalase inhibitor. Trehalose levels were increased in plant tissue after validamycin treatment (Müller et al., 1999). Validamycin A (C20H35NO13), and Zn^{2+} selectively and competitively inhibit Trehalase and increase the production of Trehalose in plant tissue. One of the most important micronutrients for plant development and productivity is zinc. Additionally, it contains a component that is needed at minute levels for the actions of a number of enzymes and proteins (Allouche, and Breitbart, 2020). Considering that it is crucial for plant growth and is involved in a wide variety of processes, rising data in recent years suggests that Trehalose and its derivatives may act as signal molecules that result in plant tolerance to a variety of stressors (Lunn et al., 2014; Mostofa et al., 2015). Better rootstocks can be created based on breeding procedures that can reduce the risk associated with the climate and other important abiotic factors (Ma and Constabel, 2019). Our understanding of how citrus interacts with these limitations will be aided by improved plant breeding and production approaches that increase plant resilience or stress tolerance.



Fig(3):Relative expression of the *Tre*gene in two citrus rootstocks sour orange and Troyer citrange. C: control plant, C25: Plants treated with 25 mMNaCl, C50: Plants treated with 50 mMNaCl, V: Val-treated plants, V25: Val- treated plants +25 mMNaCl; V50: Val- treated plants +50 mMNaCl; Z: Zn- treated plants, Z25: Zn-treated plants + 25 mMNaCl, Z50: Zn- treated plants + 50 mMNaCl. Asterisks (*) indicate statistically significant differences based on the one- way ANOVA ($p < 0.05$).

CONCLUSION:

Osmotic adjustment, a key process of salinity tolerance, is brought about by the buildup of compatible solutes like Trehalose in stressed plants. Better rootstocks can be created based on breeding procedures that can reduce the risk associated with the climate and other important biotic factors. In this work, the effect of two Trehalase enzyme inhibitors, Validamycin A and Zn^{2+}, on increasing Trehalose accumulation and inducing salinity tolerance in two citrus rootstocks, Sour Orange and Troyer citrange, was studied. Our findings suggest that application of pre-soaking of seeds with Val/ Zn alleviated the adverse effects of salinity by stimulating growth, increasing total chlorophyll content, and reducing antioxidant enzyme activity under severe salt stress conditions (50 mMNaCl) compared with the untreated control. In addition, it induces several upregulations of the *Tre*-encoding gene.

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تأثير تحضير البذور بمثبطات انزيم التريهاليز على إحداث تحمل الملوحة في أصول النارنج

و الترويز سترانج

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قسم بحوث تربية أشجار الفاكهة ونباتات الزينة والاشجار الخشبية ، معهد بحوث البساتين ، مركز البحوث الزراعية

تعد ثمار الحمضيات من أكبر محاصيل الفاكهة في العالم. تتعرض هذه المحاصيل للعديد من التحديات التي تسبب خسائر فادحة في المحصول بسبب الإجهاد اللاأحيائي كل عام. عندما يكون الإجهاد المائي عاملاً بيئيًا رئيسيًا ، تحتاج مناهج التربية إلى تطوير اصول جذرية مقاومة يمكن أن تخفف من مخاطر المناخ والعوامل غير الحيوية الرئيسية الأخرى. في هذا العمل تم دراسة تأثير اثنين من مثبطات إنزيم التريهالاز ,فاليداميسن و كبريتات الزنك على زيادة تراكم التريهالوز وتحمل الملوحة في كلا من اصل النارنج و الترويرسترانج.تشير النتائج التي توصلنا إليها إلى أن تطبيق النقع المسبق للبذور مع 30 ميكروليتر منالفاليداميسن و20 ملي مول من كبريتات الزنك قد خفف من الآثار الضارة للملوحة عن طريق تحفيز النمو ، وزيادة محتوى الكلوروفيل الكلي ، وتقليل نشاط إنزيم مضادات الأكسدة في ظل ظروف إجهاد الملح الشديدة (50 ملي مول كلوريد الصوديوم) بالمقارنة مع الكنترول غير المعالج. بالاضافة الى دراسة التغيير فى التعبير عن جين التريهالوز استجابةً لإجهاد الملح مع أو بدون معاملة بالفاليداميسن او الزنك