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Enhancement of Tolerance in Sour orange and Troyer citrangeRootstocks Challenged under Citrus Nematode (*Tylenchulus semipenetrans)* Infection

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ABSTRACT

Citrus crop production is affected by biotic and abiotic challenges that have a significant impact worldwide. Citrus nematode (*Tylenchulus semipenetrans*) is one of the most common diseases of citrus. The use of some natural or synthetic substances in plant defense mechanism or to induce a source of resistance in plants is a desirable goal, and one of these is Trehalose. In this study, the role of Validamycin A and Trehalose in inducing plant resistance against *T.semipenetrans* of sour orange (*Citrus aurantium L.*) and Troyer citrange (*Poncirus trifoliate (L) Raf. x Citrus sinensis (L) Osb,),* rootstocks was investigated. Induced resistance (IR) of such treatments was assessed through cytological, biochemical, and molecular approaches. The results showed that the total number of nematodes was significantly inhibited by each of the tested treatments, with reproduction factor (RF) ranging from 0.43 to 0.73 in sour orange, and from 0.69 to 1.2 in Troyer citrange. Among the two treatments, Trehalose significantly ($P \leq 0.05$) suppressed total nematodes with RF = 0.49 and 0.88 with reduction % 47.4 and 35.3, respectively, for sour orange and Troyer citrange. In addition, the behavior of *SA* and *PAL* genes involved in plant defense responses was determined. Our findings strongly suggest that the use of Trehalose improved the defense responses of both sour orange and Troyer citrange rootstocks against *T. semipenetrans*.

Key words: Citrus, rootstock, *Tylenchulus semipenetrans*, nematode, Trehalose.

INTRODUCTION

Citrus crop production is regularly impacted by biotic and abiotic challenges like pest infections, drought, salinity, and cold. These challenges have a significant worldwide effect. The most significant biotic stressors in Egypt are *Tylenchulus semipenetrans*, *Phytophthora*, *Fusarium* fungi, Citrus tristeza virus, and Citrus psoriasis virus (Abd-Elgawad, 2020). Egypt is the world's biggest exporter of fresh oranges and the sixth-largest producer of oranges, placing it among the top citrus-producing countries (Omar and Tate, 2018). The total area under cultivation of oranges in 2022–23 was 410,000 feddans, up by 4.5 percent from the previous year (USDA, 2022). Egypt's recently reclaimed land is being continuously expanded for agricultural use. The rise in demand for Egyptian citrus in both domestic and foreign markets is responsible for the expansion of the cultivated area. However, growing citrus seedlings in desert polders runs the danger of introducing plant-parasitic nematodes (PPNs), particularly citrus nematodes (*Tylenchulus semipenetrans*). The citrus nematode (*T. semipenetrans*) is one of the most often identified soil-borne citrus diseases. Around 40 million tonnes of fruit are lost each year, and more than half of the world's citrus-producing areas are infested with citrus nematode (Duncan and Cohn, 1990). Citrus plants slowly deteriorate because of nematode infections (O'Bannon and Esser, 1985), with the first signs being stunted development, leaf chlorosis and abscission, dieback, and small fruits. Furthermore, *T. semipenetrans* populations on citrus plants can typically reach very high levels before damage is evident. However, these hosts' capacities range from those of an extremely sensitive host to those of a low susceptibility host. No species of the genus Citrus has yet to develop immunity to these nematodes. Improved plant breeding and production techniques that enhance plant resilience or stress tolerance will aid our knowledge of how citrus interacts with these constraints. Applying molecules that increase a plant's resistance to pathogens by prompting defense mechanisms is the basis of several potential alternative control methods (Pel and Pieterse, 2013; Pushpalatha et al., 2013). The use of these natural disease control strategies may help understand the intricate processes underlying induced resistance while also offering novel, environmentally friendly disease prevention and treatment methods (Trouvelot et al., 2014). Recently, research on plant disease resistance has concentrated on sugar-mediated plant defense to determine whether it is possible to replace conventional agrochemicals with sugar-like compounds in various cropping systems (Moghaddam and Van den Ende, 2012). In plants, signaling sugars are likely to function as priming molecules for the immune responses triggered by effectors and pathogen-associated molecular pattern (PAMP)-triggered immunity. They may even facilitate immune responses against pathogens (Moghaddam and Van den Ende, 2012). For instance, Apple (*Malus domestica*) trees developed resilience to western flower thrips when 10 ppm fructose or glucose was applied topically, and the amount of chlorogenic acid and Trehalose in the leaves significantly increased (Derridj et al., 2011). This finding suggests that Trehalose may help enhance plant defense responses against the thrips. Exogenous sucrose application resulted in tolerance on apple trees to *Cydia pomonella* and in rice plants to *Magnaporthe oryzae* (30 M and 300 mM, respectively) (Ferré et al. 2008, Gomez-Ariza et al., 2007). Trehalose (1,1 α-D glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide that contains two units of glucose. It is catalyzed by trehalose-6-phosphate synthase (TPS) and, subsequently, trehalose-6-phosphate phosphatase (TPP) enzymes. Because the enzyme trehalase hydrolyzes Trehalose to glucose, Trehalose levels are typically minimal in plants. Therefore, it should be possible to increase Trehalose accumulation by lowering plant trehalase activity or by expressing the Trehalose biosynthetic genes under stress-specific regulation (Lunn et al., 2014). Trehalase is specifically competitively inhibited by Validamycin A ($C_{20}H_{35}NO_{13}$), which causes plant tissue to produce more Trehalose. Trehalose and its derivatives may function as signal molecules that cause plant resistance to a variety of stresses, according to mounting evidence in recent years (Lunn et al., 2014; Mostofa et al., 2015). Trehalose may facilitate host-microbe interactions and support plant defense, according to several studies. Trehalose, for instance, has been shown to improve the ability of *Arabidopsis thaliana* to defend itself against the green peach aphid (Singh et al., 2011), as well as the ability of wheat (*Triticum aestivum L.)* to withstand the powdery mildew brought on by *Blumeria graminis* (Reignault et al., 2000; Muchembled et al., 2006; Tayeh et al., 2014). Improved biotic and abiotic stress tolerance was induced by infusing tobacco leaves with a 10–50 mM Trehalose solution, which also increased resistance to the tobacco mosaic virus (Randoux et al., 2006). Trehalose might function as both a pathogen attack indicator and a virulence factor, as evidenced by the reduced fitness of a *Xanthomonascitri*. Trehalose synthesis mutant and the induction of defense gene expression when Trehalose was infused into citrus leaves (Randoux et al., 2010). In a different experiment, when the trehalase inhibitor Validamycin A was applied to tomato plants, it increased their resistance to bacterial and fusarium wilts as well as prompted systemic acquired resilience against a variety of other pathogens in Arabidopsis (Rausher et al., 1999; Renard et al., 2007). In addition, some tomato trehalose-6-phosphate synthase genes are silenced reducing susceptibility to *P. syringae pv*. tomato DC3000 and *Botrytis cinerea*, as well as altering the expression of defense-related, *JA*, *ET*, and *SA* signaling responsive genes (Riechers et al., 1996). By treating plants with Trehalose, many abiotic stress and plant protection genes are changed. The SA signaling mechanisms may, therefore, somehow be involved in the treatment of Trehalose.

A great way to decrease global citrus losses is to use resistant rootstocks (Anciso, 2002). The most widely used citrus rootstock in Egypt is the sour orange (*Citrus aurantium L.*), which has the best fruit and vegetative growth compatibility of all commercial citrus cultivars as well as adaptability to Egyptian conditions. In this study, we used two rootstocks, sour orange (*Citrus aurantium L.*) and Troyer citrange rootstocks, which were inoculated with *T. semipentrans* after pretreatment with 40 mM Trehalose and the trehalase inhibitor 30µM Validamycin A. The aim of the present study was of prime importance as it sought to assess whether exogenous Trehalose, and as a pre-soaked seeds with Validamycin A may enhance plant defense responses increase their efficacy and reduce the population of harmful *T. semipentrans*, which cause considerable losses in citrus trees, as well as evaluate the behavior of genes involved in plant defense responses.

MATERIALS AND METHODS

All *in vitro* and *in vivo* experiments and practical investigations were conducted at the Horticulture Research Institute and Nematodes Diseases Department, Plant Pathology Research Institute, ARC, Egypt, during 2021–2022. Seeds of two citrus rootstocks, sour orange (*Citrus aurantium L.*) and Troyer citrange (*Poncirus trifoliate (L) Raf. x Citrus sinensis (L) Osb,)*, supplemented by the Citrus Department, were used in order to study the effects of Validamycin A and Trehalose singly in comparison with Nemaphos (a commercial name for an organophosphate) on controlling *T. semipenetrans*. Fenamiphos (40 % EC) is the active ingredient that contains phosphoramidate ester. All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO).

Laboratory experiment (*in vitro*)

The effect of both Validamycin A and Trehalose substance was tested on *T. semipenetrans* juveniles survival under laboratory conditions. The chemical substance suspension and Nemaphos were separately added at a rate of 1 ml to 50 handpicked active nematode juveniles in vials (5 cm in diameter) to detect their effect on juvenile mortality of *T. semipenetrans*. The same number of juveniles received distilled water and served as controls. Each treatment was applied in four replicates. The percentage of juvenile mortality was recorded after 24 and 48 hours under a stereoscopic microscope. The bioassay experiment was replicated two times.

Greenhouse experiment (*in vivo*)

The current study was conducted under greenhouse conditions (27 ± 5 °C) to assess the role of Validamycin A and Trehalose in inducing plant resistance against *T. semipenetrans* and the consequent effect on sour orange and Troyer citrange, rootstocks. Induced resistance (IR) of such treatments was assayed through biochemical analysis and gene expression. The experiment was replicated twice over two years.

Experimental Design

Two trials were carried out using either seed soaking or foliar spraying applications, with 35 pots per each of the sour orange and Troyer citrange rootstocks. For the first trial, ten pots (diameter 35 cm, containing 7 kg sandy loam soil) received soaked seeds in 30µM Validamycin A (Lo´pez et al., 2009) for 8 hours (V), each rootstock was irrigated three times per week with tap water for three months, and then five pots per rootstock were inoculated with 1500 second-stagejuveniles (J2s)of*T. semipenetrans* (VF). Moreover, another five pots remained without inoculation (V).

However, for the second trial, twenty-five pots were planted with one seed/pot of each rootstock and irrigated with tap water for three months. Ninety days later, five pots were treated with Nemaphos (40% EC) as a conventional nematicide two days after nematode inoculation at a rate of 0.3g/pot. However, five pots received nematode alone (1500 J2) (F). Another five pots of untreated, uninoculated plants served as a control (C). Five pots were foliar sprayed twice with 40 mM Trehalose over two weeks, then inoculated with 1500 second stage (J2) of *T. semipenetrans* (TF). Moreover, five pots were left without inoculation and received foliar sprays of 40 mM Trehalose over two weeks (T). Pots were then arranged in a randomized complete block design in a greenhouse and irrigated three times with tap water.

Extraction of nematode from the soil and microscopic examination: Soil and root samples for the nematode assay of each treatment were sampled after one hundred and eighty days from germination. A compound microscope was used to count the *T. semipenetrans* that had been extracted using a modified sievingand centrifugation procedure (Grewal et al. 1999). To separate the roots from the soil and removing gravel , samples were sieved using a screen with a 4 mm aperture. The number of females and males in the soil or the number of females and eggs/egg mass in the root were used to express citrus nematode counts. The percentages of nematode decrease in either the soil or the roots (% efficiency)were calculated.

Histological studies: Roots from each treatment were washed free from soil particles, and selected portions of *T. semipentrans* infected citrus roots from untreated and treated plants from each rootstock were kept in F.A.A solution for fixation. Dehydration was made in a series of different concentrations of butanol and ethanol alcohol mixtures. Dehydrated root tissues were then infiltrated with paraffin wax at 50ºC for ten days. Then, they were sectioned at a thickness of 15 µ by a rotary microtome, stained with safranin and fast green, and mounted in Canada balsam according to the procedures of Sass (1964).

Biochemical Analysis: Using the method developed by Lichtenthaler and Buschmann (2001), the total amounts of chlorophyll a, b, and carotenes in fresh leaves were estimated. The free amino acid content was extracted using the technique outlined by Vartanain et al. (1992). Using the ninhydrin reagent approach, free amino acids were identified (Yemm et al. 1955). The Miller (1972) procedure was used to measure the concentration of reduced sugar. Trehalose content was extracted using the technique outlined by Lynch et al. (2010). The anthrone reaction was employed for Trehalose measurement, as stated by Umbreit et al. (1972). The modified Folin-Ciocalteu technique, as reported by Singleton et al. (1999), was used to calculate the total phenolic content (TPC) and the procedure outlined by Shimada et al. (1992) was followed in order to assess the antioxidant capability of using the stable radical DPPH.

RNA extraction and real-time RT-qPCR gene expression

The expression patterns of the salicylic acid (*SA*) and phenylalanine ammonia-lyase (*PAL*) genes were analyzed by real- time RT-qPCR using gene specific primers. For stress-induced expression assays, RNA was isolated from untreated and treated plants. Samples were ground to a fine powder under liquid nitrogen in a mortar and pestle, and total RNA was isolated using the Trizol reagent (Invitrogen). 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 RT-PCR was carried out using the salicylic acid gene-specific primer *SA* F: TTCTTCCACTTCGTCGGGTG, *SA* R: TGGACGCTAAGTTGTGTCTCT and Phenylalanine ammonia-lyase gene-specific primer, *PAL* F: CACAAATTGAAGCACCATCC; R: TTCTCAGGGCATAACGATCC); and the *Actin* gene F: CCAAAGGCCAACAGAGAGAAGAT, R: TGAGACACACCATCACCAGAAT.

Amplification of gene specific products from cDNA: initial denaturation at 94°C for 3 minutes, denature (94°C) for 45 seconds, anneal (54°C) for 45 seconds, extension (72°C) for 2 minutes each for 30 cycles, and final extension at 72°C for 8 minutes. The experiments were replicated at least three times. Both treatment and control samples had identical PCR efficiencies for the reference and target genes. The Applied Biosystems 7900 HT Fast Real-Time PCR System software was used to calculate CT values and relative abundance.

Statistical Analysis:

The statistical significance of the means was determined using SPSS 16 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to statistically analyze the treatment differences, and the Dunkan multiple range test was used to compare the means. For microscopic assays, logarithmic linear regression analysis followed by an analysis of covariance (ANCOVA) was carried out.

RESULTS AND DISCUSSIONS

Laboratory experiment (*in vitro*)

The effect of Validamycin A and Trehalose singly on *T. semipenetrans* survival was determined *in vitro* compared with Nemaphos (Table 1). Data showed that all tested treatments had various degrees of effectiveness towards the juvenile's survival compared with the control. Trehalose (60.0%) and Nemaphos (68.0%) treatments resulted in the best mortality percentages. Meanwhile, Validamycin A gave the lowest percentage (26.0%) after 24 hours of exposure. The same trend was achieved at 48 h as well (Table 1). The results of the two assays performed were similar.

Table (1): Mortality percentages of *Tylenchulus semipentrans* using Validamycin A and Trehalose substances compared to Nemaphos *in vitro*.

Treatments	Nematode (J2) after 4h Live	Dead	Mortality %	Nematode (J2)after 48h Live	Dead	Mortality %
Validamycin A	37.0b	13.0c	26.0	25.0b	25.0c	50.0
Trehalose	20.0c	30.0b	60.0	19.0c	31.0b	60.0
Nemaphos	16.0d	34.0a	68.0	10.0d	40.0a	80.0
Control	50.0a	0.0d	0.0	46.0a	4.0d	8.0

N= 50 second stages of *T. semipentrans* (J2).

Effects of Validamycin A and Trehalose substances singly compared with Nemaphos on controlling citrus nematode, *T.semipenetrans* in rootstocks of sour orange and Troyer citrange rootstocks were studied to figure out the most effective treatment to reduce nematode population. Data in Table (2) showed that the population density of *T. semipenetrans* juveniles drastically decreased after three months of infection. Results revealed that the total nematode population was significantly suppressed by both tested treatments, with reproduction factor (RF) ranging from 0.43 to 0.73 for the sour orange and from 0.69 to 1.2 for Troyer citrange. Among the two treatments, Trehalose significantly ($P \leq 0.05$) suppressed the total nematode population with RF = 0.49 and 0.88 and reduction % = 47.4 and 35.3in sour orange and Troyer citrange, respectively. Validamycin A had a low RF= (0.73&1.2) and Red % = (22.4 and 9.9) in sour orange and Troyercitrange, respectively, compared to the nematode alone. Nemaphos as a standard nematicide proved to be superior and showed the highest reduction in total nematode population (53.6 and 49.7%), with RF = (0.43 and 0.69) in both rootstocks, respectively. An analogous trend to the first season was noticeable in the second one.

Table (2): Effect of Validamycin A and Trehalose compared with Nemaphos on controlling *Tylenchulus semipentrans* after three months of infection on sour orange and Troyer citrange rootstocks under greenhouse conditions.

Treatments	Nematode population in * Soil (250g)	Root Developmental stages	Females	Eggs	Final population	**RF	% Reduction
Sour orange							
Validamycin A	350.0b	10.0b	30.0b	700.0b	1090.0	0.73	22.4
Trehalose	220.0c	3.0d	15.0c	500.0d	738.0	0.49	47.4
Nemaphos	100.0d	9.0c	12.0d	530.0c	651.0	0.43	53.6
Nematode alone	450.0a	21.0a	33.0a	900.0a	1404.0	0.94	0.0
Troyer citrange							
Validamycin A	810.0b	12.0b	26.0c	1000.0b	1848.0	1.2	9.9
Trehalose	570.0c	8.0c	28.0b	720.0c	1326.0	0.88	35.3
Nemaphos	350.0d	3.0d	19.0d	660.0d	1032.0	0.69	49.7
Nematode alone	900.0a	20.0a	40.0a	1090.0a	2050.0	1.37	0.0

Each value is the mean of five replicates

*Means in each column followed by the same letter(s) did not differ at P≤ 0.05 according to Duncan`s multiple range test

** Reproduction factor (RF) = Nematode population in soil + No. of developmental stages + No. of females + No. of eggs No. of the final population

Histological changes in citrus roots infested with *T. semipentrans* as affected by the addition of Validamycin A and Trehalose compared with Nemaphos.

Treated and untreated infected roots of used citrus rootstocks were processed for histological examinations. The citrus nematode, *T.semipentrans* induced pronounced alterations in cells of cortical and stellar regions in roots of citrus rootstocks, sour orange, and Troyer citrange rootstocks (Fig1. D & H) compared with Nemaphos (Fig. I & J). In both treatments, cells have dense granular cytoplasm, containing several hypertrophied nuclei scattered in the cytoplasm to various extents. In a few instances, large vacuoles were observed surrounded by dense cytoplasm. Sections showing nurse cells induced by *T. semipentetrans* in the cortex along the endodermis caused compression of endodermal and pericyclic cells. Interestingly, in the present work, the differences in feeding sites between treated and untreated roots were visible since in untreated roots, *T. semipentetrans* was able to induce healthy feeding sites in which the nurse cells occupied a considerable area in vascular parenchyma cells inducing disruption in endodermal and pericyclic cells (Fig.1 B & F) as shown with less disruption in cortical cells was found in the treatment of Nemaphos (Fig1 J & K). Mature females were observed within the tissue in apparent feeding position and the nematode's head was surrounded by nurse cells (Fig.1 A & E) compared with (Fig. C &G). Large vacuoles were also observed in the stellar region in roots treated with Validamycin A (Fig. 1E).

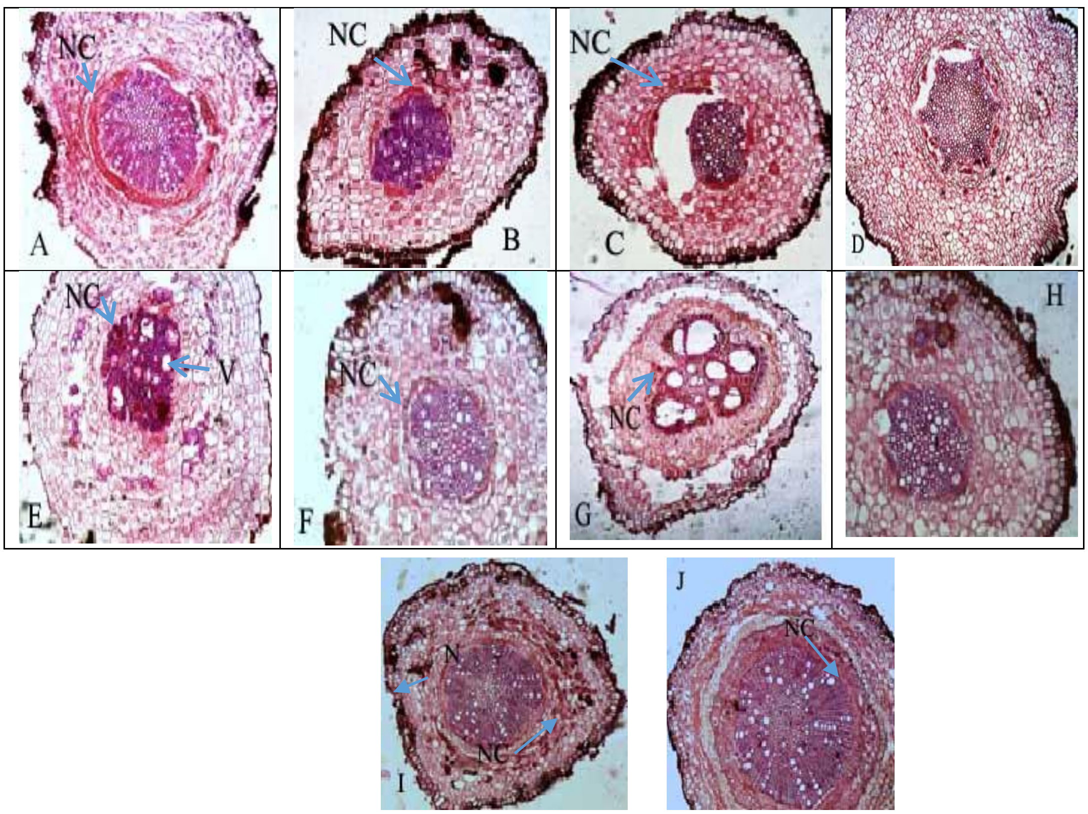


Fig (1).A-J): Histology of sour orange (A-D &I) and Troyer citrange rootstocks (E-H & J) feeder roots infested with *Tylenchulus semipentetratns* 1) Vesicle (V) formation in cortex (CO); 2) Cross section showing nurse cells (NC) induced by *T. semipentetrans* (N) in pericyclic cells comparing with Nemaphos (I &J), where is (A&E) Validamycin A; (B&F) Trehalose; (C&G) Nematode alone; and (D&H) plant health.

Influence of rootstocks and plant treatments on biochemical analysis

Photosynthetic pigments

The analysis of pigment contents, chlorophyll a, b, and carotenoids in uninfected and nematode-infected plants revealed significant variation under all treatments. The contents of Chl a and Chl b were reduced in infected plants compared with uninfected controls in the two rootstocks (Table 3). As well, a significant increase in chl a and chl b was observed in noninfected Trehalose-treated plants (T) in both rootstocks. The total carotenoid contents were not significantly changed during infection, except for noninfected trehalose- treatment plants, which recorded a significant increase (Table 3).

Amino acid, total phenol and antioxidant activity

Total free amino acid, phenol, and antioxidant activity values were represented in Fig. 2(a, b, c). Total free amino acid levels in infected plants were significantly higher than in uninfected controls in both sour orange and Troyer citrange rootstocks. Meanwhile, total free amino acids in plants treated with Val and Tre decreased significantly in infected plants when compared to inoculated plants (F). As compared to noninfected plants (C), there was a significant increase in total free amino acids in noninfected treated plants with each of Val (V) and Tre (T) (Fig. 2a).

Mechanism for scavenging oxygen free radicals during stress

The results showed a significant increase in the antioxidant activity of the DPPH radical in treated plants with both Val and Tre with nematode infection compared with noninfected (F) plants in both investigated rootstocks. There was an increase in the antioxidant activity in (F) of Troye citrange, while a slight increase in antioxidant activity in (F) of sour orange was observed.

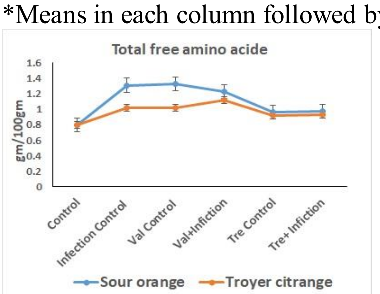
Total soluble sugars, reducing sugars, and Trehalose content

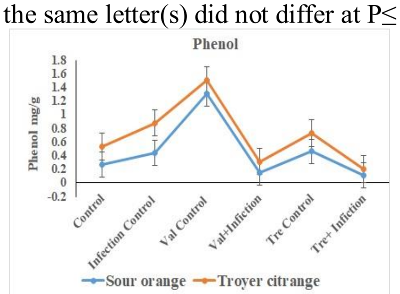
Our results also revealed that infected plants exhibited a reduction in soluble sugar content and an increase in reducing sugar compared with uninfected plants on both investigated rootstocks (Fig. 2 d). Analysis of reducing sugar obviously revealed a significant increase in pretreated plants with Val (V) and Tre (T), noninfected and infected, compared to the control plant (C) (Fig. 2e). The results in Fig. (2f) showed that accumulation of Trehalose content in infected plants compared to their uninfected peers, and treated plants on both rootstocks had greater values. Through a variety of processes, such as the accumulation of compatible solutes, plants can protect themselves from the harm caused by stress (Talaat and Shawky, 2014).

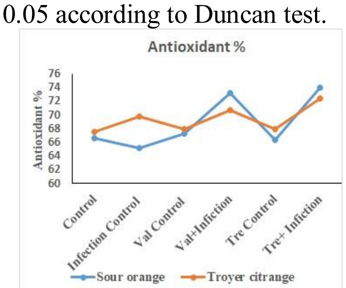
Table (3): Photosynthetic pigments of treated and untreated two citrus rootstocks plants inoculation with *Tylenchulus semipenetrans.*

Treatment	Chlorophyll a (mg/g)		Chlorophyll b (mg/g)		Carotenoids (mg/g)	
	Sour orange	Troyercitrange	Sour orange	Troyercitrange	Sour orange	Troyercitrange
C	0.62bc	0.69b	0.48b	0.46b	0.49b	0.50b
F	0.12e	0.10e	0.30c	0.29c	0.42b	0.40b
V	0.50d	0.45d	0.24c	0.25c	0.41b	0.40b
VF	0.54cd	0.51d	0.29c	0.31c	0.48b	0.46b
T	0.91a	0.83a	0.77a	0.75a	0.74a	0.72a
TF	0.53cd	0.49d	0.38bc	0.37bc	0.47b	0.42b

*Means in each column followed by the same letter(s) did not differ at P≤ 0.05 according to Duncan test.







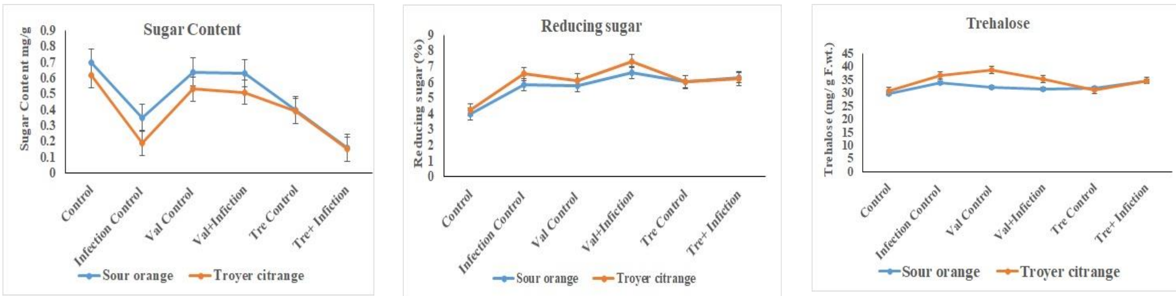
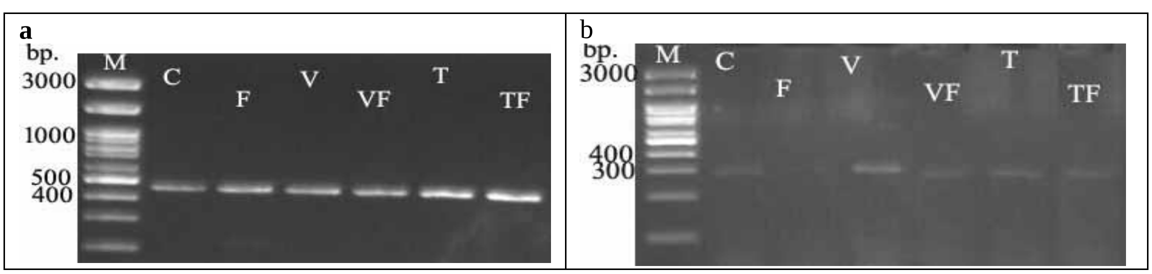


Fig. (2): Influence of Validamycin A and Trehalose treatments on biochemical analysis of citrus rootstocks under noninfectious and infectious conditions. Total free amino acids, total phenolic content, antioxidant activity, total soluble sugar content, reducing sugar content, and Trehalose content.

Expression analysis of *SA* and *PAL* genes

The relative expression of the two genes *SA* and *PAL* in sour orange and Troyer citrange rootstocks pretreated with Valdamycin A and Trehalose infected with *T. semipenetrans* is shown in Fig. 3 (a & b). The results indicated that pretreatment of both rootstocks with Val and Tre induces several upregulations of *SA* and *PAL*-encoding genes. A first significant upregulation of *SA* gene expression was recorded and resulted in a 6.6-fold increase in infected Tre-sprayed sour orange leaves (TF) and a 6.4-fold increase in infected Val-treated plants (VF) compared with control plants (C). It was followed by a second induction with a 5.8-fold increase in Troyer-infected plants (CF) and a 5.8-fold increase in infected sour orange (F) without any treatments. The *PAL* gene expression was induced on both rootstocks pretreated with Val (V), which recorded 3.0-fold increases compared to control. Moreover, Tre-spraying (T) also significantly induced a 1.8-fold increase in *PAL* gene expression compared with the control (C). While downregulation in the expression of the *PAL* gene was recorded in infected rootstocks (F) compared to control (C). Under treatment, significant inductions were observed: 1.2- and 1.8-fold increases in VF and TF, respectively, in sour orange rootstock, and a 1.5- and 1.6-fold increase in VF and TF, respectively, in Troyer citrange rootstock.



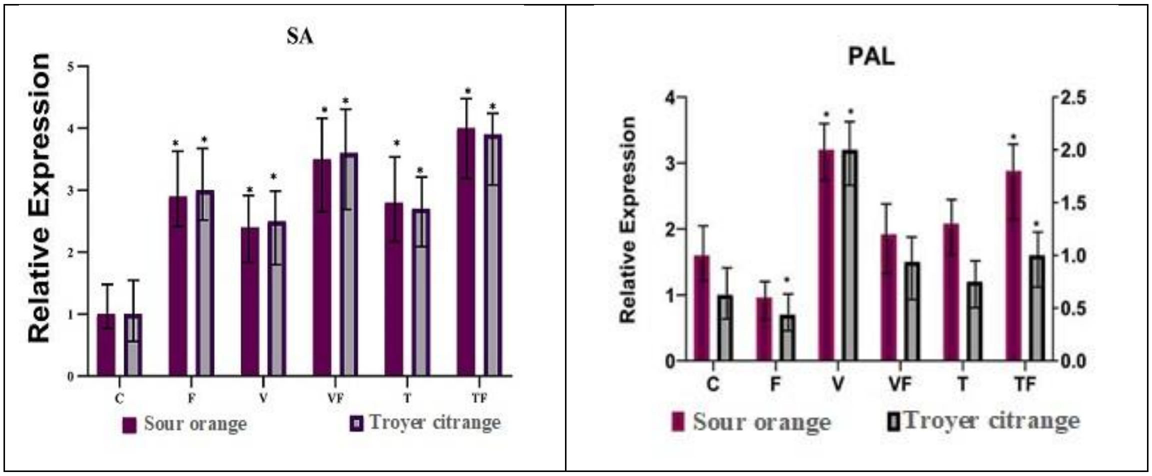


Fig. (3): Relative expression of (a) *SA* and (b) *PAL*1 genes in two citrus rootstocks inoculated with *T. semipenetrans*. C. Non-inoculated plants, F. Infected plants, V. Noninfected Val- treated plants; VF. Infected Val- treated plants; T. Noninfected Tre- treated plants, TF. Infected Tre-treated plants. Asterisks (*) indicate statistically significant differences based on One –Way ANOVA ($p < 0.05$).

Plant resistance to some pathogenic nematodes is a great way to start a nematode management program. A new strategy to control plant-parasitic nematodes relies on activating the plant's defense system through various biotic and abiotic factors. That is, organic acids, vitamins, inorganic minerals, sugars, etc. (Shalaby, 2012). The use of some natural or synthetic substances either in a plant defense mechanism or to induce a source of resistance in plants is a desirable goal. The induction of resistance through the introduction of various substances into susceptible nematode hosts has been studied by several researchers. Due to the lack of resistance in plants to most genera of plant nematodes as well as environmental limitations on the use of nematicides to control plant-parasitic nematodes, biological control and other environmentally friendly disease control measures have gained increasing attention recently. On the other hand, the use of induced resistance in plants has been suggested to have significant potential for biological control (Deverall, 1995). According to our findings, the overall nematode population was substantially reduced by both Validamycin A and Trehalose treatments, with reproduction factors (RF) ranging from 0.43 to 0.73 in sour orang and from 0.69 to 1.2 in Troyer citrange. Among the two treatments, Trehalose significantly ($P \leq 0.05$) suppressed the total nematode population with RF=0.49&0.88) and (Red. % = 47.4 &35.3) in the sour orange and Troyer citrange, respectively. Many studies on plant disease resistance have focused on sugar-mediated plant defense to observe if traditional agrochemicals can be replaced by sugar-like compounds in different cropping systems (Moghaddam and van den Ende, 2012). Signal polysaccharides likely function as precursors of pathogen-associated molecular pattern (PAMP)-inducible immunity and effector-induced immunity in plants, and may aid in immune responses against pathogens (Moghaddam and Van den Ende, 2012). It was confirmed in this study that the use of Trehalose enhanced the defense responses of both sour orange and Troyer citrange rootstocks against *T.semipenetrans*, through histological changes in citrus roots infested with *T. semipentrans* as affected by the addition of Validamycin A and Trehalose. The differences in feeding sites between treated and untreated roots were clearly visible since in untreated roots, *T.*

semipentetrans was able to induce healthy feeding sites in which the nurse cells occupied a considerable area in vascular parenchyma cells inducing disruption in endodermal and pericyclic cells (Figs B&F). Our findings are consistent with those of Pegard et al. (2005), who mentioned that root-knot nematode harm to root tissues could be reduced by treatments using inducers of resistance. According to Tordable et al. (2010), these changes may be an indication of the plant's reaction to the tested treatments as a defense against penetration of root-knot nematode. Trehalose may facilitate host-microbe interactions and support plant defense, according to several studies. Trehalose, for instance, has been shown to improve the ability of *Arabidopsis thaliana* to defend itself against the green peach aphid (Singh et al., 2011), as well as the ability of wheat (*Triticum aestivum* L.) to withstand the powdery mildew brought on by *Blumeria graminis* (Reignault et al., 2000; Muchembled et al., 2006; Tayeh et al., 2014). Trehalose might function as both a pathogen attack indicator and a virulence factor, as evidenced by the reduced fitness of a *Xanthomonas citri* trehalose synthesis mutant and the induction of defense gene expression when Tehalose was infused into citrus leaves (Randoux et al., 2010). In a different experiment, tomato plants were sprayed with the Trehalase inhibitor Validamycin A which increased defenses against bacterial and *Fusarium* wilts as well as the activation of systemic acquired defenses against a variety of other diseases in Arabidopsis (Rausher et al., 1999; Renard et al., 2007). Our findings of increased total chlorophyll content in plants pre-treated with Trehalose and Validamycin A compared to infected untreated plants suggested that PSII has adapted to the conditions caused by nematode infection. The level of carotenoids did not change significantly either, suggesting that the photosynthetic system's ability to protect itself and its antioxidant capacity were adequate during infection with *T. semipentrans* (Siefermann-Harms, 1987). Higher chlorophyll amounts under Trehalose spray treatment may be related to its ability to repair stress-dependent damage (Jun et al., 2008). Additionally, the use of 30 µM Validamycin A was effective in reducing the inhibitory effect of stress on photosynthetic pigments (Said, 2017). Analysis of the pigment contents in infected plants showed a significant decrease in Chl (a) content compared to uninfected plants (Table 3). The decreased Chl a content may be due to reduced protein synthesis in light-harvesting complexes (Allakhverdiev et al., 2008) or to Chl a degradation caused by oxidative damage to chloroplast lipids, pigments, and proteins (Tambussi et al., 2005). Photosynthesis, as the most basic and complex physiological process, is the source of reactive oxygen species in plant cells. In chloroplasts, reactive oxygen species are generated when photon absorption exceeds photosynthetic efficiency and photo protective mechanisms are perturbed (Tambussi et al., 2005). In addition, a reduction in phenol content was observed in infected plants treated with both Validamycin A and Trehalose on both rootstocks compared to infected untreated plants. These results contradicting trend was found by Labudda et al. (2016), who reported increased contents of phenols in shoots from *H. schachtii*-infected *A.thaliana*. El Hariri et al. (2010) recorded a significant increase in total phenols on onion and flax plants, respectively, under stress conditions. The use of both treatments reduced the harmful effects of *T. semipenetrans*. The ability of phenolic to act as antioxidants is primarily due to their properties as hydrogen donors, reducing agents, and singlet oxygen (O2) quenchers

(Zhang et al., 2001). Without treatments, there was an increase in antioxidant activity in the infected Troyer citrange plants, while there was a slight increase with the infected control of sour orange. According to our findings, antioxidant mechanisms were induced in infected plants compared to their uninfected peers, and treated plants on both rootstocks showed greater values. One of the early reactions of plants to pathogens is the production of ROS in plant cells (Torres, 2010). Our findings support the hypothesis proposed by (Labudda et al., 2018) in *Heterodera schachtii*-infected *A. thaliana* shoots stating that infection causes ROS production in diseased roots, shoots, and leaves in addition to the infected roots. Infected plants accumulated anthocyanins at a rate that was considerably higher (1.7-fold) than non-infected plants. Siddique et al. (2014) showed enhanced ROS generation in *A. thaliana* roots at the earliest stages of nematode infection. Bairwa and Patel (2014) discovered that nematode infection resulted in significantly higher reducing sugar content in tobacco leaves. The presentresults showed that infected plants accumulated more Trehalose content than that of uninfected plants, and treated plants in both rootstocks had higher values. Through a variety of processes, such as the accumulation of compatible solutes, plants can protect themselves from the harm caused by stress (Talaat and Shawky, 2014). Trehalose, a non-reducing disaccharide of glucose, possesses the ability to stabilize biological structures and macromolecules like proteins and membrane lipids in a variety of organisms under stress (Ali & Ashraf, 2011).

The expression profiles of defense-related genes in infectious conditions after Val pre-treatment and Tre spraying of the two rootstocks is shown in (Fig. 3 a & b). To determine the impact of Trehalose on defense gene expression, the expression of *SA* was expressed, suggesting treatment with Trehalose may have an impact on *SA* levels. The results of the qRT-PCR tests showed the investigated plants respond to Trehalose by increasing expression of the genes SA and the *PAL*-encoding gene, which are involved in SA biosynthesis pathways. The finding that *SA* and *PAL* genes were upregulated with Val and Tre treatment suggested that *SA* and PAL responses could be required for a Trehalose-mediated increase in nematode resistance. A significant upregulation of *SA* responsive rootstock defense genes in infected Tre-sprayed treatment (TF) and infected Val-treated plants (VF)was observed compared with control plants.These results concur with the findings of MacIntyre et al. (2022) who reported that Trehalose treatment upregulated *SA* dependent tomato defense genes and improved SA-insensitive NahG tomato plant resilience to bacterial wilt. Milling et al. (2011) suggest that treatment with Trehalose appears to stimulate *SA* mediated resilience pathways, which may have an effect on *Ralstonia solanacearum* infection in tomato plants. Renard et al. (2007) and Smeekens (2017) showed that spraying Trehalose on wheat leaves makes them more resistant to water stress and powdery mildew. The same gene is upregulated in infected rootstocks without any treatment. This finding might indicate that the *SA* plant hormone, which mediates *T. semipenetrans* resistance in many species, is also required for inducing plant immunity and activating systemically acquired resistance. However, under infectious conditions, the expression of the *PAL* gene was downregulated in infected plants in both investigated rootstocks, while it was upregulated with Val and Tre treatment. *SA* is generated from chorismate or phenylalanine by two key enzymes, isochorismate synthase (ICS) and phenylalanine ammonia lyase (*PAL*), respectively (Nugroho et al., 2002). Other plant species make different ICS or *PAL* inputs to the production of SA during infection. For instance, *SA* is primarily obtained from the tobacco plant's phenylpropanoid pathway (Lee et al., 1995). In comparison, when pathogens infect soybean, *SA* biosynthesis is similarly influenced by the ICS and *PAL* pathways (Shine et al., 2016).

Conclusion

The use of some natural or synthetic substances that have already been used either as a plant defense mechanism or to induce a source of resistance in plants is a desirable goal. In order to determine the most efficient method of nematode population reduction, the effects of Validamycin A and Trehalose material alone compared with Nemaphos on suppressing citrus nematodes *T.semipenetrans* in rootstocks of sour orange and Troyer citrange were evaluated. According to our findings, the overall nematode population was substantially reduced by both Validamycin and Trehalose treatments. In addition, the behavior of *SA* and *PAL* genes involved in plant defense responses was determined. Our findings strongly suggest that the use of Trehalose improved the defense responses of both sour orange and Troyer citrange rootstocks against *T. semipenetrans.*

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تعزيز المقاومة فى أصل النارنج والتروير سترانج والتى تواجه عدوى نيماتودا الموالح

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يتأثر إنتاج محاصيل الحمضيات بالتحديات الحيوية وغير الحيوية التي لها تأثير كبير في جميع أنحاء العالم. نيماتودا الموالح هي واحدة من أكثر أمراض الحمضيات شيوعًا. يعد استخدام بعض المواد الطبيعية أو الاصطناعية في آلية الدفاع عن النبات أو للحث على مصدر مقاومة في النباتات هدفًا مرغوبًا، واحد هذه االمواد هوالتريهالوز. في هذه الدراسة تم دراسة دور كلا من مادة التريهالوز والفاليداميسن فى إحداث وتحفيز مقاومة كلا من أصل النارنج والتروير سترانج ضد ديدان نيماتودا الموالح. تم تقييم المقاومة المستحثة لمثل هذه العلاجات من خلال الأساليب الخلوية والكيميائية الحيوية والجزيئية.

أظهرت النتائج أن العدد الإجمالي للديدان الخيطية قد تم تثبيطه بشكل كبير بواسطة كل من المعاملات المختبرة ، حيث تراوح عامل التكاثر (RF) من 0.43 إلى 0.73 في النارنج ، ومن 0.69 إلى 1.2 في التروير سترانج . من بين المعاملتين ، قام تريهالوز معنوياً ($P \leq 0.05$) بقمع الديدان الخيطية الكلية مع RF = 0.49 و 0.88 مع انخفاض بنسبة 47.4٪ و 35.3٪ على التوالي بالنسبة للنارنجوالتروير سترانج. بالإضافة إلى ذلك ، تم تحديد سلوك جينات SA و PAL المشاركة في استجابات دفاع النبات. تشير النتائج التي توصلنا إليها بقوة إلى أن استخدام Trehalose أدى إلى تحسين الاستجابات الدفاعية لكل من اصلالنارنج و والتروير سترانج ضد *T. semipenetrans*.