EFFECT OF TYLCV AND SALINITY ON GROWTH AND ACTIVITY OF SOME ANTIOXIDANT ENZYMES IN TOMATO PLANTS

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Tomato is a member of the Solanaceae family, genus *Lycopersicon* (Britannica, 1990). It is one of the most widely cultivated economically important vegetable in the world. Epidemiological studies suggest that intake of tomatoes or processed tomato products in particular lowers the risk of prostate cancer due to its antioxidant contents (Campbell *et al*., 2004).

Flavonoids are a diverse group of phenolic secondary metabolites that occur naturally in plants and therefore form an integral component of the human diet. Many of the compounds belonging to this group are potent antioxidants *in vitro* and epidemiological studies suggest a direct correlation between high flavonoid intake and decreased risk of cardiovascular disease, cancer and other age related diseases (Verhoeyen *et al*., 2002).

TYLCV is a member of Geminiviruses which is characterized by having a genome of a single stranded DNA contained in geminate particles. Within this group of viruses, two main subgroups can be recognized; one is transmitted by leafhoppers and the other is transmitted by whiteflies (Goodman, 1977). Members of the family *Geminiviridae* are small single stranded DNA viruses (Mart *et al*., 2009) which can be divided into three subgroups based on genome organization, host range and type of insect vector (Hamilton *et al*., 1983).

Subgroup I (genus *Masterovirus*) comprises viruses that have a single component genome, transmitted by leafhopper, and usually infect monocotyledonous plants. It includes maize streak virus (MSV) and wheat dwarf virus (WDV). Subgroup II (genus *Curtovirus*) comprises members that have a monopartite genome and are transmitted by leafhopper vector. They infect only dicotyledonous plants like beat curly top virus (BCTV). Subgroup III (genus *Begomovirus*) comprises members that have monopartite genomes (2.5 Kb) as well as bipartite ones. For bipartite viruses, the two genomic DNAs have similar size (2.6 Kb) and are designated A and B. However, they differ in sequence except for a common region of 200-250 bp that is nearly identical in the genome components of any given virus but differs between different viruses. Both

genome components are required for infectivity (Hamilton *et al*., 1983; Rojas *et al*., 2000).

Morris *et al*. (2002) stated that the detection of TYLCV in tomato plants was achieved two weeks after whitefly fumigation with improved frequency of detection at four weeks. They also found out that the PCR method is more sensitive than triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for the detection of TYLCV isolates in all known hosts. Recently, Abou-Jawdah *et al*. (2006) used the PCR reaction to confirm the presence of TYLCV in tomato plants in Lebanon.

Salt contamination in soil is one of the major challenges for agriculture and tomatoes appear as one of the most consumed fruit in the world (Pierre *et al*., 2009). A better understanding of NaCl effects on tomato plants growth is thus essential.

Excessive soil salinity is an important problem for agriculture; however, salt tolerance is a complex trait that is not easily bred into plants. Exposure of cultivated tomato to salt stress has been reported to result in increased antioxidant content and activity. Salt tolerance of the related wild species, *Solanum pennellii*, has also been associated with similar changes in antioxidants (Frary *et al*., 2010)

Numerous studies showed that the level of antioxidative enzymes increases when plants are exposed to either biotic or abiotic stresses including salinity. Comparison of the responses of cultivars and/or related species that exhibit differential sensitivity to salt stress showed a correlation between salt tolerance and increased activity of the antioxidant system (Sumalee *et al*., 2008)

Salinity stress is a common consequence of insufficient water supply and/or using poor quality water. Salinity may naturally exist particularly in arid and semi-arid regions such as Egypt (Alaa *et al*., 2009). Salinity is a well known factor affecting negative growth and production of many crops such as tomato and sweet pepper. Thus Tomato represents a good model for studying the mechanism of plant adaptation to salt. In the present work, some tomato plants had been infected with TYLCV and others were treated with different concentration of NaCl during the reproductive growth stages and the effect of salinity on some aspects of growth and physiology, including protein content and some antioxidant enzyme activity were examined.

This study was conducted to study the biochemical effects of TYLCV and salinity on nutritional quality and activity of some antioxidant enzymes in tomatoes.

MATERIALS AND METHODS

Plant Materials

Seeds of tomato were germinated in greenhouse at AGERI with 16 hr illumination per day. Plants were divided into two groups; the first group was subjected

to TYLCV infection and the second one was subjected to salinity stress.

Total DNA extraction of tomato plants

Leaves of inoculated plants were used to extract DNA with DNeasy Plant Mini Kit supplied from Qiagen Hilden Germany catalog number 69104 (Riha *et al.*, 1998). Tissues were grinded using liquid nitrogen and 400 µl of AP1 buffer and 4µl of RNase stock solution (100 mg/ml) to a maximum of 100 mg of ground tissue, the mixture was incubated for 10 min at 65°C. The tube was mixed 2-3 times during incubation, then 130 µl of AP2 buffer was added to the lysate, mixed and incubated for 5 min on ice. The lysate was applied to mini spin column (pink) and centrifuged for 2 min at 20,000 Xg then the supernatant was transferred to a new tube and 1.5 volume of AP3 buffer was added and mixed by pipetting. The supernatant was transferred to mini spin column (white) and centrifuged for 1 min at 6000 Xg then the column placed in a new 2 ml collection tube. A volume of 500 µl AW buffer was added to the column, centrifuged for 1 min at 6000 Xg and the flow through was discarded then this step was repeated. The column was transferred to a new tube and 100 µl of AE buffer were added directly on the column then incubated for 5 min at room temp. The column was centrifuged for 1 min at 6000 Xg to elute the DNA, which was stored at -20°C. Extracted DNA was subjected to (PCR) using WTGs specific primers HD1 and HD2 and TYLCV-CP specific primer.

DNA extraction from whitefly

Three to five whiteflies were placed in an Eppendorf tube containing 100 µl of 0.4% SDS, 100 µg per ml proteinase K, then it was grinded using a glass rode, following 1 hr incubation at 55°C. Extracted DNA was subjected to (PCR) using WTGs specific primers HD1and HD2.

Polymerase chain reaction (PCR)

Primers used in this work were designed from the nucleotide sequence for Tomato yellow leaf curl virus Egyptian isolate (TYLCV-Eg) (Abdallah *et al*., 1993). The oligonucleotide primers were synthesized at Agriculture Genetic Engineering Research Institute (AGERI) Agriculture Research Center (ARC, Giza, Egypt) on an ABI 392 DNA/RNA synthesizer (Applied BioSystem, Lincoln Center Drive, Foster City, CA, USA). The primers position, direction and sequence are listed in (Table 1).The PCR was carried out as described by Essam *et al*. (2004). The primers used to amplify TYLCV- Eg viral genome and position of the PCR products are shown in Table (1).

Whiteflies mediated inoculation:

Female whiteflies (*B. tabaci*) were reared on cotton plants (*Gossypium hirsutum* L.) grown in insect wooden proof cage in controlled temperature room provided with 16 hr illumination per day for one month; and tested by PCR using WTGs specific primers HD1and HD2 to be sure that they are virus free.

Group of 50-100 virus free whitefly were given an acquisition access period on infected tomato plants with TYLCV for about 48 hrs. Viruliferous whiteflies were transferred to four healthy tomato plants two month old in insect wooden proof cage for an additional 48 hrs to insure plants inoculation as 20 min is enough to infect 30%of plants and 4 hrs are enough to infect 90% of the tested plants. A latent period of 6 hrs was required for the *Bemisia tabaci* to retain its ability to infect the test plants. After exposure, the plants were sprayed with Bremor (1.5 ml per liter distilled water) (Aref *et al.*, 1995) and the plants were tested for the virus presence after 17 days of inoculation using WTG specific primers HD1, HD2 and TYLCV-CP specific primers.

Salinity treatment

Sodium chloride was added in concentrations of 0, 25, 50 and 100 mM to the second group when the plants were 45 days old. The plants were then grown for the next 75 days during which the salinized solutions were changed every 5 days under green house conditions. Four plants were grown for each salinity treatment. After 75 days of salinity treatment, growth parameters, activity of some antioxidant enzymes and total protein content in leaves of 120 day old were determined.

Growth and Yield

One hundred and twenty days old plants were harvested and their morphological characteristics such as plant height, number of lateral shoots and number of flowers and day of first flowering were determined.

Protein content

The two groups of plants were analyzed at 120 days old for their total protein content. Fully expanded mature leaves at the sixth node from the shoot apex were harvested and frozen in liquid nitrogen prior to storage at -80°C. Sample tissue (0.5 g) from each sample was ground on ice with a mortar and pestle with 5ml of 10nM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone. Crude extract was centrifuged at 12000 Xg for 30 min at 4°C and the supernatant was used as enzyme extract. The amount of protein in the enzyme extract was determined by the Bradford method's (1976).

Antioxidant enzyme activity

Ascorbate peroxidase (APX) activity was determined by measuring a decrease in optical density at a wavelength of 290 nm. The reaction mixture (3 ml) contained 50 mM ascorbic acid 0.1 mM EDTA, and 0.1 ml enzyme extract. The reaction was started by adding hydrogen peroxide to 1.5 mM. The non-enzyme extract mixture served as the blank. The ascorbate peroxidase activity was calculated using the extinction coefficient of 2.8 $nm^{-1}cm^{-1}$ (Nakano and Asada, 1981) and the activity was expressed as mmol ascorbate oxidized $mgprotein^{-1}$ $min^{-1.}$

Catalase activity was determined by measuring the initial rate of disappear-

ance of hydrogen peroxide. The reaction mixture (3 ml) contained 10 mM potassium phosphate buffer (pH 7.0) and 0.1 ml enzyme extract and the reaction was started by adding 0.035 ml of 3% hydrogen peroxide. A decrease in hydrogen peroxide concentration was followed by a decline in optical density at the wavelength of 240 nm. The non-enzyme extract mixture served as a blank. The catalase activity was calculated using the extinction coefficient of 40 mM-1cm-1 and the activity was expressed as μmol H_2O_2 reduced mg protein^{-1}min^{-1}.

RESULTS AND DISCUSSION

Polymerase chain reaction (PCR)

Whiteflies were tested by PCR using WTGs specific primers HD1 and HD2 after one month from rearing on cotton plants and it give negative results after that whiteflies reared on infected tomato plants with TYLCV for 48 hr and tested using the same primers for viral presence it give positive results, these viruliferous Whiteflies were used to inoculate two month old healthy tomato plants.

DNA extracted from inoculated tomato plants after 17 days from inoculation with whiteflies, was used in PCR analysis with WTG specific primers (HD1, HD2 and Cp-F, Cp-R). The cloned TYLCV genome was used as positive control in PCR experiments. The amplified fragments gave the expected molecular weight at 674 bp as the positive control (Fig. 1). This result indicates that the virus which infected the examined tomato plants is TYLCV compared to control plants. These results were in agreement with the results of Abou-Jawdah *et al*. (2006) and Morris *et al*. (2002) that used the PCR reaction to confirm the presence of TYLCV in tomato plants in Lebanon.

Growth parameters at the Reproductive Stage

Salinity treatment and viral infection caused retardation in growth and development of tomato plants during both vegetative and reproductive phases with different ratios. Stressed plants with 50 and 100 mM NaCl produced flowers and fruits more slowly and the fruits were smaller compared to non-stressed plants (Fig. 2) while infected plants produced flowers and fruits nearly in the same time of controlled ones due to late infection with the virus. Plant height and number of lateral shoots were reduced in infected and stressed plants with different ratios compared to control plants. The effects of TYLCV and salinity treatment on the vegetative growth and reproductive parameters; plant height, number of lateral shoots and number of mature fruits of 120-day old plants after 75 days of NaCl treatment are summarized in Table (2).

In control, infected and non stressed condition, the plants flowered at the range of 80 days in winter. Under 50 and 100mM NaCl stress, flowering was significantly delayed for 12 days. However, late infection and 25 mM NaCl had no significant effects on the vegetative and reproductive parameters

Similar observations were reported by Cuartero and Fernandez-Mu Hoz (1999). Also, Sumalee *et al*. (2008) observed that low level of salinity (25mM NaCl) had no deleterious effect on vegetative growth parameters, but caused significant decrease in the number and size of mature fruits. They reported that in the case of higher concentrations of NaCl (50 and 100 mM), both vegetative and growth parameters were drastically reduced.

Some Physiological parameters of mature leaves at the reproductive Stage

Salinity stress induced change in several physiological parameters in mature leaves of 120-days-old plants after 75 days of NaCl treatment. Leaf protein was significantly reduced in infected and stressed plants subjected to 25, 50 and 100 mM NaCl compared to the infected and non-stressed plants (Table 3).

In the investigation of the activity of antioxidative enzymes, the response of catalase to NaCl was somewhat different from APX. It is apparent that APX activity highly increased in virus-infected plants and plants treated with 25, 50 and 100 mM NaCl, respectively (Table 4). On the other hand, the activity of Catalase was enhanced in infected plants and when the plants were treated with 25 and 50 mM NaCl, respectively, but it decreased when treated with higher salt concentrations (Table 5). Also, Fig. (3) represents the effect of salinity and TYLCV on protein content, activity of ascorbate peroxidase (APX) and catalase (CAT) in mature leaves.

Similar observations were reported by Cuartero and Fernandez-Mu Hoz (1999). Also, Sumalee *et al*. (2008) observed a reduction in protein content and enhancement of APX and CAT, while CAT was reduced in case of 100 mM NaCl. Although salinity treatment caused a significant reduction in fruit yield, it could improve fruit quality by increasing sucrose content.

An important consequence of salinity stress is the generation of excessive reactive oxygen species (ROS) which lead to cell toxicity, membrane dysfunction and cell death (Sumalee *et al*., 2008). Tomato plants have been reported to be able to defend against the ROS by an increased production of antioxidative enzymes including CAT and APX. In our tomato cultivar, this raise was recorded. Similarly, Chaparzadeh *et al*. (2004) also, observed an increase activity of APX and CAT in leaves of *Caendula officinalis* L. in response to salt stress. The rise in catalase activity at low and moderate NaCl concentration is correlated with cellular defense against salt stress. Under high salt stress, CAT is inhibited and leaf cell are protected from oxidative damage by APX and superoxide dismutase (SOD) as reported by Mittova *et al*. (2002), who found that a great activity of these antioxidant enzymes resulted in less oxidative stress.

Also, there are many studies which state that stress has a preventive effect on catalase activity. They stated that cell destruction based on salt stress has a distinc-

tive role in decreasing catalase activity. According to Prasad (1997) and Shalata *et al*. (2001), catalase has a distinctive role in protection against salt stress. Prasad (1997) found that catalase provides the best relationship with salt tolerance among the enzymes he has worked with. Also, Dogan (2011) found that tolerance to salt stress changed with time, and has caused catalase activity to decrease in young and old leaves of salsola (Stenoptera) plant which is considered a halophyte, and tomato (*Lycopersicon esculentum* L.) which is considered a glycophyte.

He *et al*. (2009) reported that a high level of CAT activity obtained from rootstock tomato plants under salty conditions indicate that rootstocks act as an agent to keep active oxygen radicals. It was also stated that CAT activity acts as one of the effective enzymes in preventing cellular impacts in a manner similar to what we found in our tomato cultivar subjected to mild NaCl treatment (25 and 50 mM) and that its level decreases together with the increase in salinity (Al-Aghabary *et al*., 2004). Another research study indicated that H_2O_2 quantity increases in the leaves of rice as a result of decreased CAT activity due to salinity treatment (Khan *et al*., 2002).

Higher POX level obtained under salt conditions indicates that the plant is capable of getting rid of the H_2O_2 from the environment in a quicker manner (Kraus and Fletcher, 1994; Upadhyaya *et al*., 1989).

Oztekin and Tuzel (2011) studied some commercial tomato rootstocks for their salt tolerance levels and found out that salt stress decreased plant vigor, and rootstocks yielded better performance than non- and self-grafted treatments. In tolerant genotypes, CAT activity decreased while POX activity increased with increased salinity levels and both activities occurred higher in grafted treatments. They concluded that grafting may be considered as an alternative strategy to enhance salt tolerance in tomato (Oztekin and Tuzel, 2011).

In our experiments, both peroxidase and catalase enzyme activities have been highly induced following TYLCV infection which is in agreement with the results of Liao *et al*. (2012) who found that TMV inoculation induced the activities of SOD, APX, CAT and GPOD (glutathione peroxidase) suggesting that the TMV infection resulted in elevated ROS formation in the inoculated leaves, and the antioxidant enzyme induction under this condition seemed to function to reduce this ROS elevation (Liao *et al*., 2012).

In agreement with the elevated activity of antioxidant enzymes following viral infection, Dieng *et al*. (2011) observed an increased POD activity after TYLCV infection, during infestation and in mature leaves of *Solanum lycopersicum.* The increase in CAT and POD activity during TYLCV infection, in the present study may arise by different mechanisms, thus additional works are needed to characterize the mechanisms of

changing the concentration of catalase(s) and peroxidase(s) during infection (Dieng *et al*., 2011).

Further studies are needed to find out a suitable mechanism that enables plants to tolerate salinity with balanced levels of fruit size and quality.

SUMMARY

This study was carried out to test the effects of NaCl and TYLCV on growth, total protein and some antioxidant enzymes activity of tomato (*Solanum lycopersicon*) cultivar CastleRock. First group of plants (60 days old) were infected with TYLCV. Mature leaves were harvested and analyzed for virus presence after viral infection. While, the second group of plants at 45 days old were treated with 0, 25, 50 and 100 mM NaCl for the next 75 days. At 120 days old for both groups different growth parameters were recorded. Mature leaves were harvested and analyzed for the amount of total protein and for the level of activity of both ascorbate peroxidase (APX) and catalase (CAT).

Plants exposed to the lowest level of salinity (25 mM NaCl) and the viral infected plants showed little effect on vegetative growth parameters, total protein, catalase and ascorbate peroxidase activity, but it caused a decrease in the number of mature fruits in both cases. While at higher concentrations of NaCl (50 and 100 mM), vegetative and fruit growth parameters were drastically reduced. Salinity treatment caused a reduction in total protein content of plants and an increase in ascorbate peroxidase activity. Whereas catalase activity increased in the TYLCV infected plants and plants treated with 25 and 50 mM NaCl and decreased in plants treated with 100 mM NaCl.

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Table (1): Nucleotide sequences of the primers used to amplify the TYLCV-Eg viral genome according to Abdallah *et al*. (1993) and position of PCR product.

Primer name	Nucleotides Sequence	Added restriction site	Position of Amplified fragment	length
Cp-F	5'-CGGAATTCACATGTCGAAGCGACCAGG-3'	BamHI	467-1253	787
Cp-R1	5'-CGGGATCCTTAATTTGATAATGAATC-3'	*Eco*RI		
HD-1	5´-CGGAATTCGCCCACCAATAACTGTAGC-3´	EcoRI	1855-2528	674
HD-2	5´-CGGGATCCGCAGTCCGTTGAGGAAACTTAC-3´	*Bam*HI		

Table (2): The effect of TYLCV and salinity on vegetative and reproductive parameters of 120 days old tomato plants.

	Plant height	Number of lateral shoots	Days until first flowering	Number of mature fruits
Control plants	40.75	11.75	80.0	6.25
Infected plants	29.00	9.00	81.5	5.50
25 mM NaCl	34.00	10.25	81.5	4.50
50 mM NaCl	28.25	7.50	92.5	3.75
100 mM NaCl	20.75	5.75	92.5	2.50

These results represents average ratio between the plants

Table (3): Effect of NaCl and TYLCV on protein content of tested plants. Protein Content expressed as mg/gm FW.

Tested Plants	Control plants	Infected plants	25 mM NaCl	50 mM NaCl	100 mM NaCl
Protein Content	4.98	3.65	3.78	2.67	1.85

Table (4): Effect of NaCl and TYLCV on ascorbate peroxidase of tested plants. Ascorbate peroxidase activity is expressed as units/mg protein/gm FW.

Tested Plants	Control plants	Infected plants	25 mM NaCl	50 mM NaCl	100 mM NaCl
APX Content	0.270	0.438	0.453	0.554	1.235

Table (5): Effect of NaCl and TYLCV on catalase content of tested plants. Catalase activity is expressed as units mg protein $^{-1}$ min^{-1}.

Tested Plants	Control plants	Infected plants	25 mM NaCl	50 mM NaCl	100 mM NaCl
Catalase Content	0.004	0.057	0.0472	0.0712	0.006

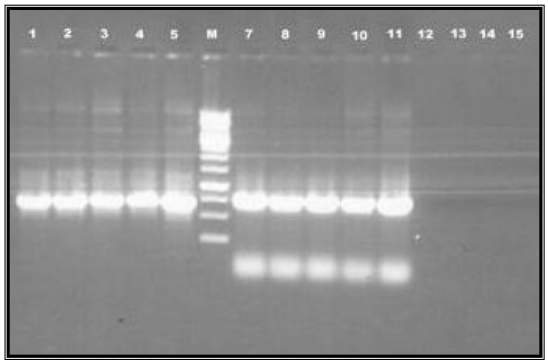


Fig. (1): Amplified PCR fragments produced from using WTGs specific primers (HD1 & HD2 and Cp-F & Cp-R) for four inoculated tomato plants with whiteflies. Infected plants were able to amplify the expected molecular weight fragment (lanes 1, 2, 3 and 4) in case of first couple of primers and lanes (7, 8, 9 and 10) for Cp-f & Cp-R, while lanes 12, 13, 14 and 15 showed negative results for control plants with HD1 & HD2.The cloned TYLCV genome was used as positive control (+) lanes (5 and 11).

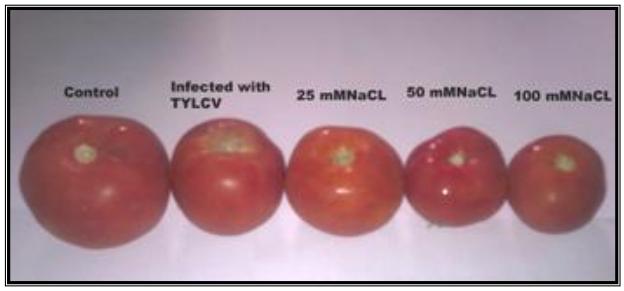
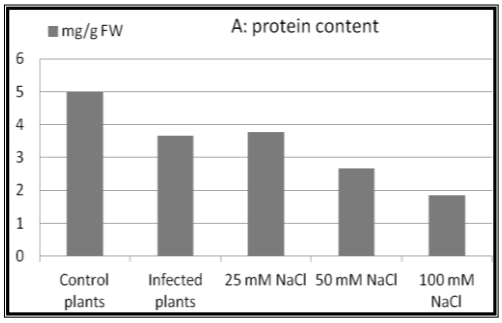
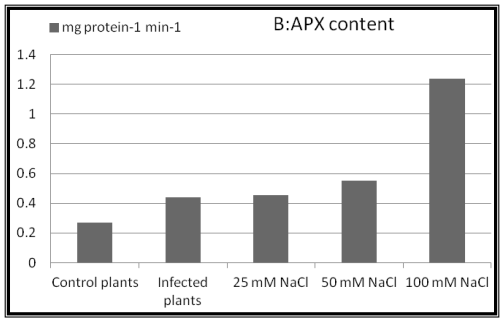


Fig. (2): Effect of salinity stress and viral infection on the size of tomato fruits.





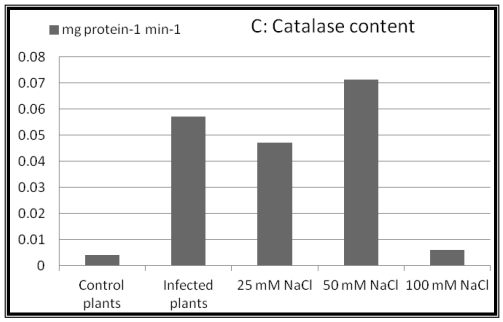


Fig. (3): Effect of salinity and TYLCV on (A) protein content, (B) activity of ascorbate peroxidase (APX) and (C) catalase (CAT) in mature leaves of 120 days old tomato plants.