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Physiological Responses to Infection by Tomato bushy stunt virus in Different Host Plants.

Awatif S. Ali[1*], Faiza A. Fattouh[2], and Radwa M. Fathy[1]

1. Department of Botany, Faculty of Science, Damanhour University, Egypt.
2. Department of Botany and Microbiology, Faculty of Science, Alexandria University, Egypt.

Corresponding Author email: awatifali95@yahoo.com.

ABSTRACT: An Egyptian isolate of Tomato bushy stunt virus, the TBSV Egh was inoculated on two different economically important hosts *Lycopersicon esculentum* and *Cucurbita pepo*. Both Elisa and RT-PCR were compared with respect to sensitivity of detection of virus infection at different days post inoculation (dpi), where the molecular technique was found to be more sensitive. At 15 dpi, systematic symptoms and some physiological responses were compared in the two hosts. Systematic symptoms varying from chlorosis, stunting to malformation on L. esculentum to chlorosis, necrotic patterns and stunting on C. pepo. TBSV Egh-infection resulted in a decline in growth of both hosts compared to control plants. At the same time, a significant increase in lipid peroxidation level of TBSV Egh-infected plants was found compared to uninfected plants. Assessment of the activities of the key enzymes for nitrogen assimilation, glutamine synthetase (GS) and glutamate synthase (GOGOAT) of TBSV Egh-infected plants revealed variable significant responses. Free amino acids composition of TBSV Egh-infected plants showed distinct disturbance compared to that of uninfected plants.
Key words: Tombusviridae, Virus symptoms, N-assimilating enzymes, host amino acids.
Abbreviations: dpi, days post inoculation; GOGOAT, glutamate synthase; GS, glutamine synthetase; MDA, malonaldehyde;TBSV, ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; TBSV, Tomato bushy stunt virus.

INTRODUCTION

Tomato bushy stunt virus (TBSV) is an isometric single-stranded plus-sense RNA virus that is the type member of the Tombusvirus genus (Family: Tombusviridae), (Russo et al., 1994). TBSV is distributed all over the world, across central and Western Europe, North Africa and the Americas. TBSV affect a restricted host range, including a few dicotyledonous species in different families and affect many vegetable crops including L. esculentum (tomato) and some Rosa sericea (Martelli et al., 2001).

Depending on the host genotype, Tombusvirus induces many symptoms varying from necrotic and chlorotic lesions, to a systemic mild or severe mosaic, or they may culminate in a lethal necrosis. The original TBSV isolates from tomato plants caused a mottle, wrinkle and downward curling of leaves which causes stunting and bushy growth, chlorotic spots, wrinkling, deformation and necrosis of leaves of tomato (Gerik et al., 1990; Luis-Arteaga et al., 1996) and pepper (Cherif and Spire, 1983) and unusually small size of fruits of both crops causing losses in the economic value of crop or may make it unmarketable (Gerik et al., 1990).

In compatible plant–virus interactions, viruses induce morphological and physiological modifications through interfering with the accumulation and/or function of host components such proteins and nucleic acids, inducing host defense reactions and altering the expression of host genes (Huang et al., 2005; Whitham et al., 2006). These alterations were suggested for the production of viral components, changes that either enhance virus multiplication or delay host defense responses, and the misregulation of genes involved in plant growth and development (Culver and Padmanabhan, 2007; García-Marcos et al., 2009).

The disease-resistant plant tissue produces reactive oxygen species (ROS) which are extremely cytotoxic and can seriously disrupt normal metabolism through oxidative damage to lipids (Alscher et al., 2002), nucleic acids and proteins (Foyer and Noctor, 2003; Herbette et al., 2003). The more susceptible to ROS is polyunsaturated fatty acids of membrane lipids resulting in peroxidation and yielding various aldehydes, alkenals, and hydroxyalkenals, including the cytotoxic compounds malonaldehyde (MDA) and 4-hydroxy-2-nonenal (HNE).

The incorporation of ammonium into the pool of N-containing molecules is first catalyzed by the glutamine synthetase (GS)-glutamate synthase (GOGOAT) cycle. Glutamate and glutamine are the N donors for the biosynthesis of major N compounds in plants including other amino acids, nucleic acids bases, polyamines, and chlorophylls. In advanced stage of viral replication, local changes in gene expression can happen such as changes in uninfected cells surrounding virus infected lesions to cease viral replication which are induced by NADP-dependent malic and catalase enzymes (Havelda and Maule, 2000).

The objective of this investigation is to perform a comparative study on the symptoms and physiological responses of both *L. esculentum* and *C. pepo* plants as a result of TBSV Egh- infection.

MATERIALS AND METHODS

Inoculation of TBSV Egh isolate onto different hosts

An Egyptian isolate of Tomato bushy stunt virus, the TBSV Egh, isolated from infected tomato L. esculentum Mill in 2009 (Hafez et aI., 2010) was inoculated on two different hosts, L. esculentum Mill (Solanum. lycopersicum) (Tomato) and C. pepo (Zucchini). Plants used for inoculation were 15 days old and in triplicates for all treatments and these at different dpi. TBSV infected leaves were ground in liquid nitrogen in a sterile mortar and pestle until powdery. Inoculation buffer (100 ml/gm 0.01M phosphate buffer pH 7.0, 0.5g Na_2 SO_3 and 1% celite) was added to the infected tissue at a ratio of 1:10 (tissue weight: inoculation buffer volume) and mixed well. Leaves were wounded during inoculation by celite to aid viral entry. Mock-inoculated plants were manually inoculated with the buffer. Following inoculation, the leaves were sprinkled with water and grown at 25-30°C. Five dpi, appearance of TBSV characteristic symptoms on the inoculated leaves was observed.

Detection of TBSV Egh by DAS-ELISA

ELISA was performed for leaf samples of each host at 0, 1, 3, 6, 9, 13, 18, 28, 37and 45 dpi according to (Clark and Adams, 1977) as modified by (Weiss and Van Regenmortel, 1989). Collected leaf samples were randomly selected to represent different leaf positions at each dpi. ELISA was to monitor the virus level in both hosts with time. ELISA kit (SRA 45400/1000-Agdia, USA) was used for performing this test according to the manufacturer's instructions.

Isolation of RNA from TBSV Egh inoculated leaf samples of different hosts

RNA was isolated from leaf samples (1, 9, 22, and 37 dpi.) for each host by the use of EaZy Nucleic acid isolation, E.Z.N.A plant RNA Kit (Omega bio-tek, USA) according to manufacturer's instruction.

Detection of TBSV Egh inoculated by RT-PCR: RT-PCR was used to monitor the infection of TBSV Egh in L. esculentum and C. Pepo at different dpi for each host.

Synthesis of cDNAs of P42, P22 and P33 genes of TBSV Egh in L. esculentum and C. pepo.

First-strand cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, USA) and its buffer (5X) [50 mM Tris-HCl (pH 8.3 at 25˚C), 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DTT], 5 µl of RNA was added to (10 µl (5x) RT-Buffer), 2 µl (10 mM) dNTPs, 5 µl (20pmol) concentrations of forwardreverse TBSV full length primer TBSV(F): 5`-ACG GGG GAT GAG CAA CTG G-3', 1 µl (200 u/µl) of RT-enzyme, 27 µl H_2O). The mixture was incubated at 37˚C for 60 minutes, then at 70˚C for 10 min (Sambrook and Russell, 2001).

Polymerase chain reaction for the detection of infection of the three genes of TBSV Egh (P42, P22 and P33) in two different inoculated hosts L. esculentum and C. pepo.

PCR reaction was performed in a total volume of 50 µl, containing 5 µl of (10x) Taq DNA polymerase Buffer (Fermentas,USA) (100mMTris-HCl pH 8.8 at 25˚C, 500 mM KCl), 1µl Taq-DNA Polymerase(5u/µl)(1min/kb) (Fermentas,USA), 3 µl $MgCl_2$ (25 mM), 2 µl (10 mM) dNTP mixture,2 µl from each primer (20 Pmol/µl), forward TBSV primer TBSV (F): 5`-ACG GGG GAT GAG CAA CTG G-3' (20 Pmol) concentration, and 2 µl from reverse TBSV primer TBSVR: 5`-ACT CGG ACT TTO GTC AGG A -3` (20 Pmol) concentration, 2 µl from TBSV P33-131(F):5'-GGG GGA TAA ATT GTA ACT TC-3'(20 Pmol) concentration, 2 µl from TBSV P33-3051(R):5'-GYT GTA ACA AAT TGC CGA C-3' (20 Pmol) concentration, and 2 µl from TBSV P22-3856 (F):5'-ATG GAT ACT GAA TAC GAA CAA G-3', 2 µl from TBSV P22-4552 (R):5'-TTC CAT ATC TCC ATC CCC-3'(20 Pmol) concentration , 2 µl of PCR product of each gene of TBSV Egh genomic cDNA, and complete the total volume by add 33 µl H_2O to a total of 50 µl.

The reaction mixture was subjected to amplification as follow: Denaturation of DNA template at 95˚C for 2 min, followed by 35 cycles of amplification with denaturation at 95˚C for 2min, annealing at 60˚Cin case of CP, for P22 it was 61˚C and for P33 it was 55˚C for 45sec, and extension at 72˚C for 3 min and ending with extension at 72˚C for 10 min. The PCR system (Gene Amp 9700 thermocycler, Applied Biosystem (ABI), USA) was used for performing PCR amplification. Resulted amplicons were run on 1% agarose gel in TBE buffer and visualized on an ultraviolet transilluminator.

Detection of physiological responses of TBSV Egh-infected L. esculentum and C.pepo plants.

Measurement of Growth Parameters

TBSV-Egh inoculated leaves of L. esculentum and *C.pepo* plants at 15 dpi were separated into shoots and roots. Root and shoot lengths of 5 plants from each group were determined. Fresh weights of root and shoot tissues were recorded, then oven-dried at 65 °C for 3 days in order to determine dry weight. The shoot : root weight ratio was calculated from the dry weights.

Determination of lipid peroxidation

The level of lipid peroxidation in leaf tissues was determined in terms of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction as described by (Andon et al., 2005). The absorbance was measured at wave length 532 nm. Correction of non-specific turbidity was made by subtraction of the absorbance value at 600 nm. The level of lipid peroxidation was expressed as µM $.g^{-1}$FW formed using an extinction coefficient 155 mM cm^{-1}.

Measurment of enzymes activities in TBSV inoculated host plants: Enzyme exctration

The frozen leaves (about 1.0 g) were ground to a fine powder in a mortar and pestle with liquid nitrogen, and then homogenized in an extraction buffer (5 ml/g fresh weight) containing 25 mM Tris-HCl (pH 7.6), 10 mM $MgC1_2$, 10 mm 2-ME, and 5% (w/v) insoluble PVP. The homogenate was filtered through two layers of cheesecloth and the resultant filtrate was centrifuged at 27,000g for 20 min. The supernatant fraction was used for the assay of both glutamine synthetase (GS) and glutamate synthase (GOGOAT) activities**.**

Assay of glutamine synthatase (GS)

Total GS activity in leaf samples was determined according to a modification of the standard biochemical assay (Canovas et al., 1991). The supernatants were used for the colorimetric determination of γ-glutamyl hydroxamate (λ-GH) at 540 nm after complexion with acidified ferric chloride. Blanks were treated like the samples, but without the addition of ATP. A minimum of three replicate determinations was undertaken for each treatment. The GS activity was expressed in µmol L^{-1} of λ-GH. min^{-1} $.g^{-1}$ FW**.**

Assay of glutamate synthase (GOGOAT)

GOGOAT activity was assayed spectrophotometrically by following the oxidation of NADH at 340 nm as described by (Chen and Cullimore 1988). Corrections were made for substrate-independent NADH oxidation. The GOGOAT activity was expressed as µM oxidized NADH $.min^{-1}.g^{-1}$ FW.

Determination of free amino acids in leaves

Leaf discs (100 mg) from TBSV Egh-infected and healthy control plants were prepared for amino acids analyzer as described by (Crabb et al., 1997). 1.0 ml of 6 M HCl was added to each sample. Samples were hydrolyzed for 17 h in a 110 °C heating block. Then, samples were cooled to ambient temperature and transferred to 1.5-mL microcentrifuge tubes and the HCl was evaporated to dryness using the SpeedVac Evaporator for approximately 2 h. Samples and blanks were reconstituted in 300 µL purified water and stored frozen until HPLC analysis. All samples were directly analyzed using a Dionex ICS-3000 manual for Dionex Amino Acid Analyzer. Samples were separated on Amino Pac PA10 analytical and guard columns (Smith, 1997 Humana press). Equilibrate the Amino Pac PA10 column with 90 mM sodium hydroxide for about 1 h at 0.25 mL/min. Injections are made with the Partial injection mode from a 25-µL loop. Run time is 10 min. Operating Backpressure: < 3,000psi. Column temperature: 30°C. Eluent: E1: 182 megohm water, E2: 250 mM NaOH and E3: 1M Sodium acetate. Eluent Flow Rate: 0.25 mL/min. Quantification was accomplished by using an external standard.

Statistic analysis

The data are presented as the mean± SE obtained from three measurements. Assuming that, the conditions of t-distribution of the difference between two means are satisfied. T-statistic was used to test the hypothesis that there is a significant difference between infected and healthy control plants. All statistically significant differences were tested at the α/2 = 0.1 level.

RESULTS

TBSV Egh inoculation resulted in the appearance of different intensity of symptoms as shown in Figs. 1 and 2. Symptoms included chlorotic lesions with different patterns, bushy growth and stunting with malformations of newly formed leaves. Symptoms were most severe on L. esculentum, and milder on *C.pepo*. Leaf samples harvested from inoculated plants at 21 dpi were stored frozen at -20^{o}C until use. All collected leaf samples were used in DAS-ELISA to detect the presence of TBSV Egh in *L. esculentum* and *C.pepo*.

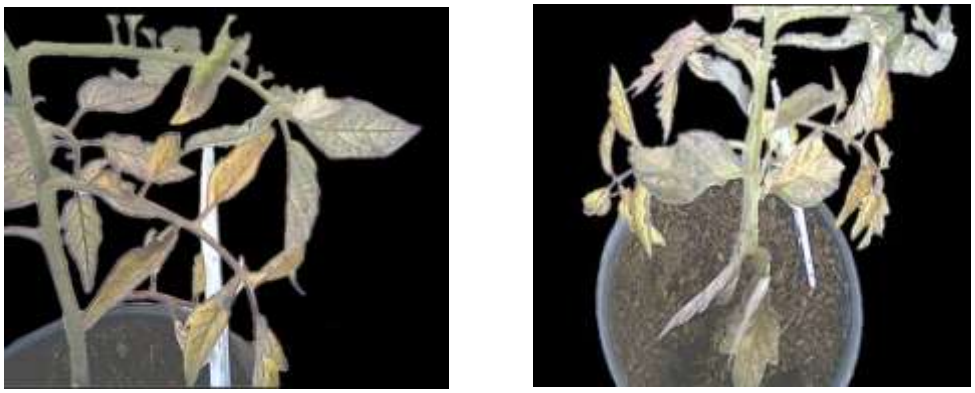

Figure 1. Diagnostic symptoms of TBSV Egh infection in *L. esculentum* Mill under green house conditions. (A) chlorosis, stunting and malformations, (B) chlorotic lesions of different patterns and leaf malformations.

Figure 2. Diagnostic symptoms of TBSV Egh infection in *C. pepo*. under green house conditions. (A) chlorosis and stunting (B) chlorotic patterns and necrosis.

Enzyme Linked Immunosorbent Assay (ELISA) for confirmation of TBSV Egh infection in different hosts.

TBSV Egh infection of both hosts L. esculentum and *C. pepo* was confirmed by the use of DAS-ELISA in different batches of harvested tissue. Positive infection was confirmed by the mean value of absorbance at 405 nm ± standard deviation of twice that of the negative control value, (2N) or higher.

Detection of TBSV Egh infection by DAS-ELISA at different days post inoculation (dpi).

Collected leaf samples of L. esculentum and *C. pepo* at different time intervals of 0 ,1 , 3 , 6, 9 , 13, 18 , 28 , 37and 45 dpi were subjected to testing by DAS-ELISA to monitor the virus level in both hosts with time.

Tested samples at each dpi represent a randomly collected sample from leaves at different positions for each dpi.In case of *L. esculentum*, the virus was not detected at 0 and 1 dpi. Positive samples were from 3dpi and on till 45dpi (Table 1). Data represent the mean value of absorbance ± standard deviation. However, the highest levels of virus detection were from samples collected at 13 and 18 dpi. A decline in virus level was also detected in the form of lower ELISA absorbance for leaf samples collected at later days 28, 37 and lowest at 45 dpi. In case of *C. pepo* the virus was not detected at 0, 1, 3 and 6 dpi. However, the virus was detected starting 9 dpi and on till 45 dpi. The highest virus titer was at 13 and 18 dpi and a progressive decline at 28, 37 and 45dpi.

Table 1. Detection of TBSV Egh isolate in *L. esculentum* and *C. pepo* in samples collected at different days post inoculation (dpi). (A1: blank, A2: negative control, A3: positive control. For *L. esculentum* inoculated samples A4: 0 dpi, A5: 1dpi, A6: 3dpi, A7: 6dpi, A8: 9dpi, A9: 13dpi, A10: 18dpi, A11: 28dpi, A12: 37dpi, B1: 45dpi. For *C.pepo* inoculated samples: B2: 0dpi, B3: 1dpi, B4: 3dpi, B5: 6dpi, B6: 9dpi, B7: 13dpi, B8: 18dpi, B9: 28dpi, B10: 37dpi, B11: 45dpi. The optical density of each well was recorded at 405nm using an ELISA reader. The cut off value for positive samples was twice that of the negative control (2N). Values are the means ± SE of three replicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.038 ±0.016	0.324 ±0.021	2.444 ±0.006	0.0720 ±0.010	0.948 ±0.008	1.251 ±0.010	1.493 ±0.003	1.723 ±0.016	1.980 ±0.006	1.945 ±0.171	1.621 ±0.004	1.481 ±0.017
B	1.299 ±0.006	0.485 ±0.010	1.091 ±0.019	1.045 ±0.007	1.280 ±0.013	1.436 ±0.032	1.918 ±0.032	1.975 ±0.016	1.711 ±0.033	1.438 ±0.168	1.301 ±0.032	

Detection of TBSV Egh infection by RT-PCR at different dpi

RT-PCR was used to monitor the infection of TBSV Egh in L. esculentum and C. pepo at different dpi. Different primers have been used, for L. esculentum P42 (Coat protein) and P22 (Movement protein) primers and for C. pepo .As shown in Fig (3) the coat protein gene P42 (600bp) was detected in L. esculentum samples at 3 and 28 dpi. No PCR product was obtained at 0 and1 dpi. The P22 (900bp) of TBSV was also detected in samples collected at 3,9,28 and 37 dpi as indicated in Fig (4). In case of C. pepo positive P42 (600bp) was detected by RT-PCR in samples collected at 3, 9 and 27 dpi as shown in Fig. (5).This indicates a higher degree of sensitivity of RT-PCR than ELISA as infection was detected by it at earlier dpi in C.pepo than in case of ELISA as outlined before.

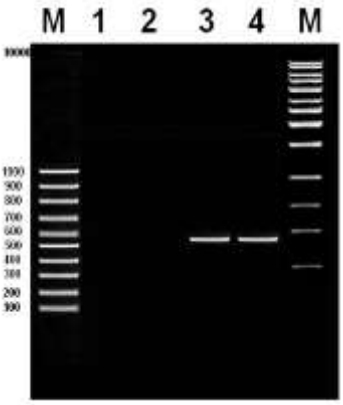

Figure 3. Electrophoretic mobility of Coat protien P42 RT- PCR product amplified from cDNA of *L. esculentum* inoculated by TBSV Egh using TBSV- 2506 F, TBSV- 4001R specific synthetic oligonucleotides primers. Lane M: Fermentase Molecular weight standard (100bp-1Kbp) DNA ladder; Lanes respectively: 1. 0 dpi , 2. 1 dpi , 3. 3 dpi , 4. 28 dpi, 5. Fermentase Molecular weight standard (100bp-10Kbp) DNA ladder.

Measurement of growth Parameters in TBSV Egh-infected L. esculentum and C.pepo.

Results showed that, TBSV Egh-infection induced a reduction in some growth parameters of L. esculentum as well as of C.pepo plants, respectively (Table 2). TBSV Egh-infection caused a significant (α/2= 0.1) decrease in root length, shoot fresh weight, shoot dry weight and root dry weight of L. esculentum plants. A significant reduction in shoot length (stunting) in case of infected C.pepo (25.33 cm) to (13.66 cm) compared to reduction of infected L. esculentum (17.9 cm) to (15.0 cm). Reduction in shoot fresh and dry weight was also relatively more in case of TBSV Egh infected C.pepo than in infected L. esculentum .On the other hand, there were no differences in shoot length, root fresh weight and shoot/root dry mass ratio of infected L. esculentum plants relative to uninfected plants

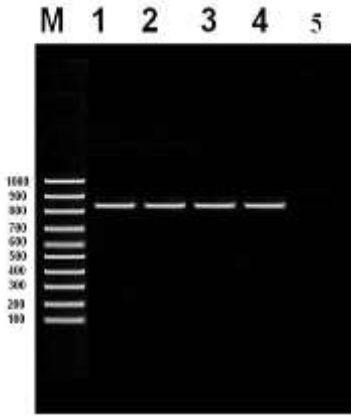

Figure 4. Electrophoretic mobility of Movement protien P22 RT- PCR product amplified from cDNA *of L. esculentum* inoculated by TBSV Egh using TBSV P22- 3856 F, TBSVP22- 4552R specific synthetic oligonucleotides primers. Lane M: Fermentase Molecular weight standard (100bp-1Kbp) DNA ladder; Lanes respectively: 1. 3 dpi , 2. 9 dpi , 3. 18 dpi , 4. 37 dpi and 5: 0 dpi.

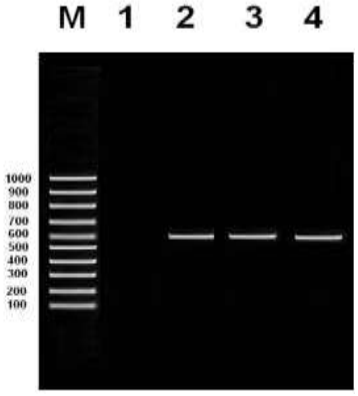

Figure 5. Electrophoretic mobility of Coat protien P42 RT- PCR product amplified from cDNA of *C. pepo* inoculated by TBSV Egh using TBSV- 2506 F, TBSV- 4001R specific synthetic oligonucleotides primers. LaneM: Fermentase Molecular weight standard (100bp-1Kbp) DNA ladder; Lanes respectively: 1. 0 dpi , 2. 3 dpi , 3. 9 dpi , 4. 18 dpi.

Table 2. Changes in growth parameters of TBSV Egh-infected leaves of *L. esculentum* and *C. pepo* plants. Values are the mean± SE of three replicates.

TBSV Egh Host Plant	Growth Parameter	Control plants	Infected-TBSVEgh plants	t
L. esculentum	Shoot length (cm)	17.9 0 ± 1.42	15.00 ± 0.73*	1.72
	Root length (cm)	8.00 ± 0.577	6.00 ± 0.50*	2.62
	Shoot fresh wt.(g)	2.72 ± 0.228	1.56 ±0.15*	4.24
	Root fresh wt.(g)	1.697 ± 0.122	0.43 ± 0.054*	9.45
	Shoot dry wt.(g)	0.265 ± 0.034	0.18 ±0.021*	2.13
	Root dry wt.(g)	0.174 ± 0.041	0.086 ± 0.007*	2.22
	Shoot/Root dry mass	1.645 ± 0.335	2.43 ± 0.324	-1.29
C. pepo	Shoot length (cm)	25.33 ±1.67	13.66 ± 1.20*	5.68
	Root length (cm)	5.67 ± 1.20	4.33 ± 0.88	0.89
	Shoot fresh wt.(g)	7.99 ± 1.66	2.77 ± 0.24*	3.12
	Root fresh wt.(g)	0.13 ± 0.02	0.05 ± 0.011*	3.57
	Shoot dry wt.(g)	0.79 ± 0.065	0.27 ± 0.008*	7.85
	Root dry wt.(g)	0.039 ± 0.009	0.023 ± 0.005	1.46
	Shoot/Root dry mass	22.26 ± 4.09	13.43 ± 3.67*	1.61

Asterisks show significant differences between infected and health leaves in t-test at (α/2= 0.1).

Effect of TBSV Egh-infection on the lipid peroxidation level

To assess the oxidative stress resulted from TBSV Egh-infection; the membrane damage integrity was determined by evaluating MDA levels in L. esculentum and C.pepo plants (Fig. 6). Results indicated that, TBSV Egh-infection caused greater oxidative stress in relative to healthy control plants. MDA levels in TBSV Egh-infected

plants showed significant (α/2= 0.1) increase comparing with healthy control plants. Comparing both hosts, it was clear that infection by TBSV Egh imposes a higher level of stress on L. esculentum than C. pepo plants as indicated by the MDA levels and that illustrate the severe symptoms showed on L. esculentum.

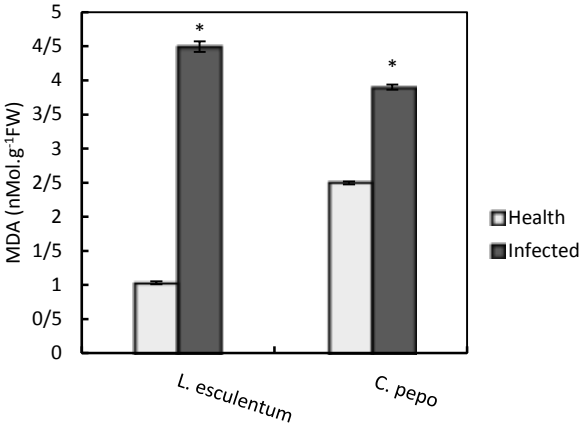

Figure 6. Changes in MDA levels of TBSV-infected L. esculentum and C.pepo leaves. Bars are the standard error of the mean of three replicates. Asterisks show significant differences between infected and health leaves in t-test at (α/2= 0.1).

Detection of TBSV Egh-infection on the activities of some nitrogen assimilating enzymes.

To assess the nitrogen assimilation efficiency in TBSV Egh-infected L. esculentum and C. pepo leaves, glutamine synthetase (GS) and glutamate synthase (GOGOAT) activities were measured and illustrated in (Fig. 7A & B). Compared to healthy control plants, GS activity in infected L. esculentum and C.pepo leaves was decreased significantly (α/2= 0.1) as shown in (Fig. 7 A). Furthermore, TBSV Egh-infection resulted in pronounced variable changes in the GOGOAT activities of L. esculentum and C. pepo leaves. GOGOAT activity showed a significant (α/2= 0.1) increase in infected L. esculentum leaves in contrast to its activity in infected C. pepo leaves which decreased significantly, as compared with the corresponding healthy control leaves (Fig. 7 B).

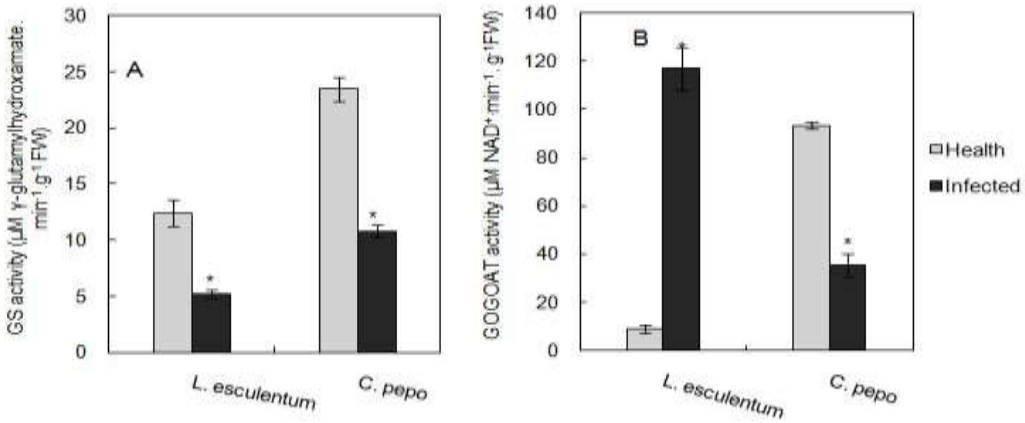

Figure (7 A&B): Enzymatic activities associated with TBSV Egh infection (A) glutamine synthetase (GS) and (B) glutamate synthase (GOGOAT) of *L. esculentum* and *C.pepo.* Bars are the standard error of the mean of three replicates. Asterisks show significant differences between infected and health leaves in the t-test at (α/2= 0.1).

Effect of TBSV-infection on amino acids composition

The concentrations of total free amino acids in leaves of TBSV-infected L. esculentum and C. pepo plants was determined by HPLC and are presented in table 3. TBSV Egh-infection caused a sharp decrease in total amino acids content to ~ 20% in L. esculentum leaves compared with healthy control plants. The most striking results were that the TBSV Egh-infection caused a drastic decrease in arginine, asparatic acid, histidine, leucine,

methionine and valine to be undetected in infected L. esculentum leaves. Alanine content was about 4-fold greater in infected L. esculentum leaves compared with healthy control plants. On the other hand, total and individual amino acids of C. pepo plants showed different response to TBSV Egh-infection. The total amino acids content decreased to ~ 91% in infected leaves compared with control plants. A slight change in concentration of threonine and valine in infected leaves (23.89 and 36.95 mM) compared with control leaves (23.72 and 34.8 mM). An obvious disappearance of glycine and phenylalanine as a result of infection was recorded. Also, infection led to increase in alanine concentration from 9.67 to 44.65 mM and the appearance of cystine at level of 21.63 mM.

Table 3. Free amino acids levels in TBSV-infected L. esculentum and C. pepo leaves.

Amino acids	L. esculentum				C. pepo			
	Health		Infected		Health		Infected	
	Amount (mM)	Relative amount %	Amount (mM)	Relative amount %	Amount (mM)	Relative amount %	Amount (mM)	Relative amount %
Ala	3.91	4.39	17.33	100.0	9.67	5.88	66.52	44.65
Arg	59.59	66.93	NA	NA	NA	NA	NA	NA
Asp	7.39	8.30	NA	NA	NA	NA	NA	NA
Cys	NA	NA	NA	NA	NA	NA	21.63	14.52
Gly	NA	NA	NA	NA	86.72	52.80	NA	NA
His	5.66	6.35	NA	NA	NA	NA	NA	NA
Leu	9.93	11.16	NA	NA	NA	NA	NA	NA
Met	1.60	1.80	NA	NA	NA	NA	NA	NA
Phe	NA	NA	NA	NA	9.27	5.64	NA	NA
Thr	NA	NA	NA	NA	23.72	14.44	23.89	16.04
Val	0.95	1.07	NA	NA	34.88	21.24	36.95	24.80
Total A.A	89.04		17.33		164.25		149.0	
Percentage %			19.46				90.71	

Ala, alanine; Arg, arginine; Asp, asparatic acid; Cys, cystine; Gly, glycine; His, histidine; Leu, leucine; Met, methionine; Phe, phenylalanine; Thr, threonine; Val, valine; NA, not available.

DISCUSSION

In Egypt, evidence of TBSV infection has been reported where infection by the virus was detected in 7 different governorates in Egypt in 2008 (Hafez et al., 2010). Incidence of virus infection was 23.7% in selected fields. An Egyptian isolate of the virus TBSV Egh has been isolated from field infected tomato samples collected from EL Fayoum governorate. The isolate has been purified, partially characterized and sequenced. In this work, TBSV Egh is further characterized with respect to its natural host (L. esculentum) and also an experimental host (C. pepo) that is as well an economically important crop in Egypt which if infected may cause serious losses. More recently, it has been known that TBSV is the cause of severe epidemics in several horticultural crops; it cause epidemic outbreaks in tomato and eggplant crops in southeastern Spain and has spread with the expansion of nipplefruit cultivation in Japan (Luis-Arteaga et al., 1996). Severe yield losses associated with TBSV have been reported in peanut, tobacco, tomato, pepper and potato as well as in some ornamental crops (Martelli et al., 2001; Jones, 2009). In Korea in 2007, virus disease incidence of tomato cultivating fields were about 20% in a cultivated area.

DAS-ELISA and RT-PCR were used to monitor TBSV infection at different days post inoculation in both hosts L. esculentum and C. pepo. Three different primers of the virus namely the coat protein P42 gene primer, the movement protein P22 gene primer and the replicase subunit P33 gene primer were used for virus monitoring in total RNA extract of both hosts tissue samples. Results indicated a higher degree of sensitivity by RT-PCR in TBSV infected samples at 1 dpi compared to the detection by ELISA at 3 dpi in L. esculentum and C. pepo. For some viruses detection by ELISA only is sufficient, however in case of TBSV detection by RT-could be essential for lower level of virus infection. The higher degree of detection for RT-PCR has been documented in studies on different plant viruses (Mekuria et al., 2003; Gaglayan et al., 2006; Berniak et al., 2009). TBSV RNA accumulation was reported to occur very rapidly (2-2.5 h p.t) in N.benthamiana protoplasts (Qiu and Scholthof, 2001).

Virus infection can cause comprehensive physiological changes in some plant hosts, such as altered water content of tissues and the synthesis and translocation of metabolites and as a consequence reduction the biomass

of plant hosts (Hull, 2002; Kazinczi et al., 2006). In this study, TBSV Egh-infection resulted in a reduction in biomass of L. esculentum and C. pepo plants which are represented in table 2. However, TBSV Egh-infection had a greater negative effect on C. pepo plants compared with L. esculentum plants. There was more decrease in shoot length (stunting) in TBSV Egh-infected C. pepo plants and a reduction in shoot fresh and dry weights of infected plants to 34.5 and 34.6% of the corresponding healthy control plants, respectively. In agreement, PVY infected D. metal plants showed a decrease of 31% in leaf area and the weight of fresh and dry shoots were decreased by 38 and 54% respectively (El-Dougdoug et al. 2007).

Results showed sever reduction in shoot fresh and dry weights of infected L. esculentum to about 57% and 68.3 % of healthy control plants. At the same time, root fresh and dry weights of infected L. esculentum plants were decreased to 76.6 % and 46.7 % of healthy control plants. Consistent with the previous findings, the disruption of the circadian system of the plant combined with infection by CaMV significantly perturbs the accumulation of shoot biomass (Dodd et al. 2005; Handford and Carr, 2007). The decline in growth indicates the involvement of physiological changes of the infected plants. Firstly, the decrease in photosynthetic pigments content may be responsible. Second possibility may be impaired translocation (Olesinski et al., 1995). Third possibility is that viral infections reduced the content of protein (Taiwo and Akinjogunla, 2006).

TBSV Egh-infection triggered extensive cellular damage appeared in the high level of MDA in L. esculentum and C. pepo plants (Fig. 6). MDA content increased significantly from 1.033 in healthy to 4.49 $nMol.g^{-1}$ FW in infected L. esculentum plants and from 2.49 in healthy to 3.29 $nMol.g^{-1}$ FW in infected C. pepo plants. In a range of different host–pathogen interactions, the hypersensitive response (HR) is associated with a rapid production and accumulation of ROS which causes unprompted oxidative damage to lipids, proteins and nucleic acids and antioxidant strategies are compulsory for maintenance of cell redox homeostasis (Li and Burrit, 2003; Gayoso et al., 2004). ROS produced during viral disease could be responsible for the partial activation of the senescence programme during viral diseases. Lipid degradation is another catabolic process present in senescent leaves (Lim and Nam, 2005) and was represented by the induction of genes for hydrolases during the compatible viral infections.

Nitrogen plays an essential role in the nutrient relationship between plants and pathogens. The development of a pathogen which is a stress causes induction of some physiological process related to nitrogen remobilization via the induction of enzymes involved in amino acid metabolism, especially GS and GOGOAT which are the key enzymes in nitrogen assimilation (Solomon et al., 2003).

Results represented in (Fig. 7A) showed that GS activity interaction in TBSV Egh-infected L. esculentum as well as in C. pepo displayed similar response, respectively. GS activity was decreased to ~ 42 % in infected L. esculentum leaves and to ~ 46% in infected C. pepo leaves of its activity in corresponding control leaves. Surprisingly, GOGOAT activity showed different trend in interaction with TBSV Egh-infection in both host plants (Fig. 7B). Since, GOGOAT activity was reduced in infected C. pepo leaves to about 38% of its activity in healthy control leaves; it increased in infected L. esculentum to ~13 fold its activity in healthy control leaves. However, viruses have differential effects on both GDH and GS activities. In a similar manner as during senescence, virus infection cause a decrease in the total GS activity and an increase in GDH activity (Pageau et al., 2006). Such findings were suggested that N-remobilization was favored in virus infected leaves (Tavernier et al., 2007).

The results displayed in table 3, indicated that individual amino acids could be modified by host metabolism changes such as photosynthetic efficiency, respiratory pathway and carbohydrate metabolism which induced by TBSV Egh-infection (Kisaka and Kida 2003; Mohamed, 2011). Total free amino acids of TBSV Egh-infected L. esculentum leaves was decreased to 19.47 % and in C. pepo leaves was reduced to 90.71% of the total free amino acids of the corresponding uninfected leaves. The concentration of arginine, asparatic acid, histidine and valine are drastically decreased in TBSV-infected L. esculentum leaves to be negligible.

Interestingly, alanine was the major amino acid in TBSV-infected L. esculentum (relative amount, 100%) and in C. pepo leaves (relative amount, 44.65%). Indeed, alanine can be modulated depending on the stress (hypoxia) (Miyashita et al., 2007) and photorespiratory conditions (Igarashi et al., 2006). The depletion of arginine, asparatic acid, histidine, leucine, methionine and valine might be due to virus replication as well as to amino acid inter-conversion through plant transamination. It was observed that in other plant–pathogen interactions like Brassica napus/Leptosperia maculans, the necrotrophic phase also seems to start when the plant begins to mobilize nitrogen (Rossato et al., 2001). TBSV Egh infection might be expected to drain nutrient from a host plant.

In conclusion: TBSV Egh-infection has serious and varied effects on L. esculentum and C. pepo plants. L. esculentum plants showed more severe symptoms and physiological alterations especially in biomass and MDA content compared to C. pepo plants.

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