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Differentiation among three Egyptian isolates of *Citrus psorosis virus*

El-Dougdoug, Kh.A.[1], S.A. Ghazal[2], A.A. Mousa[2], H. Fahmy[3] and A.R. Sofy[2]

Three Egyptian isolates of *Citrus psorosis virus* (CPsV-EG), namely ARC, TB and TN were obtained from citrus cvs. Grapefruit, Balady and Navel, respectively. These isolates were differed in some of their external symptoms. The CPsV-EG isolates were detected by biological indexing, giving rise to oak leaf pattern (OLP) on Dweet tangor. The three isolates were differentiated using double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA), woody indicator plants, differential hosts, peroxidase isozymes and activity, total RNA content and reserves transcription-polymerase chain reaction (RT-PCR). The severe isolate (ARC) gave the highest OD. value (2.204) in ELISA, followed by the mild isolate (TB) (1.958) and the last latent isolate (TN) (1.669). These isolates differed also in incubation period, intensity of symptoms and response to sensitivity of woody indicator plants and differential hosts. The CPsV-EG isolates showed differences in isozymes fractions, R_F value and intensity as compared with healthy plant. Results were confirmed by peroxidase activity where the level of peroxidase activity was considerably higher in ARC leaves than TB and the last TN. The total RNA content in infected leaves gave the highest content in ARC followed by TB isolate while the lowest was recorded in TN isolate. Finally, RT-PCR showed differences between CPsV-EG isolates of PCR-products using specific primer (Ps66 & Ps65) where base number of coat protein gene ARC isolate 571 bp; TB isolate 529 bp and TN isolate 546 bp.

Keywords: CPsV, Egyptian isolates, DAS-ELISA, Hosts, Isozyme, RT-PCR.

[1] Microbiology Department, Faculty of Agric., Ain Shams University. [2] Botany and Microbiology Department, Faculty of Sci., Al-Azhar University. [3] Citrus Certified Centre, Agricultural Research Center (ARC), Giza, Egypt.

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INTRODUCTION

Fawcett and Klotz (1938) classified psorosis into two types: Psorosis A and Psorosis B. Both types produced similar bark lesion symptoms but different leaf and fruit symptoms.

The more aggressive form of psorosis disease, called psorosis B (**Fawcett, 1932; Fawcett and Klotz, 1938**), additionally caused chlorotic blotching in old leaves with gummy pustules on the leaf underside and blisters on twigs (**Roistacher, 1991; 1993**).

Among graft transmissible diseases that have been reported from Egypt *Citrus psorosis virus* (**Roistacher, 1991; Fahmy *et al.*, 2002**).

Particles of *Citrus psorosis virus* (CPsV), type species of the genus *Ophiovirus* (**Milne *et al.*, 2000**), have been partially purified from several psorosis isolates (**Derrick *et al.*, 1988; Garcia *et al.*, 1991,b; Navas-Castillo *et al.*, 1993**), and later associated with additional isolates of different origins by Western-blot detection of the coat protein (*CP*) using an antiserum to the isolate CPV-4 (formerly CRSV-4) from Florida (**Da Graca *et al.*, 1991; Garcia *et al.*, 1994; Navas-Castillo and Moreno, 1995**).

Roistacher (1991) reported that, there was much variability in symptoms found in citron, Dweet tangor and sweet orange among the various isolates, and only two isolates were mechanically transmitted. On the other hand, psorosis might remain symptomless in certain host trees, the virus could be transmitted from a symptomless host and induce symptoms in progeny trees.

CPsV isolates frequently are differed in the intensity and kind of symptoms induced in different citrus species (**Derrick *et al.*, 1988; Garcia *et al.*, 1991,a; Navas-Castillo *et al.*, 1991; 1993; Garcia *et al.*, 1994; Legarreta *et al.*, 2000**).

Variation in the epitopes present in the *CP* of different CPsV isolates (**Djelouah *et al.*, 2000; Alioto *et al.*, 2003**) makes it advisable to use a mixture of MAbs (**Martin *et al.*, 2002**).

Shelly-Praveen *et al.* (2003) reported that, the failure to amplify the coat protein gene of Indian *Citrus ringspot virus* (ICRSV) by the specific primers of *Citrus psorosis virus* (CPV) suggested that these 2 viruses were quite distinct.

The present work was carried out to compare among CPsV Egyptian isolates through

biological, serological and molecular characters.

MATERIALS AND METHODS

Source of Virus Isolates

Three CPsV-EG isolates namely ARC, TB and TN were obtained from citrus cvs. Grapefruit (at the farm of Agric. Res. Cent.), Balady and Navel (Tersa) respectively where show different external symptoms (Fig. 1).

Double antibody sandwich Enzyme-linked immuonosorbent assay (DAS-ELISA) was used for CPsV-EG isolates detection in shoots of infected trees, using specific monoclonal antibody for CPsV (Agritest, Italy) regarding **Clark and Adams (1977).**

Grapefruit (ARC), Navel (TB) and Balady (TN) trees were for the presence of *Citrus tristeza virus* (CTV) using direct tissue blot immuno-assay (DTBIA) according to (**Garnsey *et al.* (1993),** *Spiroplasma citri* using Diene's stain according to **El-Dougdoug *et al.* (1993)** and *Citrus excortis viroid* (CEVd) using direct tissue blot hybridization according to **Sambrook *et al.* (1989).**

Source of Virus Isolates

Infected trees of Grapefruit, Balady and Navel which gave +ve DAS-ELISA were used as a CPsV-EG isolates source. The virus isolates were isolated and propagated by graft inoculation with eye buds from infected materials on Sour orange. The inoculated plants were kept under greenhouse conditions (24-27°C/16 light day).

The three CPsV-EG isolates were differentiated based on external symptoms, DAS-ELISA, woody indicator plants, differential hosts, peroxidase isozymes and activities, total RNA content and RT-PCR as follow:-

DAS-ELISA

As mentioned before in detection of CPsV-EG by DAS-ELISA (**Clark and Adams, 1977).**

Woody indicator plants

Three CPsV-EG isolates samples were indexed by graft inoculation with two blind buds, using different woody indicator plants for each isolate (Dweet tangor, Madam vinous, New hall, Navel parent Washington, Balady, Navelina and Valencia) grafted on Volkameriana lemon as rootstock. The grafted plants were kept under insect proof greenhouse at 24-27°C (Max. day) / 18-21°C (Min. night) and recorded the symptom development for 1 month.

Differential hosts

The CPsV-EG isolates were mechanically inoculated onto healthy herbaceous plants (*Gomphrena globosa, Chenopodium amaranticolor L., Ch. quinoa L., Datura metel L.* and *Nicotiana rustica*) via forefinger.

Peroxidase isozymes

The present work was carried out using native-polyacrylamide gel electrophoresis (Native-PAGE) to detect peroxidase isozymes variation among the three Egyptian virus isolates. The enzyme was extracted from citrus leaves of the three virus isolates, and separated in 9 % polyacrylamide gel electrophoresis according to **Stegemann *et al.* (1988)**.

The extraction buffer solutions for extract of the enzyme was prepared by dissolving 1 g tris-base (pH 7.5), 5 ml glycerol and 100 µl mercaptoethanol in 94.9 ml distilled water.

A half ml of extraction buffer was added to each sample. The samples were centrifuged at 10000 rpm for 5 min. The supernatant was transferred into new eppendorff tubes. Forty µl of each sample was mixed with 10 µl bromophenol blue, and then loaded in each well.

Acrylamide stock solution (30%) was prepared by dissolving 30 g of acrylamide and 1 g of bis-acrylamide in 50 ml distilled water. Then the solution was completed to 100 ml by distilled water.

Tris-borate buffer (pH 8.4) was prepared by dissolving 2.12 g of tris and 0.62 g of boric acid in 50 ml distilled water. HCl adjusted the pH to 8.4. Then the solution was completed to 100 ml by distilled water.

Vertical slab gel electrophoretic apparatus was used. The gel solution was prepared by mixing the chemical components (32 ml acrylamide 30%, 26 ml TB buffer, 47 ml distilled water, 1 ml APS and 50 µl TEMED). The prepared gel solution was poured immediately between the two glass plates. The comb was placed immediately. The gel was left to polymerize.

A volume of 5 liters was prepared by mixing 27.25 g tris, 8.5 g boric acid in 5 L distilled water.

The apparatus was connected to the power supply (100 volts). Electrophoretic apparatus was placed inside a refrigerator during running duration.

After electrophoresis, the gels were stained. The staining gels

were carried out by 0.025 g of benzidine dihydrochloride was dissolved in 0.5 ml glacial acetic acid and 3:4 ml of distilled water was added in dark room. The volume was completed up to 100 ml with distilled water and poured on the gel. A half ml of H_2O_2 was added to the gel. The gel was left on a shaker at room temperature until the appearance of the bands (**Gottlieb, 1977**).

After the appearance of the enzyme bands, the reaction was stopped by washing the gel two or three times with tap water. This was followed by adding the fixative solution, which consists of ethanol and 20% glacial acetic acid (1:11), respectively. The gel was kept in the fixative solution for 24 hr and then stored in the refrigerator until ready to be photographed. The gels were analyzed using AlphaEaseFC 4.0 software.

Peroxidase activity

The peroxidase activity was directly determined of the crude enzyme extract according to a typical procedure (**Hammerschmidt *et al.*, 1982**).

Total RNA content

Nucleic acids were extracted according to the method of **Marmur (1961)** as modified by **Mohamed and Capensius (1980)**. RNA determination was carried out according to the method described by **Schneider (1957)**.

RT-PCR

Total RNAs were extracted from infected Grapefruit, Balady and Navel plants, by using High Pure RNA Tissue Kit (Roche) as described by the manufacturer.

The two sets of oligonucleotide primers used to amplify the coding sequence of CPsV 3' end of the coat protein region were commercially obtained from OPERON (Qiagen company, 1000 Atlantic Avenue, suite 108 Alameda, CA 94501). The following RT-PCR primers (Ps66 and Ps65) were selected according to **Martin *et al.* (2004)**.

The Ps66 primer (TCGAAGCTGTATGATGGTGA) and Ps65 primer (TGCCATCTGGAGTGAGGCCT) cDNA synthesis and PCR amplification were performed according to **Martin *et al.* (2004)** for PS66 and PS65 primers.

The PCR Product of the *CP* gene was determined by electrophoresis onto 1% agarose gel in 1XTAE to examine for the actual size of the PCR products. The gels were analyzed using AlphaEaseFC 4.0 software.

RESULTS

The CPsV-EG isolates were detected in infected citrus trees, by DAS-ELISA which gave different range with specific Mab of CPsV where 2.204, 1.958 and 1.669 for ARC, TB and TN respectively compared with negative 0.302-0.137 and positive DAS-ELISA control 2.248-2.213. Also, they were detected by indexing on healthy citrus Dweet tangor seedlings which gave oak leaf pattern (OLP) on Dweet (Table 1) (Fig. 2). On the other hand, these trees were found free from CTV (Fig. 3), CEVd and *Spiroplasma citri* (Fig. 4).

CPsV-EG isolates exhibited external symptoms that could be differentiated into three groups (Fig. 1):

Isolate **ARC** exhibited severe psorosis-bark scaling, bark lesions which appeared as pimples or small flakes of the outer bark, and gum symptoms were observed on the two sides of the main trunk above grafting union.

Isolate **TB** produced mild symptoms where bark scaling on the two sides of the trunk above grafting union and branches.

Isolate **TN** produced no symptoms (latent) with decreasing of the fruits numbers.

The tested citrus woody indicators (Dweet tangor, Madam vinous, New hall, Navel parent Washington, Balady, Navelina and Valencia) (Table 2), were differed in response to the three CPsV-EG isolates. These isolates were differentiated from each other through the symptoms initiated into various woody indicator plants and different incubation periods. Oak leaf pattern developed on Dweet, New hall, Navel and Valencia inoculated with the three isolates but were differed in incubation periods. On the other hand, oak leaf pattern developed on Balady inoculated with the isolates ARC and TB except isolate TN non-symptoms developed. Shock symptoms developed on Madam vinous inoculated with the isolates ARC and TB except isolate TN non-symptoms developed.

Oak leaf pattern and shock symptoms developed on Navelina inoculated with ARC while isolate TB and TN developed oak leaf pattern differed in incubation periods. These results were confirmed serologically by DAS ELISA against specific CPsV Mab where as DAS-ELISA gave positive results.

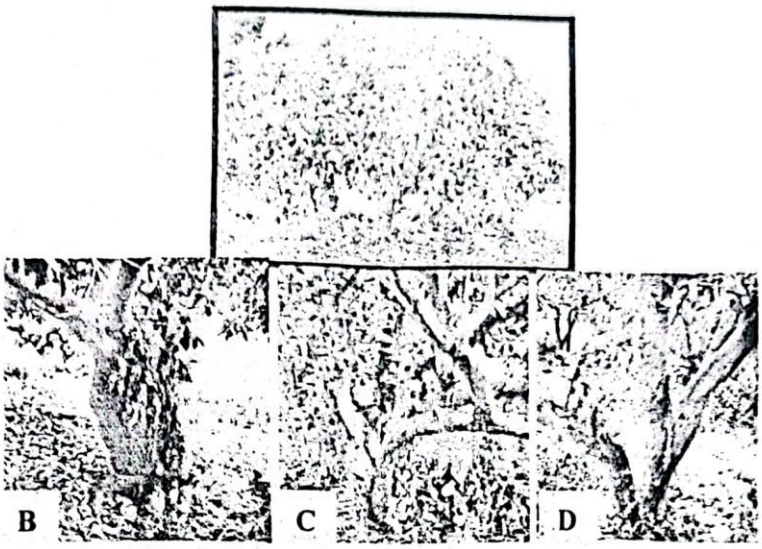

Fig. 1. Citrus trees of different varities presumed to be CPsV infected or CPsV free on the basis of the symptoms showing:

A) Healthy tree. **B)** ARC isolate. **C & D)** TB isolate

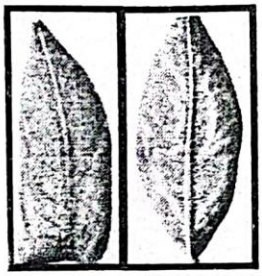

Fig. 2. Oak leaf pattern (OLP) on Dweet tangor leaf as woody indicator plant.

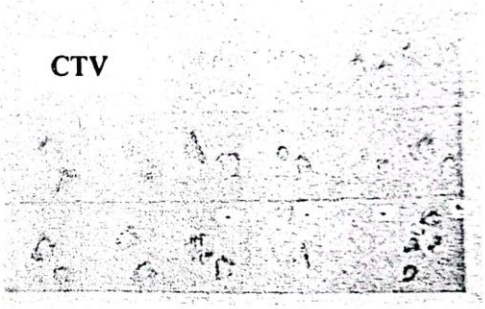

Fig. 3. Immunoprinting assay for CTV detection in six citrus trees cv. Navelina, Balady and Grapefruit infected with CPsV (source of CPsV-EG isolates) using specific monoclonal antibodies against CTV.

Fig. 4. Diene's stain for *Spiroplasma citri* detection showing:

A) A section of healthy petiole and blade in which the xylem and phloem remains unstained.

B) A section of infected petiole and blade (Positive control) containing *Spiroplasma citri* in which the xylem colored bright turquoise blue and the phloem stained a distinct blue.

Table 1. Detection of CPsV infected citrus cultivars* by indexing on woody indicator plants (Dweet tangor / Volkameriana).

Samples locations	Variety	No. of inoculation	Inoculation success	Observaions	
				Symptoms	Period (day)
Tersa 2 samples	Navelina	2	2	OLP	28
Tersa 2 samples	Balady	2	2	OLP	30
ARC 2 samples	Grapefruit	2	2	OLP	27

(OLP) Oak leaf pattern.
(*) six samples out of 120 samples.

Table 2. Response of woody indicators to graft inoculation with three CPsV-EG isolates from different locations.

Indicator plants		CPsV-EG isolates											
		ARC				TB				TN			
		Symptoms	Incubation period (day)	Sensitivity	DAS-ELISA	Symptoms	Incubation period (day)	Sensitivity	DAS-ELISA	Symptoms	Incubation period (day)	Sensitivity	DAS-ELISA
Woody indicator plants	Dweet tangor	OLP	27	+	+	OLP	28	+	+	OLP	30	+	+
	Madam Vinous	Shock	30	++	+	Shock	30	++	+	-	-	-	+
	New hall	OLP	28	+	+	OLP	29	+	+	OLP	29	+	+
	Navel	OLP	28	+	+	OLP	28	+	+	OLP	30	+	+
	Balady	OLP	30	+	+	OLP	30	+	+	-	-	-	+
	Navelina	OLP & Shock	25-35	+++	+	OLP	26	+	+	OLP	29	+	+
	Valencia	OLP	25	+	+	OLP	28	+	+	OLP	30	+	+
Field inspections symptoms		Bark scaling and Gum symptoms were observed on the trunk on the 2 sides of the main trunk (Severe).				Bark scaling was observed on the trunk and branches on the 2 sides of the tree (Mild).				Tree symptomless with decreasing of fruits (Latent).			

Six samples were tested from isolates +: Response (OLP) ++: Sensitivity (Shock)

+++: Hypersensitivity (OLP & Shock) - : No response (Non symptoms)

On the other hand, the three CPsV-EG isolates reacted with different responses at 28 ± 2°C under greenhouse conditions on 5 host plants belonging to 3 families (Table 3) as follow; *Gomphrena globosa* exhibited chlorotic local lesions followed by epinesity and deformation of leaves (systemic infection) with ARC isolate whereas TB isolate exhibited chlorotic local lesions followed by epinesity only (systemic infection). On the other hand, TN isolate gave chlorotic local lesions only (local infection) after 15 days (Fig. 5). *Ch. amaranticolor* L. exhibited local vein chlorotic lesions on inoculated leaves followed by necrotic veins and rugosity (systemic infection) with ARC isolate only. On the other hand, TB isolate gave local chlorotic lesions only (local infection). TN isolate developed non-symptoms (no response) (Fig. 6). *Ch. quinoa* L. exhibited chlorotic local lesions (local infection) with isolates ARC, TB and TN but with different incubation periods (Fig. 7). *Datura metel* L. exhibited chlorotic lesions (local infection) followed by deformation of leaves (systemic infection) with isolate ARC. On the other hand, exhibited local chlorotic lesions (local infection) followed by deformation of leaves (systemic infection) with isolate TB whereas, exhibited chlorotic lesions followed by necrosis (systemic infection) with isolate TN (Fig. 8). *Nicotiana rustica* exhibited vein clearing (systemic infection) with isolates ARC, TB and TN but with different incubation periods. These results were confirmed serologically by DAS-ELISA against specific CPsV antiserum. DAS-ELISA gave positive results with all host plants (except *Ch. amaranticolor* with isolate TN) inoculated with all isolates but with different ELISA reading where ARC isolate gave the highest value, then the TB isolate and the last TN isolate.

The total RNA content values in the leaves of the three CPsV-EG isolates compared with healthy are recorded in Table (4). From the results, the highest value of 328 µg/g was recorded in ARC isolate, followed by the value of 300 µg/gm in TB isolate. While the lowest value of 280 µg/g was recorded in TN isolate, compared with the value of 240 µg/g in healthy (Fig. 9).

Table 3. Comparison between the three CPsV-EG isolates.

Family and plant species	CPsV-EG isolates					
	ARC		TB		TN	
	Symptoms	Incubation period (day)	Symptoms	Incubation period (day)	Symptoms	Incubation period (day)
Family : Amaranthaceae *Gomphrena globosa*	L.Ch.L, E & De.	10-18	L.Ch.L & E.	13-25	L.Ch.L	15
Family : Chenopidiaceae *Ch. amaranticolor* **L.**	L.Ch.L., NV. & R.	12-30	L.Ch.L.	18	0	-
Ch. quinoa L.	L.Ch.L.	11	L.Ch.L	14	L.Ch.L.	15
Family: Solanaceae *Datura metel* **L.**	L.Ch.L & De.	12-30	L.Ch.L & De.	17	L.Ch.L & N.	25
Nicotiana rustica	VC.	12-20	VC.	14-21	VC.	17

Three replicates of each host were mechanically inoculated by infectious sap of infected citrus plants.

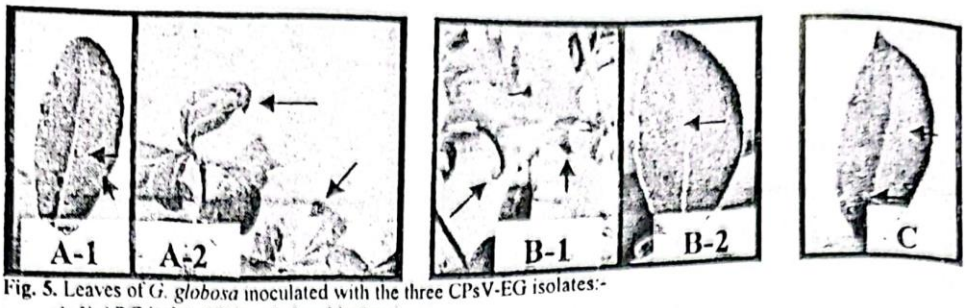

Fig. 5. Leaves of *G. globosa* inoculated with the three CPsV-EG isolates:-
A-1) ARC isolate (Chlorotic local lesions).
A-2) ARC isolate (Epinisty and deformation of leaves).
B-1) TB isolate (Chlorotic local lesions).
B-2) TB isolate (Epinisty of leaves).
C) TN isolate (Chlorotic local lesions).

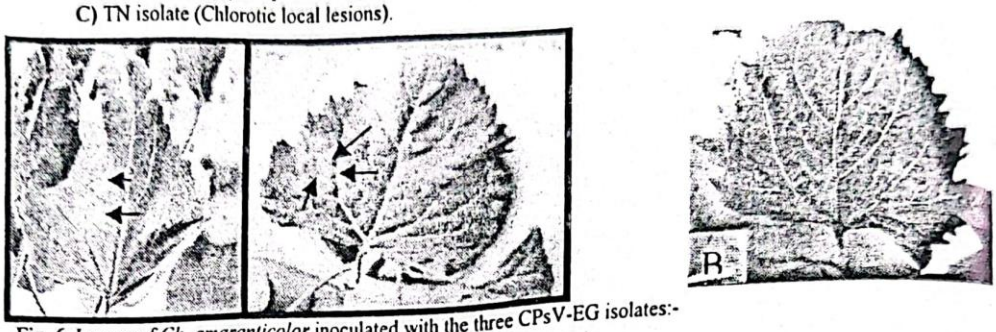

Fig. 6. Leaves of *Ch. amaranticolor* inoculated with the three CPsV-EG isolates:-
A-1) ARC isolate (Vein chlorotic local lesions).
A-2) ARC isolate (Necrotic veins and rugosity).
B) TB isolate (Chlorotic local lesions).

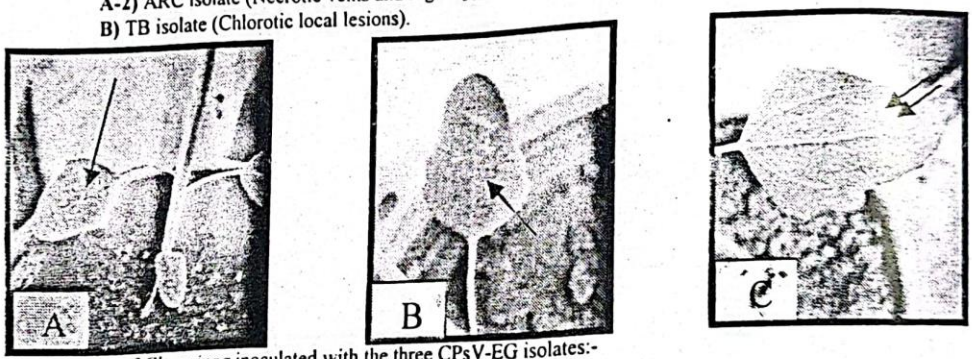

Fig. 7. Leaves of *Ch. quinoa* inoculated with the three CPsV-EG isolates:-
A) ARC isolate (Chlorotic local lesions).
B) TB isolate (Chlorotic local lesions).
C) TN isolate (Chlorotic local lesions).

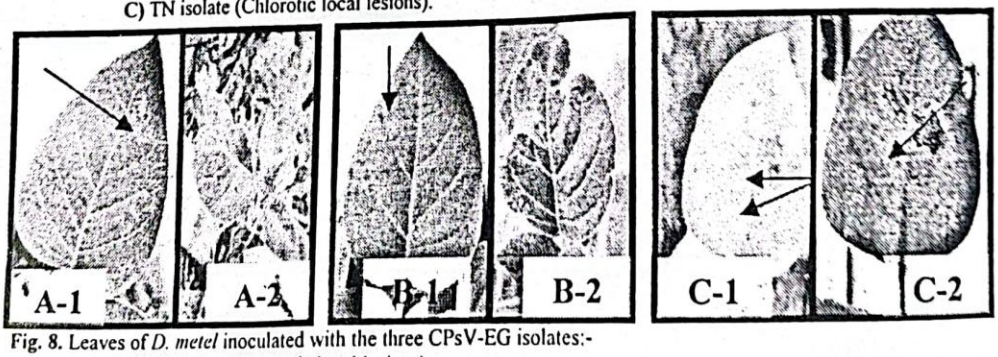

Fig. 8. Leaves of *D. metel* inoculated with the three CPsV-EG isolates:-
A-1) ARC isolate (Chlorotic local lesions).
A-2) ARC isolate (Deformation of leaves).
B-1) TB isolate (Chlorotic local lesions).
B-2) TB isolate (Deformation of leaves).
C-1) TN isolate (Chlorotic local lesions).
C-2) TN isolate (Necrosis).

Table 4. Comparison between RNA content three CPsV-EG isolates comparing with healthy.

Isolates	Healthy	ARC	TB	TN
O.D	0.799	1.105	1.034	0.972
Conc. (µg/g)	240	328	300	280

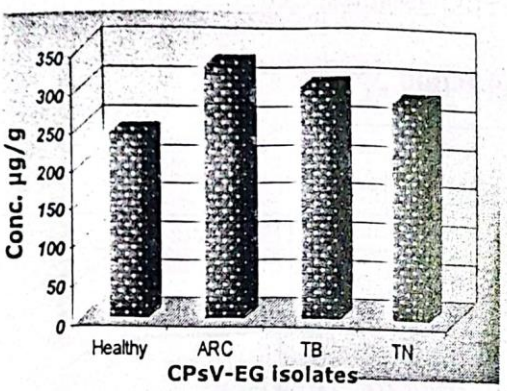

Fig. 9. The RNA content of the three CPsV-EG isolates.

The total number of peroxidase isozymes shown in all samples (ARC, TB, TN and healthy) were 7 isozymes. The healthy plant has one pattern of peroxidase isozyme (Lane no. 4). The CPsV-EG isolates affect the fraction of peroxidase isozyme. The CPsV-EG isolates (ARC, TB and TN) appeared 2 isozymes (Lane no. 1, 2 and 3 respectively) but differed in density of isozyme fractions (Fig. 10). TB near to TN isolate in R_F value but was differed in density of isozyme fractions. On the other hand, ARC isolate was differed from TB and TN isolates in R_F and density of isozyme fractions Table (5) and Fig. (11). These results were confirmed by peroxidase activity where *Citrus psorosis virus*-Egyptian-infection in citrus plants altered peroxidase activity. Data from fig. (12) showing that, the level of peroxidase activity was found to be considerably higher in infected leaves than healthy ones. Where the ARC isolate gave the highest level of peroxidase activity, then the TB isolate and the last TN isolate.

Finally, RT-PCR appeared differences between CPsV-EG isolates of PCR products (Table 6), (Fig. 13). It was found that, base number of coat protein gene ARC isolate 571 bp; TB isolate 529 bp and TN isolate 546 bp. On the other hand, it was appeared different in height of peak 253, 255 and 252 and area under peak 2783, 5100 and 4536 for ARC, TB and TN isolates respectively. In addition, it was appeared different of band density for three isolates. Whereas, TB isolate more density than ARC and TN isolates (Fig. 14).

Fig. 10. SDS-polyacrylamide gel electrophoresis illustrating peroxidase isozyme of the studied CPsV-EG isolates extracted from citrus leaves.
1) ARC isolate. 2) TB isolate.
3) TN isolate. 4) Healthy.

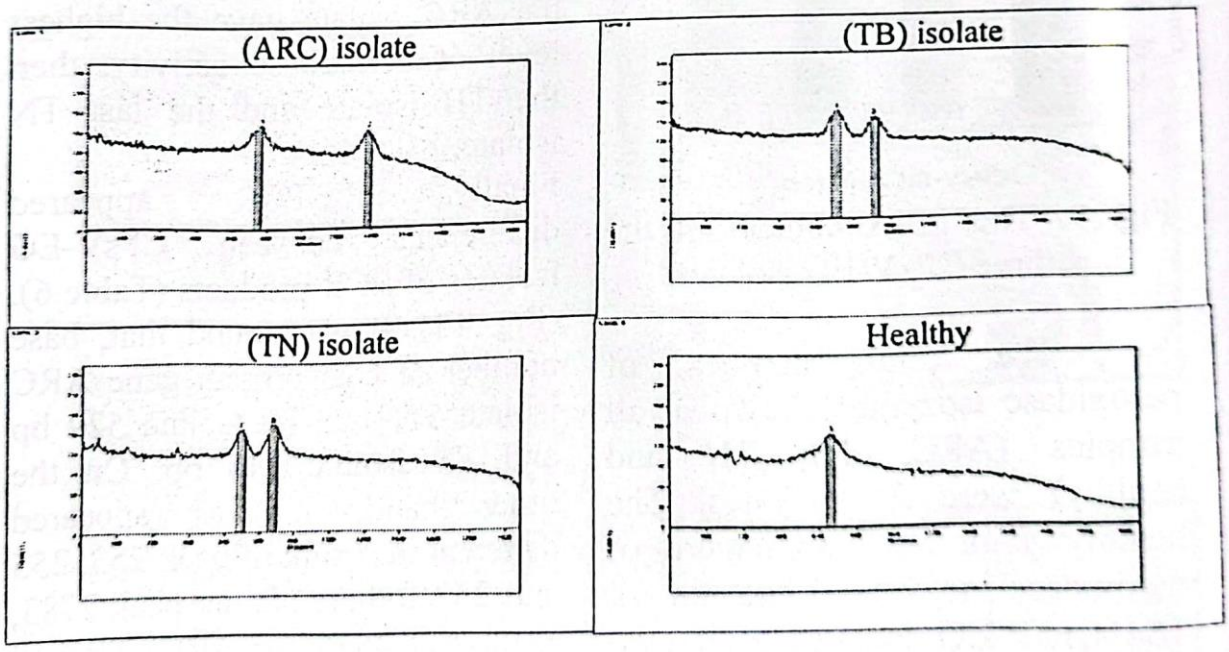

Fig. 11. Gel documentation software analysis (AlphaEaseFC 4.0 oftware) showing peroxidase isozyme patterns, distance and intensity of bands for CPsV-EG isolates compared with healthy.

Table 5: Electrophoretic banding patterns of peroxidase isozymes in healthy and infected CPsV-EG isolates.

R_F	CPsV-ARC				CPsV-TB				CPsV-TN				Healthy			
	Fraction %	Width mass	Height	Area* Calc.	Fraction %	Width mass	Height	Area* Calc.	Fraction %	Width mass	Height	Area* Calc.	Fraction %	Width mass	Height	Area* Calc.
0.35	-	-	-	-	-	-	-	-	-	-	-	-	100	37	139	5143
0.36	-	-	-	-	-	-	-	-	45.1	42	154	6468	-	-	-	-
0.37	-	-	-	-	46.5	41	166	6806	-	-	-	-	-	-	-	-
0.38	58.5	51	162	8262	-	-	-	-	-	-	-	-	-	-	-	-
0.43	-	-	-	-	-	-	-	-	54.9	44	162	7128	-	-	-	-
0.45	-	-	-	-	53.5	38	155	5890	-	-	-	-	-	-	-	-
0.64	41.5	50	151	7550	-	-	-	-	-	-	-	-	-	-	-	-

(*): Width mass x Height.

Fraction %: Relative to the total protein contents in each sample.

ARC isolate: Agricultural research center.

TB isolate: Balady from Tersa.

TN isolate: Navel from Tersa.

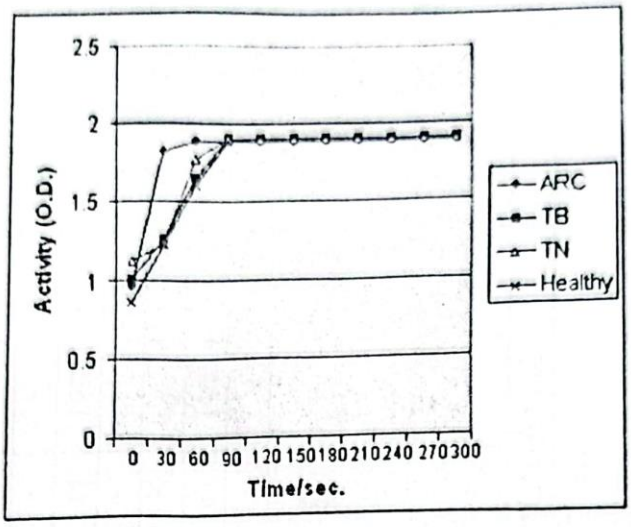

Fig. 12. Peroxidase activity curve showing the level of peroxidase activity in infected citrus leaves with CPsV-EG isolates and healthy.

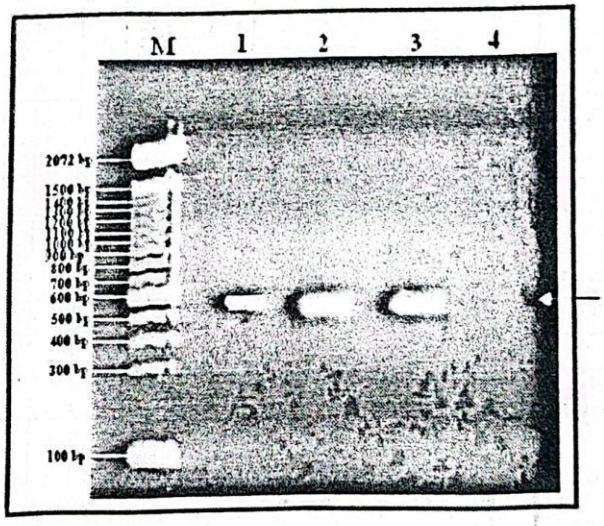

Fig. 13. Agarose gel electroporesis stained with ethidium bromide showing the PCR products after amplification. Arrow indicated the correct sizes of the amplified products (571, 529 and 546 bp) by using downstream and upstream primers (PS66 & PS65).

Lane (M): DNA molecular weight marker (100 bp ladder).

Lane (1): Citrus Grapefruit leaves sample infected with CPsV-EG isolate (ARC).

Lane (2): Citrus Balady leaves sample infected with CPsV-EG isolate (TB).

Lane (3): Citrus Navel leaves sample infected with CPsV-EG isolate (TN).

Lane (4): healthy citrus Grapefruit leaves sample.

Table 6: PCR products differences among three CPsV-EG isolates.

CPsV-EG isolates	MW. (bp)	Distance	Width	Height	Area Calc.	R_F
ARC	571	139	11	253	2783	0.56
TB	529	144	20	255	5100	0.58
TN	546	142	18	252	4536	0.57

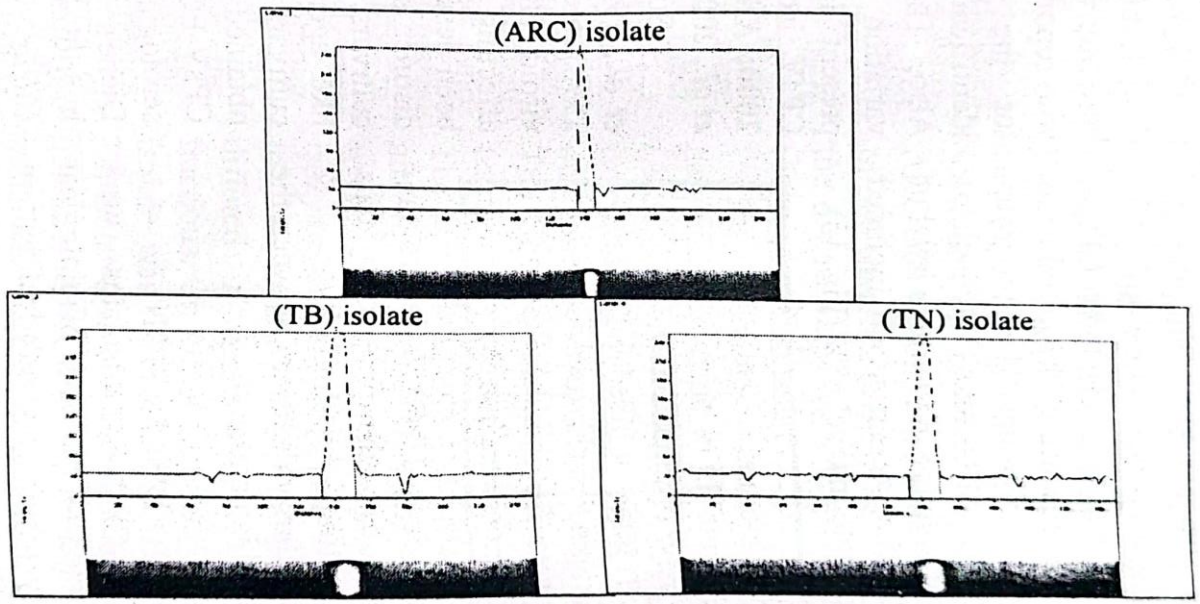

Fig. 14. Gel documentation software analysis (AlphaEaseFC 4.0 software) showing scanning profile of stained agarose gel containing of PCR product of nucleic acid for CPsV-EG isolates.

Finally, according to the dendrogram produced from cluster analysis based on the previous data it was found that, the studied isolates have been an average dissimilarity percentage of 68% and at this level the three CPsV-EG isolates were delimited into two distinct groups. The first group included ARC and TB isolates linked together in the same level of 33%. The second group included TN isolate in level 68% (Fig. 15).

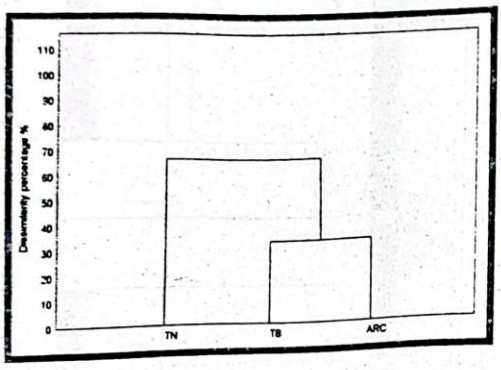

Fig. 15. UPGMA-dendrogram based on the previous data illustrating similarity & dissimilarity distances between the studied isolates.

DISCUSSION

CPsV-EG isolates were detected on the basis of biological assay which gave oak leaf pattern on Dweet tangor and serological assay using specific Mab specific CPsV by DAS-ELISA. Where ARC, TB and TN were differed in external symptoms and DAS-ELISA reading where the severe isolate (ARC) gave the highest rate (2.204), then the mild isolate (TB) (1.958) and the last latent isolate (TN) (1.669). These results are in harmony with **Barthe *et al.* (1998)** who reported that, Mabs are useful for the differential serological identification of CPsV strains. Also, These differences due to variation in the epitopes which present in the *CP* of different CPsV isolates (**Djelouah *et al.*, 2000; Alioto *et al.*, 2003; Fahmy *et al.*, 2003**).

Some authors (**Duran-Vila *et al.*, 1991; El-Dougdoug *et al.*, 1993**) mentioned that, more one viroid, phytoplasma and 5 viruses affecting the genus *Citrus* have been described. The CPsV induced distinct symptoms in commercial cultivars. Because virus and virus like agents infected many citrus cultivars in commercial farms, the obtained samples which using for CPsV-EG isolates were examined serologically by immunoprinting, Diene's stain and tissue print hybridization for the presence of *Citrus tristeza virus* (CTV), *Spiroplasma citri* and *Citrus excortis viroid* (CEVd). The results showed that, the tissues of Grapefruit, Balady and Navel

samples gave negative results using specific monoclonal antibodies of CTV, *Spiroplasma citri* using Diene's stain and CEVd using tissue print hybridization. These results indicate the samples were virus and virus like agents tested and infected with CPsV-EG only **(El-Shorbagy, 2007)**.

The woody indicators (Dweet tangor, Madam vinous, New hall, Navel parent Washington, Balady, Navelina and Valencia) citrus plants were differed in response to three CPsV-EG isolates. These isolates were differentiated from each other through the symptoms initiated into various woody indicator plants and different incubation periods. These results are in agreement with **Roistacher (1991)** who reported that, twenty-one isolates of psorosis have variability in symptoms found in citron, Dweet tangor and sweet orange among the various isolates. Also, CPsV isolates frequently differ in the intensity and kind of symptoms induced in different citrus species (**Derrick *et al.*, 1988; Garcia *et al.*, 1991, a; Navas-Castillo *et al.*, 1991; 1993; Garcia *et al.*, 1994; Legarreta *et al.*, 2000**).

On the other hand, the three CPsV-EG isolates reacted with different responses at 28 ± 2°C under greenhouse conditions on 5 host plants belonging to 3 families and they are differd in symptoms and incubation period.

The total RNA content values in the leaves of three CPsV-EG isolates compared with healthy are recorded where the highest value of 328 µg/g was recorded in ARC isolate, followed by the value of 300 µg/gm in TB isolate. While the lowest value of 280 µg/g was recorded in TN isolate.

The total number of peroxidase isozyme in all samples (ARC, TB, TN and healthy) were 7 isozymes. The healthy plant has one pattern of peroxidase isozyme. The CPsV-EG isolates affect the fraction of peroxidase isozyme. The CPsV-EG isolates (ARC, TB and TN) appeared 2 isozymes but were differed in density of isozyme fractions. TB isolate near to TN isolate in RF but was differed in density of isozyme fractions. On the other hand, ARC isolate was differed from TB and TN isolates in R_F value and density of isozyme fractions. These results indicated that, the number of peroxidase fractions increased in the infected plants and this due to the plant resistance could be due to

increasing peroxidase fractions. Also, the level of peroxidase activity was found to be considerably higher in infected leaves than healthy ones. Whereas, the ARC isolate gave the highest level of peroxidase activity, then the TB isolate and the last TN isolate. These results are in harmony with **Ali *et al.* (2006)** who reported that, virus infection in hypersensitive host induced oxidative stress which enforces the host to produce high levels of reactive oxygen speces (ROS) which have high oxidative power activity to oxidize and attack any oxidisable material in infected cell and included organelles besides phenolic compounds in the cell. In addition, destroying membranes or organelles i.e. lysozyme causing flowing out of its contents due to hydrolytic enzymes which hydrolyze the bio-molecules in the cell. This finally, leads to cell death in infected area. Thus, increased cellular H_2O_2 levels and subsequent redox imbalances may be responsible for cell death in plants.

RT-PCR showed differences between CPsV-EG isolates of PCR products where base number of coat protein gene ARC isolate 571 bp; TB isolate 529 bp and TN isolate 546 bp. On the other hand, it was appeared different in height of peak 253, 255 and 252 and area under peak 2783, 5100 and 4536 for ARC, TB and TN isolates respectively. These results indicated that, molecular variabilities between the three CPsV-EG isolates and this due to variability of *CP* and these are in harmony with **Alioto *et al.* (2003)** and **Fahmy *et al.* (2003)** who reported that, variability of the coat protein (*CP*) gene of *Citrus psorosis virus* (CPsV).

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