Incidence and Detection of *Citrus Tristeza Virus* in Egypt by ELISA, RT-PCR and real-time PCR (TaqMan®)

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ABSTRACT—*Citrus tristeza virus* (CTV) has been previously reported in citrus growing regions of Egypt. Incidence, distribution of CTV and extensive surveys were conducted in citrus growing areas of Qalyubia, Ismailia and Sharkya Governorates during 2012-13 to determine the occurrence of the virus, to evaluate its incidence, to identify and characterize the CTV isolate. A total of 3630 citrus seedlings and trees from nurseries and orchards were sampled from more than 10300 Feddans of citrus in three governments. Leaf samples were collected and symptom intensity of the trees was recorded. Field observations including stem pitting, stunting, vein clearing and leaf curling symptoms revealed the existence of CTV. Double Antibody Sandwich - Enzyme Linked Immunuo-Sorbent Assays (DAS-ELISA), one step RT-PCR and TaqMan real time- RT-PCR were used to index and confirm the existence of CTV in different locations in Egypt. Isolate genotyping was conducted using reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR and the real-time RT-PCR assays developed were more sensitive and less time consuming than DAS-ELISA.

keywords— Citrus, *Citrus tristeza virus* CTV, Survey, DAS-ELISA, RT-PCR.

1 INTRODUCTION

The Egyptian citrus industry has a high economic value and annual production is close to 3.2 million tons, from 420 thousand Feddans. Egypt is the leading exporter of fresh citrus and the ninth most important producer of the world, export are estimated at 820.000 Tons **(Statistic of Ministry of Agric. 2013)**. Citrus also has a very high social importance because it provides much employment and additional income to small growers. *Citrus tristeza virus* (CTV) was first reported in Kanater region, Qalyubia government in Egypt in 1957 **(Nour-Eldin and Bishay 1958).** Starting from 1990, a large-scale CTV survey was carried out, reporting several CTV outbreaks, in different governorates. Preliminary data based on CTV molecular characterization suggest that some CTV isolates are very similar to the severe strain from Florida, that causes quick decline and stem pitting **(Abou-Zeid *et al.*, 1990; Abdel-Salam; Fahmy *et al.*, 2009).** Tristeza disease caused by *Citrus tristeza virus* (CTV), is one of the most destructive and economically important viral diseases limiting commercial citrus production worldwide **(Bar-Joseph *et al.*, 1989; Rocha-Pena *et al.*, 1995)**. CTV is able to infect most species, varieties and hybrids of *Citrus* as well as some close relatives of *Citrus* **(Muller and Garnsey, 1984)**. While most commercial *Citrus* varieties are sensitive to CTV, a citrus relative, *Poncirus trifoliata,* and some of its hybrids are resistant to this virus. Citrus Tristeza diseases spread by infected plant material and insect vectors *Toxoptera citricidus*, the most efficient CTV-vector, has been recently introduced in Portugal and Spain, seriously threatening the Mediterranean region. Citrus tristeza virus (CTV) virions are flexuous illaments, ~2000 x 11 nm, with a genomic RNA (gRNA) molecule and two capsid proteins of 25 and 27 kDa, which coat 95 and 5% of the particle length, respectively **(Bar-Joseph and Lee, 1989; Pappu *et al.*, 1994; Febres *et al.*, 1996).** The CTV gRNA, single-stranded with positive polarity, is 19226 to 19296 nucleotides in size, and is organized into 12 open reading frames. It potentially encodes at least 19 protein products, with untranslated regions (UTRs) of-108 and 290 nucleotides at the 5'- and 3'-termini,respectively**(Karasev,*et. al.*,1995;Mawassietal.,1996; Vives *et al.*, 1999; Yang *et al.*, 1999).** Variations in serological reactivity, peptide maps of the coat protein, double-stranded RNA patterns, restriction fragment length polymorphisms, and single-strand conformation polymorphisms (SSCPs) have been utilized in an attempt to differentiate CTV isolates **(Orita *et al.*, 1989; Rubio *et al.*, 2001; Sambade *et al.*, 2002).** Among these methods, nucleotide sequence analysis is the most accurate procedure for identifying and analyzing genetic variants in geographically restricted regions, as well as among countries **(Rubio *et al.*, 2001; D'urso *et al.*, 2003)**. Several techniques have been developed for the detection and differentiation of CTV isolates. Indexing on a standard host range **(Garnsey *et al.*, 2005)** detects and categorizes the different biological types of CTV, but it is laborious and expensive for large-scale tests. Serological techniques have been used since the 1970s for detecting CTV **(Bar-Joseph *et***

***al.*, 1979; Garnsey *et al.*, 1993).** Enzyme-linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) are the most common because of their reliability, rapidity and low relative cost. More recently, polymerase chain reaction (PCR) has been adapted for detection of CTV: two-step reverse-transcription RT-PCR **(Metha *et al.*, 1997; Hilf and Garnsey, 2000; Huang *et al.*, 2004)**; one-step RT-PCR **(Hung *et al.*, 2000);** immunocapture (IC)-RTPCR **(Cambra *et al.*, 2002)**; and multiplex RT-PCR **(Roy *et al.*, 2005)**. Although PCR techniques described above can detect a low titer of virus, they are not quantitative. Real-time PCR allows rapid detection of target-specific amplicons and accurate quantification when used with a standard curve. Real-time RT-PCR has been reported for the detection of different woody plant-infecting viruses **(Marbot *et al.*, 2003; Schneider *et al.*, 2004; Varga and James, 2005,2006; Osman and Rowhani, 2006; Osman *et al.*, 2007)** as well as for viruses in different insect vectors **(Boonham *et al.*, 2002; Fabre *et al.*, 2003; Olmos *et al.*, 2005).** We report results of incidence, distribution and detection of *Citrus Tristeza Virus* in three governorates using DAS-ELIZA, RT-PCR and real-time reverse transcription-PCR (TaqMan®) to confirm the presence of CTV infection in tested samples.

2MATERIALS AND METHODS

2.1 Field Surveys:-

Field survey and inspection were conducted in Qalyubia, Ismailia and Sharkya Governorates in addition to other newly regions with large scale citrus cultivation. Therty-one field visits covered about 964.21 acres at Qalubia Governorates represented on 48 farms. Whereas, Ismailia governorate was visited within 25 field trips and investigated 6855.9 acres in 39 farms. In addition to, 958.5 acres were inspected at Sharkia governorate in 20 farms. Field survey aimed to select the representative governorates and areas of citrus production and nurseries and build up technical protocols for monitoring and diagnosis of CTV. Survey was carried out by visiting different locations and throughout the externally symptoms that subjected to CTV disease. Farm information was recorded by reporting the farm area by Feddan, variety and rootstock of citrus and tree age. Field inspection was done either random or regarding to the farmer's notes. The diseased trees were sampled and marked. Each sample of one diseased tree was obtained from the 4 directions of tree as young branches (east, west, and north, south). On some other cases, a piece of phloem trunk was sampled. The tree was marked (red spray on trunk) and photographed. The location of each tree was recorded and subjected disease mentioned. Field inspection team work consisted of specialists (multi disciplines) on horticulture, pathology (virology & bacteriology) and entomology. Tools were provided to each teamwork when field inspection done by digital camera, plastic bags, scissors, and red color spray, marker and block notes. Then, samples were transferred to laboratory (Plant Pathology Research Institute).

2.2 Monitoring of CTV and insect vector:

The main Egyptian citrus growing areas of Qalyubia (Qaha, El Kanater, Kafr El Arbein, Toukh, Kafr Shokr and Moshohor), Ismailia (Wadi Al Molak, El Kassaseen, Al Salhya, Al Quntara East, Abou Soweer and Sarabium) and Sharkya (Belbeis and Abou Hammad) governorates were surveyed for the presence of CTV and its vectors. The choice of citrus species and/or cultivars surveyed was based on their relative economic importance. As to CTV, field monitoring was carried out during the two successive seasons 2012 and 2013 especially in autumn and spring seasons. Trees were sampled, according to the hierarchic method of Gottwald and Hughes (2000), in each selected farm and in the two stands of bud-wood sources. Apical bud-sticks (10-15 cm long) were collected from the quadrant of each tree and stored at 4ºC. In nurseries, sampling was carried out in the outside row of each homogeneous block (variety/rootstock and age). Monitoring for aphid species was conducted in March-July in 18 citrus groves located in Qalyubia, Ismailia and Sharkya governorates. The number of collected aphid samples was proportional to the citrus species present in the visited farms.

2.3 Serological test:

Doubled antibody sandwich- enzyme linked immune-sorbent assay (DAS-ELISA) using ELISA-kit provided from Agritest were used. Signal develops by alkaline phosphates reaction with P-nitro phenyl **(Bar-Joseph *et al.*, 1979).** All positives were confirmed using commercial kits (Agritest, Italy).

2.4 Molecular characterization:

Viral RNA preparation and one-step RT-PCR amplification:

Phloem tissue from young shoots was scraped and powdered in liquid nitrogen. About one hundred mg of each sample was used for total RNA extraction using the Plant Total RNA Mini Kit, according to the manufacturer's protocol (Real Biotech, Corp., Taiwan). RNA was finally eluted with 50 µl of RNase- free water, and stored at -20ºC. RT-PCR was carried out on RNA preparations with Reverse-iT™One-Step RT-PCR Kit (ABgene®UK). This allows RT and amplification to be performed sequentially in the same tube. A 353 bp fragment at the carboxy terminal half of the coat protein gene of CTV was amplified using 64 anti sense primer (5′ TGACATTAGTAACTACGACATCATCAGCCC 3′) and 65

sense primer (5'ATGACGACGCCACGGGTATAACGTACACTC3') as described by **Lair *et al.*, 1994**. In particular, 2.5 µl of target RNA was mixed with 12.5 µl 2x RT-PCR Master mix containing 1.25U/50µl Thermo prime Plus DNA Polymerase; 1.5mM $MgCl_2$, 0.2 mM each dNTPs, 10µM specific sense and antisense primers; 0.5 µl Reverse-iT™ RTase Blend (50U/µl); RNase/DNase-free water to a volume of 25 µl. Synthesis of cDNA was performed at 47ºCfor 30 min, followed by denaturation at 94ºC for 2 min. Amplification cycles consisting of 95C/1'; 50C/1'; 72C/2' followed by a final 5 min at 72 C.

2.5 Agarose gel electrophoresis analysis:

5-7 µl of PCR-amplified DNA fragments were separated by agarose gel electrophoresed in 1-2% agarose (FMC,Rockland,Maine) minigels in 0.5x TBE buffer (Trisborate-EDTA, 90 mM tris-acetate, 90 mM boric acid, 2 mM EDTA) at 120 volt, 100bp DNA molecular weight markers were used to determine the size of PCR products and visualized with UV light after staining for 10-15 min with ethidium bromide (10 µg/ml) **(Sambrook *et al.*, 1989)** and photographed using BioRad Gel Documentation System.

2.6 TaqMan real time- RT-PCR detection:

TaqMan real time reverse transcriptase (RT)-polymerase chain reaction (PCR) using purified RNA target was developed to detect (CTV) RNA-targets in plant tissues. With this method all CTV isolates from different hosts and origins could be detected. RNA was extracted and purified from citrus infected tissues as described above. TaqMan assays for real time RT-PCR were performed in CFX96 BioRad real-time system. TaqMan Universal PCR Master Mix, 1XMultiScribe and RNase Inhibitor Mix (BioRad), 1µM primer 3'UTR1(5'CGTATCCTCTCGTTGGTCTAAGC3') and 1µM 3'UTR2 primer (5'ACAACACACACTCTAAGGAGAACTTCTT3'), 150nM TaqMan Probe (FAM-TGGTTCACGCATACGTTAAGCCTCACTTG-TAMRA) (**Edson *et al.*, 2008)** and 5µl of extracted RNA from citrus plant tissues. An internal control NADH was used as described by **Menzel *et al* (2002).** Real-time RT-PCR protocol consisted of one step at 48C for 30 min and 95C for 10 min followed by 45 cycles of amplification (95C for 15 s and 60C for 1 min). Data acquisition and analysis were performed with BioRad software. The default threshold set by the machine was slightly adjusted above the noise to the linear part of the curve at its narrowest point according to the CFX96 BioRad Manufactures.

3RESULTS

3.1 Disease symptoms:

Naturally occurring of Tristeza disease is varied considerably in the ability of cause symptoms on different host plants and in the intensity of the symptoms expressed. Some causes are mild and produce noticeable effect on most commercial citrus varieties that distributed over all Egyptian farms. Then usually causes mild vine, clearing and stem pitting. However, most causes could one or more of the symptoms indicated. An attention must be paid to the seedling yellow, that are cholorosis and dwarfing occurred. Decline results from viral effect on the phloem just below the bud union. The decline may occur over a period of several years or very rapidly (Regarding the notes that obtained from the farmer). Trees that decline slowly generally have a bulge above the bud union and honey combing in present on the inner face of bark flaps removed from the orange rootstock. However when the trees decline rapidly, honey combing does not occur, but brown line may be observed in the bud union after the bark is removed. In addition, severely affected trees are stunted and may have a bushy appearance. Leaves are chlorotic and the twigs are brittle and break easily when bent. When the bark is removed from these twigs, elongated pits are apparent in the wood and gum associated with fine pits in other cases. Of course, the tree is reducing vigor. Symptoms severity of CTV is varied considerably depending on citrus varieties and locations.

3.2 CTV incidence and insect vector:

As shown in **Table (1)** 175 trees of 3630 tested collected from the commercial fields were CTV-positive (4.8 % infection rate) by ELISA. Surveys of insects related to CTV were started by external observation during field inspection. Few samples were identified as two species of aphids: cotton (melon) aphid *(Aphis gossypii)* and citrus aphid (*Toxopter spp*.). Frequencies of cotton aphids are more than those of citrus aphids. Some difficulties are faced the action of surveys due to multi-spraying with chemical insecticides by citrus growers in spit the existence of honey dew with black sooty moulds on upper surface of leaves and young shoots of citrus trees. Indexing of CTV by DAS-ELISA was carried out on CTV samples. Each sample represented on seedling (1-2 years old) or citrus tree that obtained from the 4 directions of tree. Data presented in Table, 1 showed that 175 samples were found to be clear positive reaction with ELISA. That gave 4.8 % infection as average for all farms. However, such infection of CTV ranged between 7and 31 according to samples obtained of diseased trees value of each one gave more than 3 fold of the O.D. value of negative controls (healthy one). In this respect, some samples gave no clear positive reactions,

which their values recorded little more of 2 fold of negative controls. These samples will be taken to do PCR to confirm their infection. However, most samples (175 of 3630 tested ones) revealed to infection with CTV. Out of 29 tested farms, only 12 farms were CTV-free each farm was investigated during the period of field monitoring (6 months) except only one farm which was investigated twice. More extensive surveys are needed to cover the other growing areas of citrus, especially in Behera and Monufia Governments and Upper Egypt which are an important production area.

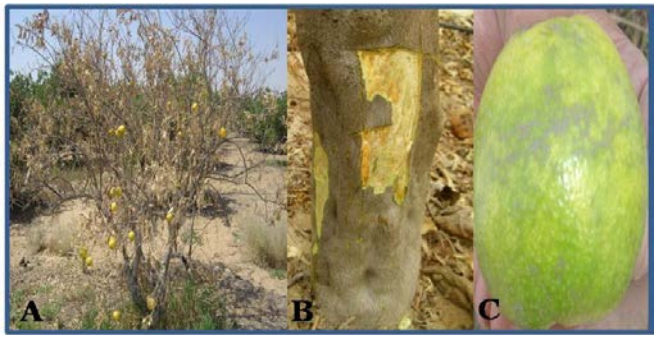

Fig.(1): CTV Symptoms appeared during filed survey, **A**)declining symptoms on sweet orange trees grafted on sour orange rootstock infected by CTV collected from Ismailia Governorate. **B)** Bud-union of sweet orange CTV-infected tree grafted on sour orange rootstock, and pin-holing or honeycombing in the inner face of the bark of the sour orange rootstock below the bud union of the *Tristeza*-infected tree collected from Ismailia and Qalyubia Governorates. **C)** *Tristeza* aggressive isolate-induced some deformation on fruits.

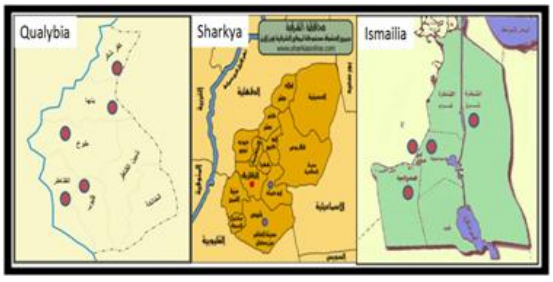

Fig. (2). A map showing some of the locations in Qualybia, Sharkia and Ismailia governments surveyed for the incidence and severity of *Citrus tristeza virus* (CTV).

Table, 1: CTV incidence in different governments in commercial fields during the two successive seasons 2012 and 2013.

Governments	Location	Area (Fed.)	Tree Age (Year)	ELISA Reaction		
				Tested No.	Infected No.	% of Infected
Ismailia	Wadi Al Molaak	1117	4-20	500	00	00
	El Kasaseen	1527	2-25	500	00	00
	Al Salhya	3692	4-20	500	00	00
	Al Quntara East	165	4-5	80	25	31.3
	Abou Sower	3756	3-10	500	00	00
	Sarabium	40	2-10	100	00	00
Sharkya	Beleis	555.5	2-50	500	89	17.8
	Abou Hammad	143	2-7	300	00	00
Qalyubia	Qaha	28.71	2- 10	50	5	10
	Al-Kanater	86	40-60	90	13	14.4
	Kafr ElArbein	108	2-9	100	16	16
	Toukh	293	2-50	100	9	7
	Banha	135	30-80	90	00	00
	Kafr Shokr	264	15-100	150	11	7.3
	Moshtohor	30	40	70	7	10
Total				3630	175	4.8

3.3 Molecular Detection:

RT-PCR amplification of 353 bp fragment at the carboxy terminal half of the coat protein gene (Fig.3) of some representative CTV samples collected from different locations surveyed in this study according to data presented in Table (2) showing the presence of CTV.

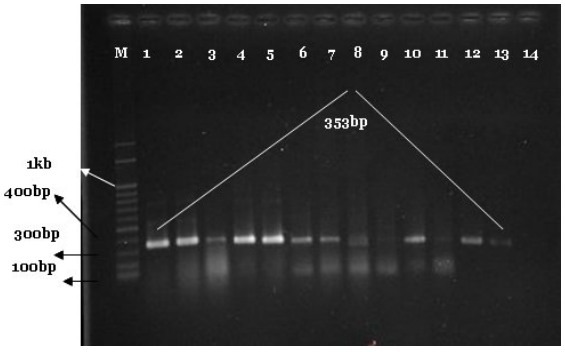

Fig.(3). Agarose Gel Electrophoresis of RT-PCR amplification of 353 bp fragment at the carboxy terminal half of the coat protein gene of some representative CTV samples collected from different regions according to Table (2). M,100 bp Ladder DNA Marker Real Biotech Corp.

Table. (2). Source of samples and variety/Rootstock of representative samples in Fig.(3).

Lane	Source of sample	Variety/Rootstock	PCR Results
1	Qaluybia	Orange / Sour orange	+

2	Qaluybia	Orange / Volka Mariana	+
3	Garbia	Valencia / Sour orange	+
4	Qaluybia	Orange / Sour orange	+
5	Qaluybia	Orange / Sour orange	+
6	Sharkia	Valencia / Volka Mariana	+
7	Qaluybia	Mandarin / Volka Mariana	+
8	Qaluybia	Navel / Sour orange	+
9	Ismailia	Mandarin / Volka Mariana	+
10	Ismailia	Lemon / Volka Mariana	+
11	Minofya	Mandarin / Sour orange	+
12	Sharkia	Valencia / Volka Mariana	+
13	Sharkia	Valencia / Volka Mariana	+
14		Negative control	

3.4 Development of TaqMan PCR assay for CTV detection.

CTV TaqMan® assays successfully detected CTV isolates. Furthermore, the assay detected CTV in all infected samples from greenhouse as well as from CTV-infected field trees. All CTV isolates which included mild stem pitting, seedling yellows and decline, were successfully detected, demonstrating that the primers used detected a broad spectrum of CTV isolates. The designed primer and TaqMan probe were able to recognize all tested CTV sources that included several reference isolates as well as a large number of samples. No amplification was obtained from healthy plants used as control or from plant tissue infected with other viruses (Fig. 4).

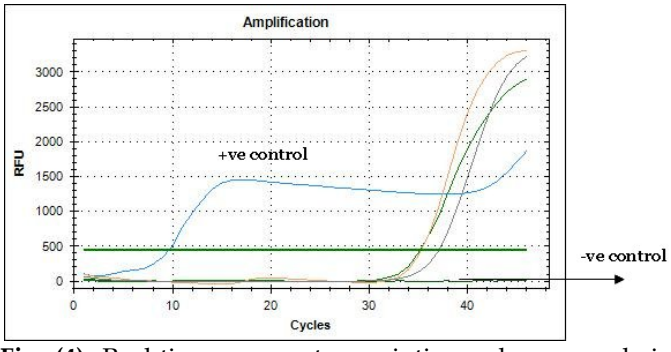

Fig. (4). Real-time reverse transcription polymerase chain reaction (RT-PCR) for CTV. (+ve control) CTV positive control, singleplex amplification of total RNA from CTV isolated from naturally infected trees (-ve control) healthy control.

4 Discussion

Data presented in this study confirmed the presence of CTV in citrus growing areas of Qalybia, Sharkia and Ismailia governments with a molecular technique, thus supporting earlier studies by other authors **(Fahmy *et. al.*, 2009)**. Field symptoms usually associated with CTV infections were also observed **(Bar-Joseph *et al.*, 1989; Roistacher, 1991; Moreno *et al.*, 2008; Atta *et al.*, 2012)**. CTV-infected trees were observed in the **8** locations from 15 locations surveyed in the three governments and in all scion/rootstock combinations sampled. This implies that CTV is widespread in citrus growing areas of North and East Egypt. **Atta *et al.* (2012)** indicated that CTV affects almost all known species of citrus and it can occur even in non-citrus host species. Symptom intensity ranged from mild or no symptoms to severe in the orchards surveyed suggesting the presence of different CTV strains.

Several authors **(Roistacher, 1991; Moreno *et al.*, 2008; Atta *et al.*, 2012)** have previously identified strains of CTV able to induce seedling yellows, stem pitting and quick decline/tristeza. Mixtures of different CTV strains have been detected in infected trees **(Nickel *et al.*, 1996)** and thus detailed characterization and differentiation of local isolates is needed in order to properly indentify the CTV strains present in Egypt. This could be verified via biological indexing on indicator test plants **(Roistacher, 1991)**, with monoclonal antibodies **(Permar *et al.*, 1990)** or by using molecular techniques like bi-directional PCR and others **(Cevik *et al.*, 1996; Roy and Ramachandran, 2002)**. In the present study, primers and TaqMan® probes for CTV detection in planta RT-PCR and real-time RT-PCR assay were developed. The TaqMan® RT-PCR assay incorporated an internal control based on the mRNA of the mitochondrial gene NADH dehydrogenase (nad5). This provided a check on the quality of extracted RNA and helped to assess if it was sufficient, hence, reducing chances for false negative results **(Menzel *et al.*, 2002)**. The primers and TaqMan® probe detected all geographically and biologically different CTV isolates tested. The degeneracy included in the CTV primers and TaqMan® probe assured that the assay was broad spectrum. In contrast, serological methods based on the recognition of specific epitopes by monoclonal and some polyclonal antibodies may not detect some isolates due to coat protein variability among strains. In these cases, a mixture of monoclonal or polyclonal antibodies is usually needed **(Cambra *et al.*, 1990)**. In conclusion, DAS-ELISA, RT-PCR and real-time RT-PCR were used successfully to detect CTV in infected plants. Whereas, Rapid and accurate detection of CTV achieved by real-time RT-PCR can now be used as a tool to support control measures to limit CTV disease spread. In addition, it can be used in quarantine, eradication and certification programs in addition to RT-PCR. Throughout the increasing international demand for olive plants and legislation enacted require that all citrus propagative material produced in nurseries must be free of all viruses.

This led to the development of sensitive diagnosis techniques to assist in selection, improvement and sanitary certification of citrus planting material. Reliable virus detection is also needed in epidemiological studies and in-establishing strategies for control and certification programs.

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REFERENCES

[1.] Abdel-Salam A.M.,1998. Sanitary status and certification of citrus in Egypt. Proceedings of the Mediterranean Network on Certification of Citrus. Options Méditerranéennes, Series B 21, CIHEAM Pablications: 95-97.

[2.] Abou-Zeid A.A., Ibrahim I.A., Nasr El-Din T.M., Khalil E.M.,1990. Detection of unusual isolates of citrus tristeza virus (CTV) using visual symptoms, indicators and Enzyme-linked immunosorbent assay (ELISA). In: Proc. of 6th congress of Phytopat. Cairo, 1990: 645- 656.

[3.] Atta S., Chang-yong ZHOU, Yan ZHOU, Meng-ji CAO, Xue-feng WANG, 2012. Distribution and Research Advances of Citrus tristeza virus. Journal of Integrative Agriculture, 11: 346-358.

[4.] Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F., Loebenstein, G., 1979. The use of enzyme-linked immunosorbent assay for detection of Citrus tristeza virus. Phytopathology 69, 190– 194.

[5.] Bar-Joseph M, Lee RF (1989). Citrus tristeza virus. In: Descriptions of Plant Viruses, No. 353. Commonwealth Mycological Institute/ Association of Applied Biologists, Kew, Surrey, England.

[6.] Bar-Joseph M, Marcus R, Lee RF (1989). The continuous challenge of citrus tristeza virus control. Annu. Rev. Phytopathol. 27:292-316.

[7.] Boonham, N., Smith, P., Walsh, K., Tame, J., Morris, J., Spence, N., Bennison, J., Barker, I., 2002. The detection of tomato spotted wilt virus (TSWV) in individual thrips using real-time fluorescent RT-PCR (TaqMan). J. Virol. Methods 101, 37–48.

[8.] Cambra, M., Garnsey, S.M., Permar, T.A., Henderson, C.T., Gumpf, D.,Vela, C., 1990. Detection of Citrus tristeza virus (CTV) with a mixture of monoclonal antibodies. Phytopathology 80, 103.

[9.] Cambra, M., Gorris, M.T., Olmos, A., Mart´ınez, M.C., Rom´an, M.P., Bertolini, E., L´opez, A., Carbonell, E.A., 2002. European diagnostic protocol (DIAGPRO) for Citrus tristeza virus in adult trees. In: Duran-Vila, N., Milne, R.G., da Grac¸a (Eds.), Proceedings of the 15th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 69– 77.

[10.] Cevik, B., Pappu, S.S., Pappu, H.R., Benscher, D., Irey, M., Lee, R.F., Niblett, C.L., 1996. Application of bi-directional PCR to Citrus tristeza virus: detection and strain differentiation. In: da Grac¸a, J.V., Moreno, P., Yokomi, R.K., Proceedings of the 13th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 17–24.

[11.] D'Urso, F., Sambade, A., Moya, A., Guerra, J., Moreno, P., 2003. Variation of haplotype distributions of two genomic regions of Citrus tristeza virus populations from eastern Spain. Mol. Ecol. 12, 517–526.

[12.] Edson,B., Moreno,A., Capote,N., Olmos,A., Vidal,E., Pérez-Panadés, J., Cambra,M., 2008. Quantitative detection of Citrus tristeza virus in plant tissues and single aphids by real-time RT-PCR.Eur J Plant Pathol 120:177–188.

[13.] Febres V.J., L. Ashoulin, M. Mawassi, A. Frank, M. Bar-Hoseph, K.L. Manjunath, R.F. Lee and C.L. Niblett, 1996. The p27 protein is present at one end of Citrus tristeza virus particles. Phytopathology 86, 1331–1335.

[14.] Fabre, F., Kervarrec, C., Mieuzet, L., Riault, G., Vialatte, A., Jacquot, E., 2003. Improvement of barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real-time RT-PCR. J. Virol. Methods 110, 51–60.

[15.] Garnsey S. M., Civerolo E. L., Gumpf D. J., Paul C., Lee R. F., Brlansky R. H., et al. (2005). Biological characterization of an international collection of Citrus tristeza virus (CTV) isolates. Int. Organ. Citrus Virol. 16, 75–93

[16.] Gottwald T.R. and G. Hughes, (2000). A new survey method for citrus tristeza virus disease assessment. In: Proceedings of the 14th Conference of the International Organization of Citrus Virologists, 77–87.

[17.] Hilf, M.E., Garnsey, S.M., 2000. Characterization and classification of Citrus tristeza virus isolate by amplification of multiple molecular markers. In: da Grac¸a, J.V., Lee, R.F., Yokomi, R.K., Proceedings of the 14th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 18–27.

[18.] Huang, Z., Rundell, A.P., Guan, X., Powell, A.C., 2004. Detection and isolate differentiation of Citrus tristeza virus in infected field trees based on reverse transcription-polymerase chain reaction. Plant Dis. 88, 625–629.

[19.] Hung, T.H., Wu, M.L., Su, H.J., 2000. A rapid method based on the onestep reverse transcriptase-polymerase chain reaction (RT-PCR) technique for detection of different strains of Citrus tristeza virus. J. Phytopathol. 148, 469–475.

[20.] Karasev, A. V., V. P. Boyko, S. Gowda, O. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. Cline, D. J. Gumpf, R. F. Lee, D. J. Lewandowski, and W. O. Dawson 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520. 25. Lee, R. F., P. S. Baker, and M. A. Rocha-Peña.

[21.] Lair, S.V., Mirkov,T.E., Dodds,J.A., and Murphy,M.F. 1994. A single temperature amplification technique applied to the

detection of citrus Tristeza viral RNA in plant nucleic acid extracts. J. Virol. Methods 47:141-152.

[22.] Marbot, S., Salmon, M., Vandrame, M., Huwaert, A., Kummert, J., Dutrecq, O., Lepoivre, P., 2003. Development of real-time RT-PCR assay for detection of prunus necrotic virus in fruit trees. Plant Dis. 87, 1344–1348.

[23.] Mawassi M., Gafney R., Bar-Joseph M., 1993. Nucleotide sequences of the coat protein gene of citrus tristeza virus: comparison of biologically diverse isolates collected in Israel. Virus Genes 7: 265-275.

[24.] Mehta, P., Brlansky, R. H., Gowda, S., and Yokomi, R. K. (1997). Reverse transcription polymerase chain reaction detection of Citrus tristeza virus in aphids. Plant Disease. 81(9), 1066-1069.

[25.] Menzel, W., Jelkmann, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99 (1/2), 81–92.

[26.] Moreno P., S. Ambrós, M.R. Albiach-Martí, J. Guerri and L. Peña, 2008. Citrus tristeza virus: a pathogen that changed the course of the citrus industry. Molecular Plant Pathology 9, 251–268.

[27.] Muller, G. W., and Garnsey, S. M. (1984). Susceptibility of citrus varities, species, citrus relatives, and non-rutecous plants to slash-cut machanical inoculation with Citrus tristeza virus. Garnsey, S. M. Timmer, L. V. And Dodds, J. A. (eds). In: 8th Conf. Int. Organ. Citrus Virol., Riverside, CA. 62-65.

[28.] Nickel, O., Santos Filho, H.P. and Vilarinhos, A.D. 1996. Segregation of citrus tristeza strains by graft propagation. p.64-70. In: J.V. da Graça, P. Moreno and R.K. Yokomi (eds.), Proc. 13th Conf. Int. Organ. Citrus Virol. IOCV, Riverside, CA, USA.

[29.] Nour Eldin F., Bishay F., 1958. Presence of the tristeza virus disease in Egypt. FAO Plant Protection Bulletin. 6: 153-154.

[30.] Olmos, A., Bertolini, E., Gil, M., Cambra, M., 2005. Real-time assay for quantitative detection of non-persistently transmitted Plum pox virus RNA targets in single aphids. J. Virol. Methods 128, 151–155.

[31.] Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi. 1989. A rapid and sensitive detection of point mutations and genetic polymorphisms using polymerase chain reaction. Genornics 5: 874-879.

[32.] Osman, F., Rowhani, A., 2006. Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). J. Virol. Methods 133, 130–136.

[33.] Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2007. Real-time RT-PCR (TaqMan®) assays for the detection of Grapevine Leafroll associated viruses 1–5 and 9. J. Virol. Methods 141, 22–29.

[34.] Pappu H.R., A.V. Karasev, E.J. Anderson, S.S. Pappu, M.E. Hilf, V.J. Febres, R.M.G. Eckolff, M. McCaffery, V. Boyoko, S. Gowda, V.V. Dolja, E.V. Koonin, D.J. Gumpf, K.C. Cline, S.M. Garnsey, R.F. Dawson and C.L. Niblett, 1994. Nucleotide sequence organization of eight 3′ open reading frames of the Citrus tristeza closterovirus genome. Virology 199, 35–46.

[35.] Roistacher, C.N., 1991. Graft-Transmissible Diseases of Citrus: Handbook for Detection and Diagnosis. IOCV and FAO, Rome, p. 286.

[36.] Roistacher C.N. and P. Moreno, 1991. The worldwide threat from destructive isolates of citrus tristeza virus. A review. Proceedings of the 11th Conference of the International Organization of Citrus Virologists. IOCV, Riverside, CA, USA, 7–19.

[37.] Roy, A. and Ramachandran, P. 2002. Bi-directional PCR- a tool for identifying strains of Citrus tristeza virus. Indian Phytopathol. 55:182-186.

[38.] Rubio L., M.A. Ayllon, P. Kong, A. Fernandez, M.L. Polek, J. Guerri, P. Moreno and B.W. Falk, 2001. Genetic variation of Citrus tristeza virus isolates from California and Spain: evidence for mixed infections and recombination. Journal of Virology 75, 8054–8062.

[39.] Rocha-Pena M.A, R.F. Lee, R. Lastra, C.L. Niblett, F.M. Ochoa-Corona, S.M. Garnsey and R.K. Yokomi, 1995. Citrus tristeza virus and its aphid vector Toxoptera citricida. Plant Disease 79, 437–445.

[40.] Roy, A., Fayad, A., Barthe, G., Brlansky, R.H., 2005. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. J. Virol. Methods 129, 47–55.

[41.] Sambade A., L. Rubio, S.M. Garnsey, N. Costa, G.W. Müller, M. Peyrou, J. Guerri and P. Moreno, 2002. Comparison of viral RNA populations of pathogenically distinct isolates of Citrus tristeza virus: application to monitoring crossprotection. Plant Pathology 51, 257–265.

[42.] Sambrook J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

[43.] Schneider,W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D., Frederick, R.D., 2004. Specific detection and quantification of plum pox virus by real-time fluorescent reverse transcription-PCR. J. Virol. Methods 120, 97–105.

[44.] Varga, A., James, D., 2005. Detection and differentiation of plum pox virus using real-time multiplex PCR with SYBR green and melting curve analysis: a rapid method for strain typing. J. Virol. Methods 123, 213– 220.

[45.] Varga, A., James, D., 2006. Real-time RT-PCR and SYBR green I melting curve analysis for the identification of plum pox virus strains C, EA, andW: effect of amplicon size, melt rate, and dye translocation. J. Virol. Methods 132, 146–153.

[46.] Vives, M.C., Rubio, L.,Sambada, A., Mirkov, E., Moreno, P., Guerri, J 2005. Evidence for multiple recombination events between 2 RNA sequence variants within a citrus tristeza virus isolate. Virology 331 :232-237.

[47.] Yang, Z.N., Mathews, D. H., Dodds, J.A., Mirkov, T. E. 1990. Molecular characterization of an isolate of citrus tristeza virus that cause severe symptoms in sweet orange. Virus Genes 19: 131-142.