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Detection and Characterization of *Apple Chlorotic Leaf Spot Virus* (ACLSV) Isolate from peach orchards in Egypt

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Apple Chlorotic Leaf Spot virus (ACLSV) was isolated from symptomless peach trees and then identified with a specific antiserum (Sanofi comp.) using Double Antibody Sandwich ELISA (DAS-ELISA) and Dot Immunobinding Assay (DIBA). Survey was carried out during 2001 to 2003 in different locations on commercial peach orchards. Percentages of infection were 6.3, 6.7, 12.3and10.9 in Monofia(Khatatba) , EL-Behira (EL-Nobria), North Sinai (Rafah) and Dakkahlia(Metgamer) respectively.ACLSV was transmitted mechanically from peach trees those showing less vigorous and some abnormalities on growth without leaf symptoms to different herbaceous hosts including Chenopodium quinoa willd, Ch. amaranticolor (Cost&Reyyn) and Phaseolus vulgaris L.cv. Pinto.,Typical chlorotic spots were appeared on the woody indicator GF305 leaves one month after chip-budding of infected buds. Reverse transcription polymerase chain reaction (RT-PCR) procedure was used to amplify fragments using coat protein gene primers (358bp) of the viral coat protein gene. PCR product was used to generate ACLSV-specific probe to detect the virus in infected plants using non-radioactive molecular hybridization methods. The result showed that it is more sensitive than DAS-ELISA and can be used for large scale detection. PCR product was cloned and sequenced. Comparison between local isolate sequence and other published sequences of the same virus shows 79.4% - 84% Homology

INTRODUCTION

Apple Chlorotic Leaf Spot Virus (ACLSV) is one of the most economically important viruses affecting both stone and pome fruits. The virus was originally considered a definitive closterovirus, then became a tentative closterovirus, but is now the species of the trichovirus genus(Martelli *et al.*, 1994). The virus appears as elongated very flexuous particles of 720×12 nm encapsidating a single stranded positive sense genomic RNA. The genomic RNA is 7549 nucleotide long (Lister and Barjoseph, 1981).ACLSV is known to be able to infect most fruit trees species including Apple, Pear, Peach, Plum, Cherry and Apricot. The virus caused some serious diseases in stone fruit including False plum pox (Jelkman and Kunze, 1996) ,Plum bark split (Dunez *et al*, 1972),Viruela in Apricot(Pena and Ayuso, 1973) and Butteratura in Apricot and cherry (Ragozzino and pugliano,1981).The virus appears to be world wide distribution. Its spread has been reported in Eastern Asian Region, Eurasian Region, North American Region, Pacific Region, Australia, New Zealand, and Mediterranean Region. The virus is transmitted by mechanical inoculation, by grafting and not transmitted by seed or by insects. There are two strains of the virus; peach strain &apple strain. The virus was first described by (Mink and Shay, 1959) in the United states, and by(Luckwil and Campbell, 1995) in the United Kingdom. Later virus was discovered in Yugoslavia by (Babcvic and Delibasic, 1986), in Egypt by (Ghanem *et al*, 2002).The aims of this study are to identifi the occurrence of the virus in Egypt and to establish a standard procedure for rabid and

accurate detection of the virus in peach trees.

MATERIALS AND METHODS

Field surveys

A survey was carried out during 2001to 2003 in spring and early summer on peach orchards to determine ACLSV occurrence using DAS-ELISA technique. Samples were collected randomly from several commercial peach orchards in four Governorates [Monofia (Khatatba), El-Behira (EL-Nobria), North Sinai (Rafah), Dakkahlia (Metgamer)]. Six leaves from different directions of each tree were collected and stored at 5°C until used for virus detection.

Virus isolation and identification

Samples from peach trees showing less vigorous and some abnormalites on growth without clear symptoms on the leaves were collected and examined in the lab. Using specific antiserum (Sanofi, Sante Animal ,Paris ,France) for *Apple chlorotic leaf spot virus* (ACLSV), *Plum pox virus* (PPV), *Prunes necrotic ring spot virus* (PNRSV), *Prunes dwarf virus* (PDV), *Peach rosette mosaic virus* (PRMV) and *Tomato ring spot virus* (ToRSV) were used as a first step for detection of the virus infection. The samples reacted positively only with the ACLSV antiseram were used for identification of the virus by biological reaction on herbaceous and woody hosts. Different serological and molecular techniques were also applied.

Biological test

1- Mechanical transmission and host range:

leaves of infected peach plants(positively reacted with ELISA test) were homogenized with 2.5%nicotine solution in 0.01M phosphate buffer, PH 8.0 and inoculated onto corborandom dusted leaves of *Chenopodium quino* L. (wild), *Ch. amaranticolor* cost&Reyng, *Phaseolus vulgaris* cv. Pinto , *Cucumis sativus* L. cv Beit Alpha, *Nicotiana glutinosa* L. , *N. benthamiana* and *N. tabacum* L. cv.white Burley . Ten plants of each host were inoculated with the prepared inoculum while the control plants were inoculated with buffer only. All treated plants were kept in the greenhouse at (23-30oC) for a month. The inoculated plants were examined by visual symptoms and DAS- ELISA assay.

2- Graft transmission

Ten healthy GF305 peach seedling were inoculated by double chip budding from naturally infected peach cv. Swelling. Grafted plants were kept in the greenhouse at (23-30° C) for month .Plants was examined visually and serologically using ELISA test.

Serological Tests

1- Double antibody sandwich enzyme linked immuno-assay (DAS-ELISA):

The procedure used as described by (Clark and Adams, 1977). DAS-ELISA was performed by using commercially available kits specific for ACLSV .The kits were purchased from (Sanofi, Sante Animal ,Paris ,France). Reactions were read at 405 nrn in a microtiter plate reader (Tecan Spectra). ELISA readings were considered positive when the absorbance of sample wells was at least two times greater than the mean absorbance reading of healthy controls.

2- Dot-immunobinding assay (DIBA)

The technique of indirect immuno dot blotting was used according to (Knappet *et al.*, 1995)).

62 peach samples were tested using both DIBA and DAS-ELISA techniques for ACLSV infection. The membrane was immersed in substrate solution containing one tablet [BCIP/NPT (Nitroblue tetrazolium, 5bromo-4-chloro-3-indol phosphate)] (Sigma) in 10 ml (0.1M tris, 0.1m Nacl, 5mM MgCL2, PH9.5) to obtained the calorimetric reaction. Results indicated by the development of purple color on the tissue blotted membrane and negative reaction developed no color on the blot.

Molecular Methods

1- Extraction of Total RNA

Total RNA was extracted from infected peach tissues using cetyltrimethylammonium bromide (CTAB) procedure as described by (Gibbs and Mackenzie, 1997).The obtained RNA was used for PCR procedures and hybridization tests.

2- RT-PCR amplification

Reverse transcription polymerase chain reaction (RT-PCR) was done with Qiagen®onestep RT-PCR Kit according to the manufactory instructions. The oligonucleotide primers selected according to (Candrese, *et al.*, 1995). The reverse transcription primer (A52) has the sequence 5\ -CAG ACC CTT ATT GAA GTC GAA-3\ (position 7213-7233 0n ACLSV P863),while the sense return primer (A53) has the sequence 5 \ - GGC AAC CCT GGA ACA GA--3\ (position 6875-6891 on ACLSV P863). Expected amplification product of 358bp of the viral coat protein gene can be obtained. RT-PCR reaction was performed in 50µl volume .The thermal cycling was performed after 30 min of reverse transcription at 50°C followed by 15mins at 94°C. Then35 cycles were applied for template denaturation at 95°C for 1 min, primer annealing at 50°C for1 min.and DNA synthesis at 72°C for 1 min. A fi.al extension step at 72°Cfor 7mins.PCR amplification product was analyzed by electrophoresis in 1% agarose gel in 1/2 x TBE buffer(Tris-HCl 90mM,Boric acid 90mM,EDTA 2nM,pH8).The product was stained with 0.01% ethidium bromide for 10 min. The gel was visualized under UV light and photographed using (Bio-Rad Gel-Doc2000).

3- Cloning of PCR amplified fragments of ACLSV

Amplified DNA segments were cloned using the TOPO -TA Cloning® invetrogene system according to the manufacturer's instructions developed by Invitrogen Corporation, San Diego, California. The plasmid has ampicillin and kanamycin resistance genes for selection purposes and has also incorporated a lac-z gene in the location of the polylinker region. The inclusion of the viral fragment disrupts the lac-z gene sequence and prevents it expression. Escherichia coli strain DH5α (Stratagene) was transformed with the recombinant plasmid. Expression and non-expression are visualized using media containing of 5-Bromo-4Chloro-3- Indolyl-p-D-Galactopyranoside (x-gal) and Isopropyl Thio-β-D-Galactoside (IPTG). DNA was prepared from selected white colonies, digested with Eco RI and fractionated on agarose gels. Restriction enzyme digestion, ligation reactions, transformation of *E. coli* and other molecular biology techniques were done according to Sambrook *et al.* (1989).

4- Sequencing and computer analysis

The nucleotide sequences of PCR amplified fragments inserts were

done using ABI PRISMTM Dye Terminal Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (University of Wisconsin, Madison. Biotechnology Dept) and performed on 377XL automated DNA Sequencer (Applied Biosystem Co.). The nucleotide sequences were compared and analyzed using DNAMAN Sequence Analysis Software (Lynnon BioSoft. Quebec, Canada) with those of ACLSV isolates available in GenBank

5- Non-Radioactive molecular hybridization

Total RNA from 52 tissue samples were extracted using CTAB methods. Samples were denaturited by heating in a boiling water bath for 10 min and blotted on a nylon membrane. Non-radioactive alkaline phosphatase-labeld cDNA probe were generated from ACLSV PCR product using the eneimagesTM system (amersham pharacia biotec) and according to manufacturer's directions. The chemiluminescent signal was generated and detected on a autoradiography film using CDP-Star(amersham pharacia biotec). The same samples were simultaneously tested by DAS-ELISA technique.

RESULTS AND DISCUSSION

Field surveys

Survey was carried out during 2001 to 2003 to determine ACLSV occurrence in peach orchards in different Governorates. In spite of the absence of visible symptoms, the virus was detected using DAS-ELISA on random samples .Data presented in Table (1) indicated that the average of infection in peach cultivars Florida Prince, Desert Red. Swelling, Early grand and Metgamer were 8.1%, 9.2%, 9.5%10% and 8.7% respectively. The survey also indicated the dissemination of the virus was varied according to locations, the data show that the percentages of infection were 6.3, 6.7, 12.3and10.9 in Monafia (Khatatba) , EL-Behira (EL-Nobria), North Sinai (Rafah) and Dakkahlia(Metgamer) respectively. These results indicated that the virus was distributed in the country at different levels. Also, it appeared that all tested cultivars were susceptible to infection. The obtained data agreed with (Diekmann and Putter, 1996) and (Ghanem *et al.* 2002) they described that most peach cultivars latently infected with the virus .The percentage of infection with ACLSV in orchards was mainly due to contamination with infected vegetative propagating materials such as buds or germplasm and rootstocks.

Virus Identification

Biological test

1- Mechanical transmission and host range

Eight species and cultivars belonging to four families were mechanically inoculated with ACLSV to determine the host range and differential host reactions. The data presented in (Table2) and (Fig.1) showed that ACLSV infection produced Chlorotic local lesions on the inoculated leaves of *Chenopodium quino* L. (wild) and *Ch. Amaranticolor* cost&Reyng, and local brown necrotic spots on *Phaseolus vulgaris* cvs Pinto. Results were confirmed by ELISA tests .The symptoms were appeared after 5-10 days from infection, inoculated Vigna unguiculata Walp, *Cucumis sativus* L. cv *Beit Alpha*, *Nicotiana glutinosa* L., *N. benthamiana* and *N. Tabacum L.* cv. White Burley wre not infected with the virus and ELISA tests failed to detect ACLSV from these hosts. The data reviled that ACLSV Egyption

isolate had limited host range, these results agreed with those of Chairez and Lister (1973), Lister and Bar-Joseph (1981) and Ghanem *et al.* (2002) they reported that ACLSV infect restricted species outside family Rosaceae and some of them are symptomless.

2- Graft transmission

One month after inoculation, GF305 peach seedlings showed chlorotic spots on leaves (Fig. 2) and no symptoms were observed on control plants grafted with healthy buds. Results were confirmed by DAS-ELISA. Data presented confirmed the result of (Ghanem *et al*, 2002) who recorded that the virus caused light colored rings or sunken spots on leaves of GF305 peach seedlings inoculated with infected buds of ACLSV.

Serological Tests

Dot-immunobinding assay (DIBA)

Data presented in Fig. (3) showed that 20 samples were infected with ACLSV. also the data indicated that DIBA has the same sensitivity of DAS-ELISA. However it needs very simple equipment. also eliminate the need for a plate reader. The antibody is added only once, however In ELISA antibody is added twice. These results agreed with (Wang *et al*. 1998) they discussed that DIBA is simple rapid and economically also it can be visualized without any instruments and nitrocellulose membrane cheaper than ELISA plates and can be stored for later reading.

Molecular Methods

1- PCR amplification of ACLSV:

An amplified fragment of the expected size (358bp) from the coat protein gene was obtained in RT-PCR from the total RNA isolated from infected plants using specific primers (A52, A53) and no fragments were amplified from the RNA extracted from healthy plants. Results showed a high sensitivity for the detection of ACLSV in low concentration (Fig. 4). These results agree with (Candrese *et al.*, 1995), Malinwski *et al.*, (1998), Pasquini *et al.*, (1998).

2- Cloning, sequencing and computer analysis

The RT-PCR product was cloned into pCR® II -TOPO cloning vector. The clone was used to determine the nucleotide sequence of the coat protein gene. The sequence comparisons with published sequences indicated sequence homology levels between the Egyption isolates (Eg-ACLSV) and other published sequence available at the gene bank. Presented data showed the ACLSV-Egyption isolate genome is 84.0%, 83.4%and80.6% homologous to those in the AF251275, AJ243438 and D14996 respectively and 79.4% to the X99752 nucleotide sequence homology (Table3). The results clearly indicated that the Egyption strain of ACLSV was far from other previously isolated strains recorded in the gene bank. This data is very valuable for breeding programs for resistance bank.

3- Non-Radioactive molecular hybridization

Total RNA of 52 peach plant samples were extracted using CTAB as described before .The total RNA were doted onto nylon membrane and hybridized with specific probe for ACLSV.

Table (1): Distribution of *Apple chlorotic leaf spot virus* (ACLSV) infection in Peach cultivars in different locations

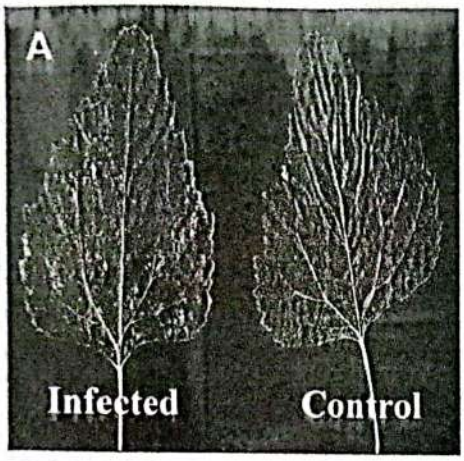
Location / Cultivar	Monofia (Khatatba)			El-Behira EL-Nobria			North Sinai Rafah			Dakkahlia Metgamer			Total		
	*Tested	infected	%	*Tested	infected	%	*Tested	infected	%	*Tested	infected	%	*Tested	Infected	%
FP	330	34	10.3%	600	27	4.5%	354	44	12.4%	390	30	7.7%	1674	135	8.2%
Dr	420	33	7.9%	480	34	7.1%	300	43	14.3%	-----	-----	-----	1200	110	9.2%
SW	300	0	0%	345	34	9.9%	-----	-----	-----	324	58	17.9%	969	92	9.5%
Eg	-----	-----	-----	-----	-----	-----	300	30	10%	-----	-----	-----	300	30	10%
Metgamer	-----	-----	-----	-----	-----	-----	-----	-----	-----	450	39	8.7%	450	39	8.7%
Total	1050	67	6.3%	1425	95	6.7%	954	117	12.3%	1164	127	10.9%	4593	406	8.3%

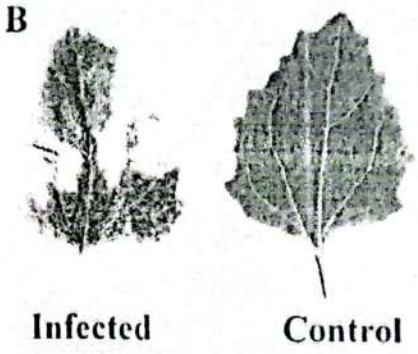
- FP. Florida Prince. Dr: Desert Red. SW: swelling. EG. Early gra
- * No. of tested plants in 3 years from 2001

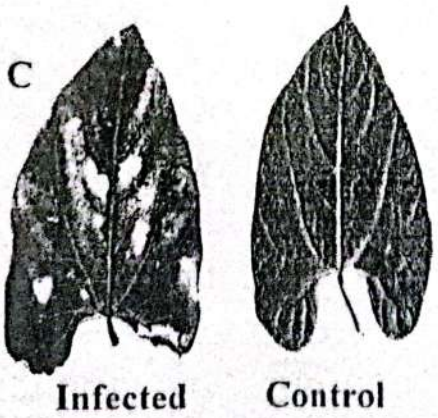
Table (2): Reaction of some indicator host to Apple Chlorotic Leaf Spot Virus (ACLSV) infection

Tested host	Family	Reaction	ELISA Test
Chenopodium quinoa	Chenopodiaceae	*C.L.L.	+
Ch. amarnticolor		*C.L.L.	+
Cucumis sativus cv.Beitalpha	Cucurbitaceae	---	-
Phaseolus vulgaris cvs Pinto	Fabaceae	**B.N.S.	+
Vigna unguiculata Walp		---***	-
Nicotiana benthamiana Domin	Solanaceae	---	-
N. Glutinosa L.		---	-
N. tabcum L.cv.White Burle		---	-

*C.L.L. Chlorotic local lesion * L.B.N.S local Brown necrotic spots *** --- No symptoms







(Fig.1):
A: Chlorotic local lesion on inoculated *Ch. Amarnticolor.*
B: Chlorotic local lesion on inoculated *Ch. quinoa*
C: Purple-brown necrotic spots on inoculated coteledon leave of *Phaseolus vulgaris* cvs Pinto

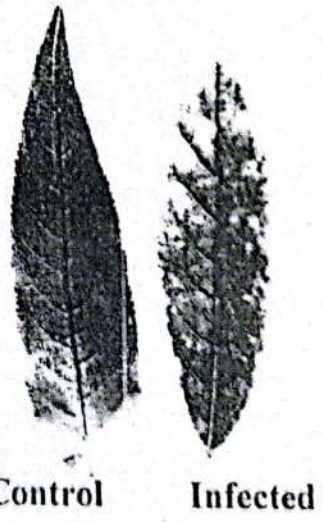
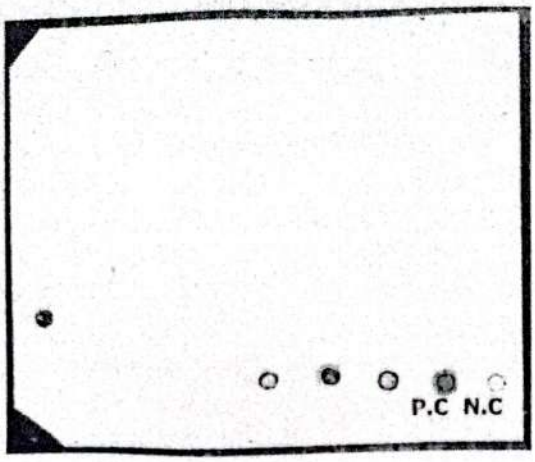


Fig. (2): Chlorotic leaf spots or sunken spots Caused by Apple Chlortic Leaf .Spot virus (ACLSV) on grafted GF305 peach seedling



Fig(3): Detection of ACLSV using (DIBA). pink color indicates positive reaction

The result showed that 10 plants from 52 showed positive for ACLSV Fig (5), while the same samples were simultaneously tested by DAS-ELISA technique only 7 plants of the 52 were showed positive reaction. This data clearly showed that non-radioactive molecular hybridization methods were more sensitive than DAS-ELISA and can be used for large scale detection. These result agreed with (Dominguez 1998) who found that non-radioactive molecular hybridization was more sensitive than DAS-ELISA for detection of PNRSV.

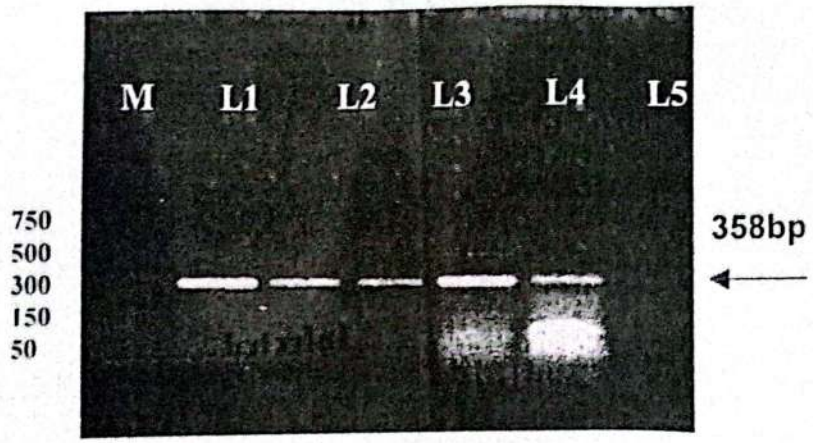


Fig. (4): Electrophoresis analysis of RT-PCR products of ACLSV. M: DNA molecular marker (promega cat G3161). (Lane1) positive control for ACLSV (Lane 2, 3, 4, 5) infected samples while (Lane 6) showing the healthy control

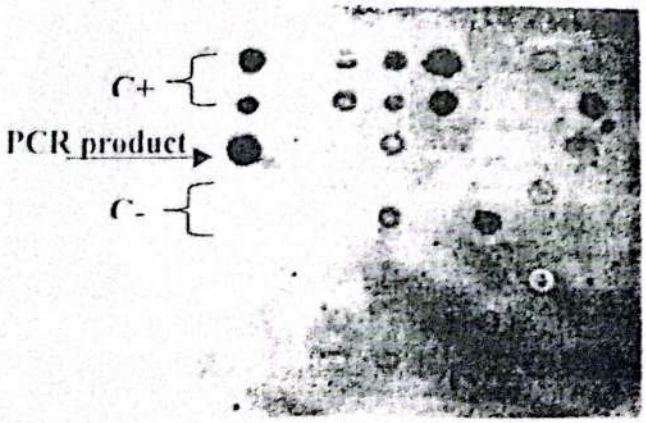


Fig. (5): Dot Blot Hybridization with specific Probe for ACLSV. C+: Positive control for ACLSV, C- Negative control for ACLSV, and PCR Product for the virus.

Table (3): Nucleotide sequence of the amplified 358≈bp fragment from the coat protein gene of the Egyption ACLSV isolate with other ACLSV isolates available in Gen bank using DNAMAN software

Virus isolates Gene Bank Accession No.	Homology identity percentages %	References
AF251275 Plum Poland	84.0%	(Malinowski, *et al*.,1998)
X99752 Cherry Hungary	79.4	(German, *et al*.,1997)
AJ243438 Apricot Germany	83.4%	(Jelkmann,1996)
D14996 Apple Japan	80.6%	(Sato,1993)

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