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Occurrence of *Apple Chlorotic Leaf Spot Trichovirus* in Egypt

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Apple chlorotic leaf spot Trichovirus (ACLSV) isolated from apple trees was successfully transmitted by dagger nematodes (Xiphinema spp). It was transmitted to bait plants grown in soil containing *Xiphinema spp.* nematodes. The virus was identified according to host range, mode of transmission, particle morphology, serological tests and SDS-Polyacrylamide gels. Purification of ACLSV was performed using bentonite/polyethylene glycol. Electron micrograph of purified virus preparation revealed flexuous filamentous virus particles of 700 nm length and 12 nm wide. The average yield was 1.14-1.7mg/100g tissue of Chenopodium quinoa Willd. Purified virus preparation was used for rabbit immunization. The polyclonal antibodies raised against the local isolate of ACLSV had a specific titer of 1: 8000. The concentration of IgG and IgG conjugate with alkaline phosphatase was 1: 1000. The prepared antiserum was used for detection of ACLSV using ELISA dot immunobinding assay (DBIA). The presence of the virus was checked in blossom parts. (petioles, stigma, and flower cup).

INTRODUCTION

Apple is a one of the most widely grown fruit crops worldwide. *Apple chlorotic leaf spot virus* (ACLSV) is the type species of *Trichovirus* genus (Martelli *et al.*, 1994 and Lister, 1996). It was identified for first time in Egypt by Ghanem *et al.* (2002). ACLSV is probably the most common virus of apple. The virus is mechanically transmissible to *Chenopodium quinoa* willd and *C. amaranticolor* Costs&Reyn. and has been transmitted from *C.quinoa* willd plants back to apple (Lister *et al.*, 1965). An inhibitor in *C. quinoa* willd tissue extracts prevented local lesion development on *Phaseolus vulgaris*. The inhibitor could be removed from *C.quinoa* willd extracts by adsorption on magnesium bentonite (Saksena and Mink, 1969a and b). The virus particles are flexuous filamentous (Lister *et al.* 1964). Geographical distribution of the virus is present in all parts of the world wherever stone and pome fruits are cultivated. *Xiphinema spp* (dagger nematodes) has a long needle like hollow stylet which enables them to feed from cells deep within plant roots (Wyss, 1981). It is one of the most serious pests commonly found in orchard soils. Dagger nematodes feed along root surfaces and serve as vectors for transmitting *Tomato ring spot virus* (TmRSV) and *Tobacco ring spot virus* (TbRSV), and *Grapevine fan leaf virus* (GFLV). Moreover Brunt *et al.* (1996) and Sutic *et al.* (1999) mentioned that the transmissibility of the virus by nematodes requires confirmation. The objectives of the present study were to purify ACLSV, and ELISA Kit production, which can be used to confirm the results of nematodes transmission of ACLS

MATERIALS AND METHODS

Source of the virus

ACLSV isolated from apple trees Malus/ 106 M.M rootstock collected from South Tahrir farm showing

chlorotic lesions symptoms was used for production ELISA kit. The virus was propagated and maintained on *C.quinoa willd*, which was used as a source for host range study, virus purification, and nematodes transmission.

Virus purification

The virus was purified by bentonite/ polyethylene glycol procedure described by DeSequeira and Lister (1969) using 100 g of *C.quinoa* Willd fresh leaves.

Electron microscopy

Purified virus preparation was negatively stained with 1% phosphotungestic acid and mounted on Formvar coated grids and examined with an electron microscope (JEOL-JEM-1200 EX II).

Serological studies

a: Production of polyclonal antibodies against ACLSV.

Newzealand white rabbit was given six subcutaneous injections each of 1.7 mg of purified virus emulsified with an equal volume of Freund's incomplete adjuvant at weekly intervals. The blood was collected 10 days after last injections. The antiserum titer was determined using indirect ELISA test at dilutions of 1/500 to 1/8000 with antigen buffer (coating buffer pH 9.6). Anti-ACLSV immunoglobulin was purified from the antiserum according to the method described by Steinbuch and Audran (1969) and it was conjugated with alkaline phosphatase, using the method described by Brattey and Burns (1998). The concentration of IgG and IgG conjugated with alkaline phosphatase was determined using the check board test (Coverse and Martin, 1990).

b: Detection of ACLSV using dot immunobinding assay (DBIA).

DBIA technique on nitrocellulose membrane was used for ACLSV detection in leaves and blossom parts according to the methods described by Hsu and Lowson (1991), using the prepared antiserum.

Polyacrylamide gel electrophoresis (PAGE).

Total proteins were extracted from *C.quinoa* willd leaves infected with ACLSV. Electrophoresis of the protein of ACLSV was performed in 10% polyacrylamide gels in phosphate buffer, pH 7.2, containing 0.1% SDS and 0.1% mercaptoethanol according to Maniatis *et al.* (1982).

Nematodes transmission.

It was performed according to Dijkstra and De-Jager (1998). One hundred grams of soil containing 75 Xiphinema sp /pot 10 cm (previously isolated from infected apple rhizosphere) and each pot planted with one seedling of *C.amaranticolar* Costs&Reyn and *C.quinoa* Willd. Twenty seedlings were inoculated mechanically with ACLSV. One month after inoculation plants were removed and replaced by healthy *C.amaranticolar* Costs&Reyn and *C.quinoa* willd seedings. Roots, shoots, and leaves 30 days later were checked for virus infection using ELISA test. In the case of roots, before tested by ELISA, it should be washed away the soil from them, cut the roots to pieces and ground thoroughly in a mortar with (1:5 w/v) phosphate buffered saline-Tween (PBST). In

control treatment, the soil was autoclaved before used.

RESULTS AND DISCUSSION

It is clear that the virus under study was transmitted mechanically to the tested host plants. The symptoms produced on *Malus sp.* were chlorotic local lesions, which became necrotic after 2-3 days on *Chenopodium amaranticolar* Costs&Reyn (Fig.1) ahd *Chenopodium quinoa* Wild. These results were confirmed by ELISA and were in agreement with those reported by Ghanem *et al.* (2002).

Host range and symptomatology

Results obtained in Table (1) clearly proved that the virus isolated from South Tahrir farm exhibiting chlorotic spots and stunting on infected apple trees as well as other characteristics viz., modes of transmission, particle morphology, serological reactions and polyacrylamide gels electrophoresis (PAGE) is ACLSV

Virus purification

Using the purification method, one clear zone was observed 2 cm below the meniscus of the sucrose density gradient column. The ultraviolet absorption spectrum of ACLSV was characteristic of a nucleoprotein, having a maximum absorbance at 260 nm, a minimum absorbance at 250 nm and a 260/280 nm ratio of 1.17 (uncorrected for light scattering). Fig (2A). The above result

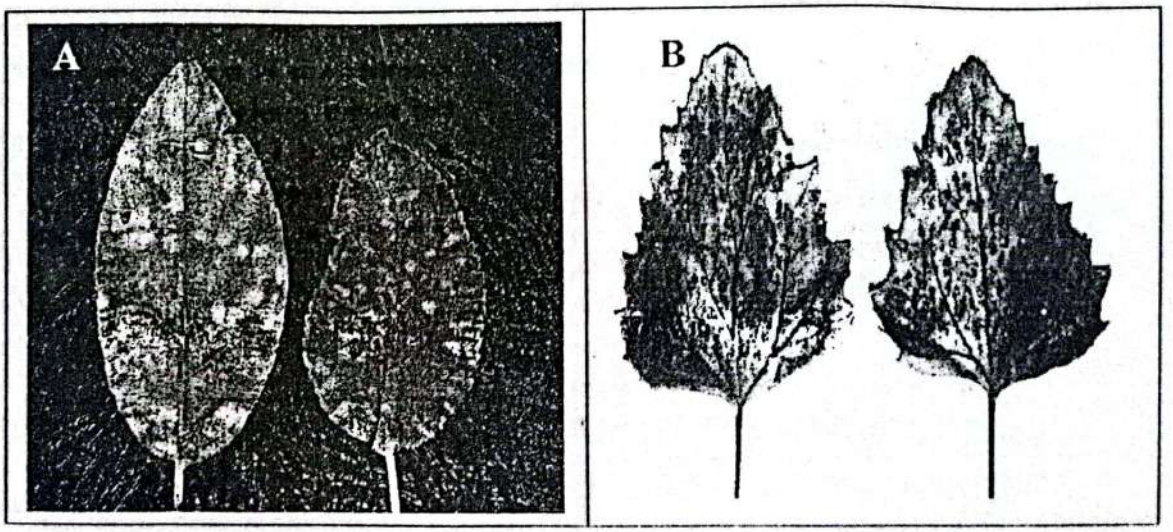

Fig (1) A: Chlorotic spots induced naturally by ACLSV on Malus' rootstock of apple trees. B: Necrotic local lesions developed on *Chenopodium amaranticolar* Costs&Reyn artificially inoculated by ACLSV

Table (1): Symptoms on different plant species inoculated with the virus isolate obtained from *Malus* sp. family Rosaceae

Test plant	Commons name	The main symptoms
Chenopodiaceae:		
Chenopodium amaranticolar Costs&Reyn	Goose –foot	NLL
Chenopodium quinoa Wild		NLL
Rosaceae:		
Malus sp.	Malus'106 rootstock	VB, CLL

NLL= Necrotic local lesion CLL= chlorotic local lesion VB= Vein banding

was in agreement with that obtained by Saksena and Mink (1969b). On the other hand DeSequeira&Lister (1969) and Saksena& Mink (1969b) found that the concentration of ACLSV rises to a peak in *C.quinoa* Willd plants five days after inoculation. These agree very well with our results. Virus yield was 1.14-1.7 mg/100g tissue of infected *C.quinoa* willd. *C.quinoa* Willd seems to have some unique susceptibility to many plant viruses; it is probably the best of the few herbaceous hosts to which viruses latent in apple can directly to be mechanically transmitted. Whether this extreme sensitivity of *C.quinoa* Willd to virus of woody plant implies a similarity in type of inhibitor in the extract or inoculated tissues is not clear (Fulton, 1965) so the purification of ACLSV was performed by magnesium bentonite, clarification followed by differential centrifugation and Saksena and Mink (1969a)

Electron microscopy

Electron microscopy of purified virus revealed the presence of filamentous flexuous particles. The particles were 700×12 nm (Fig 2). These results are similar to those reported by DeSequeira&lister (1969), Pasquini *et al.* (1998) and Ghanem *et al.* (2002) for ACLSV.

Serological studies.

a: Production of ACLSV antiserum

Titer of the induced antiserum measured with indirect ELISA was1/8000. when 1:10 dilution of ACLSV infected *C.quinoa* Willd plant extracts was used. Dilution 1/6000 for 1 St and 2 nd bleedings were the best usable dilution for producing high absorption values at 405 nm (Fig.3). Positive reactions were obtained when the concentration of purified IgG and IgG conjugate with alkaline phosphatase was 1:1000. Indirect ELISA could be useful for the routine detection of plant viruses, virus disease diagnosis and survey where accurate quantitation is not of concern. One of the major goals in the present work is to produce ELISA kit which can be used a rapid method for ACLSV detection in stone fruit, pear and

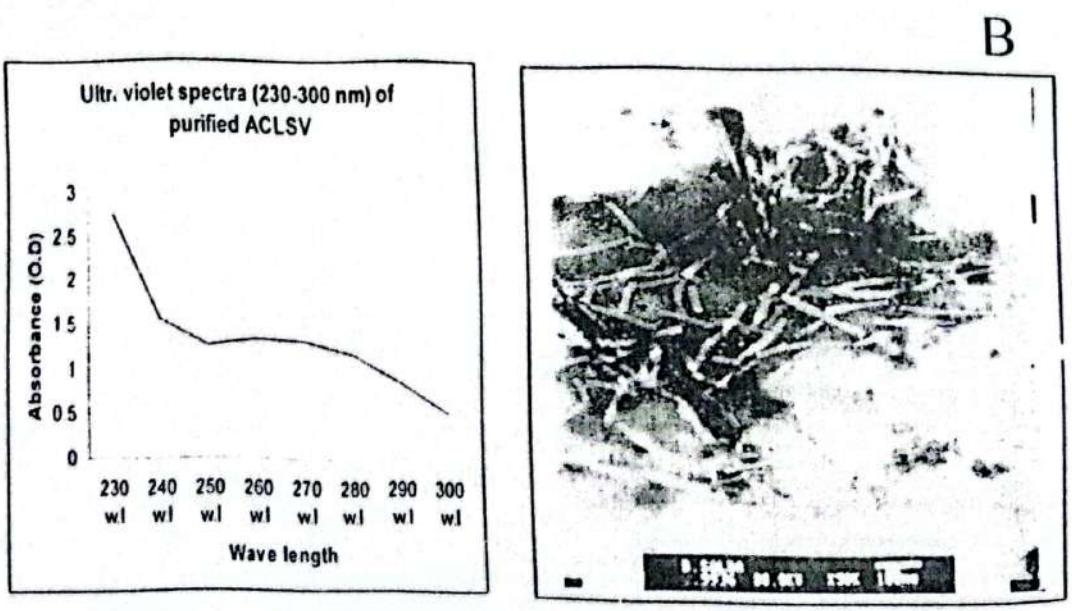

Fig (2):A: Ultraviolet spectrum of purified ACLSV. B: Electron microscopy of ACLSV particles showed flexuous particles, negatively stained with 1% phosphotungestic acid.

apple. The antiserum was also used to check the presence or absence of the virus in nematode transmission experiments.

b: Detection of ACLSV using dot blot immunobinding assay (DBIA)

Results presented in Fig (4) show that positive reaction was obtained with ACLSV- infected tissues when the antiserum was used at 1/1000 titration as stronge pink colour appeared, while negative reaction was obtained with samples of healthy plants. Positive reaction indicating the presence of ACLSV in leaves and blossom parts of apple by DBIA technique, apple flower petals offer advantage in freedom from chlorophyll pigments and with a high virus content (Lister *et al*., 1965). The advantage of nitrocellulose membrane (NCMs) for ELISA have been used for their high affinity for proteins coupled with a large surface area permitting the detection of small amounts of protein with appropriate methodology. These result show the efficiency of DBIA for detection of ACLSV (Ghanem *et al*., 2002) as well as other viruses (Smith and Banttari, 1987; Makkouk *et al*., 1993 and Fegla *et al*., 2001).

SDS-polyacrylamide gel

C.quinoa Willd leaves infected with ACLSV and healthy ones were analyzed by electrophoresis in 10% polyacrylamide-SDS gel. ACLSV protein was appeared in Fig. (5). No such protein band was showed in samples of healthy plants. The same results were obtained by Pasquini *et al*. (1998).

Nematodes transmission

ACLSV was detected in the roots, shoot, and leaves of twenty seedlings of both *C. quinoa* Willd and *C. amaranticolar* Costs & Reyn using ELISA test. The virus was detected in three out of ten leaves, shoots and one out of ten in roots of *C.quinoa* Willd. No infection occurred in roots, shoot, and leaves of *C. amaranticolar* plants. (Table 2). There is considerable virus vector specificity. The efficiency of transmission may be also influenced by the plant host as well as by nematode species and virus strain. (McGuire and Douthit, (1978) suggested that ACLSV is transmitted by *Xiphinma* to *C.quinoa* Willd but not *C.amaranticolar* therefore *C.quinoa* Willd is a propagated host for ACLSV. The mechanism of transmission by nematode presumably attach to the stylet guiding sheet, pharynx, or esophagus when the infected sap passes through the food canal and the virus dissociate from these surface when saliva flows toward the punctured cell (Matthews, 1981).

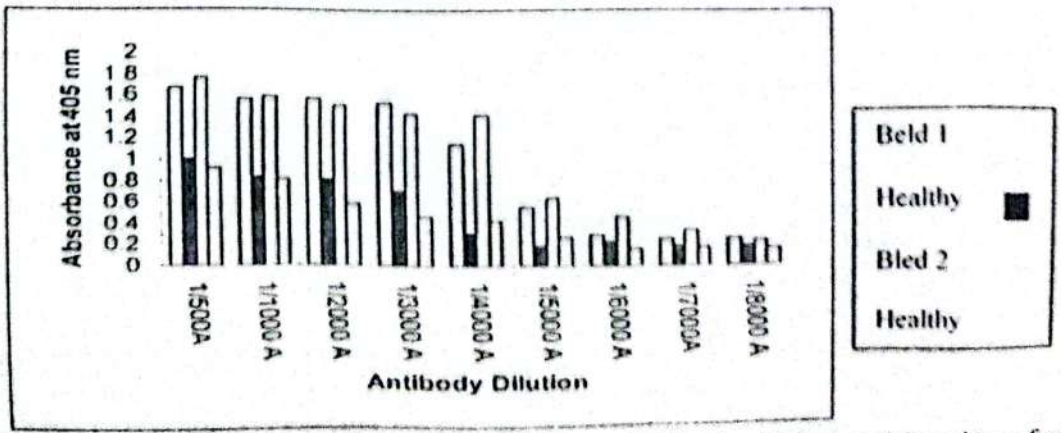

Fig (3): Titer of ACLSV antiserum measured with indirect ELISA for bleds 1 and 2 antigens from diseased and healthy *C.quinoa* Willd plant. Tissues were extracted in 1:10 of coating buffer

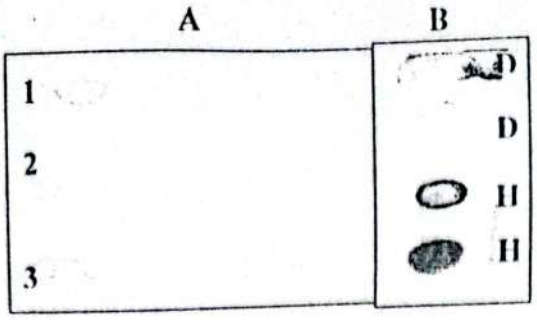

Fig (4): DBIA of ACLSV of ACLSV. A blossom parts 1. petioles, 2.stigma, and 3 flower cup) B= leaves D= Diseased and H Healthy

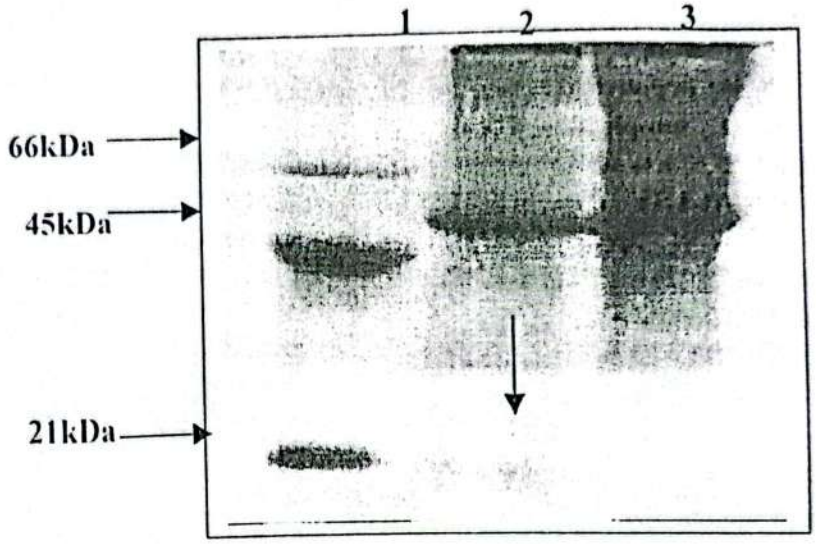

Fig (5): Electrphoresis of the protein of ACLSV. 1):Marker protein were: Bovine serum albumin 66,000 (BSV) egg albumin 45,000 (EG) trypsin inhibitor 21,000(TI) (2): protein bands from *C.quinoa* infected with ACLSV (3): C. *quinoa* (healthy). ACLSV protein upon staining with Commassie brilliant blue. Arrow indicates a coat protein of ACLSV.

Table 2: Infection of bait plants grown in infected soil with *Xiphinma* nematodes

Bait plant	Infection of		
	Root	Shoot	leaves
Chenopodium .quinoa Willd	1/10*	3/10*	3/10*
Chenopodium amaranticolar Costs& Reyn	0/10*	0/10*	0/10*

*= number of plants in which virus was detected by ELISA test

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