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Characterization of *Tobacco Rattle Tobravirus* from Kaki *(Diospyros Kaki)*

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Kaki *(Diospyros kaki)* trees, showing vein banding, upward rolling of leaves and stunting of growth were observed in the farm of South Tahrir. Isolation trials revealed that these symptoms were induced by *Tobacco rattle virus*. The virus was transmitted by mechanical inoculation. Symptoms were reproduced in the greenhouse on *D.Kaki* seedlings and tested host plants. Host range of the virus studied was restricted in *Chenopodiceae, Cucurbitaceae, Ebenaceae, Leguminosae and Solanaceae*. The identity of the virus to TRV was confirmed by mode of transmission, particle morphology and serological typing. Examination of the purified virus preparation by electron microscopy revealed rod-shaped particles with average length of 48-114 nm and 22 nm wide. Purification was performed to purify the Egyptian isolate of TRV. The yield of the virus was 2.6-5.7 mg/ 100 g tissue of *Nicotiona rustica* plants. The polyclonal antibodies raised against the Egyptian isolate of TRV had a specific titer of 1: 10000. The concentration of IgG and IgG conjugated with alkaline phosphatase was 1: 1000. The prepared antiserum was used for detection of TRV using dot-blot immunobinding assay (DBIA). This is the first report for isolation of *Tobacco rattle virus Tobravirus* (TRV) from *D.Kaki* under the Egyptian conditions.

INTRODUCATION

TRV has probably the widest host range of any plant virus. Over 100 plant species are infected in nature, and under laboratory condition more than 400 species have been infected. Many serotypes of TRV are known. They differ in type and severity of symptoms produced (Visser *et al*., 1999). TRV has been found throughout Europe, NewZealand, in North America and in Japan (Visser *et al*., 1999). TRV can cause economic losses in bulbs, such as tulip, narcissus, crocus and gladiolus. TRV was isolated from infected gladiolus in Egypt by Sabek (1973). TRV infects also red and black currant trees (van der Meer, 1987). It is not surprising to isolate this virus from other fruit trees like kaki trees. The advantage of DBIA technique for detection of small amounts of antigen over standard ELISA and also provides simplicity, rapidity and sensitivity and convenience for large numbers of samples. This result had already been declared by many authors (Lin *et al*., 1990; Fegla *et al*., 2001and Ghanem *et al*., 2002).

Gels electrophoresis is a rapid method for detecting viral coat proteins. Provided they occur in high enough concentration in leaf extracts (Alper *et al*., 1984). In the present study TRV was isolated and identified as the causal virus of the symptoms naturally observed on Kaki trees. Purification of the virus was achieved and the induction of specific antiserum against the isolated virus was done to produce ELISA regents, which can be used as a rapid method for TRV detection.

MATERIALS AND METHODS

Source of virus

Kaki trees growing at the South Tahrir farm, showing up-ward rolling and vein banding were used as source for virus isolation.

Mechanical Transmission

D.Kaki seedling, one year age were dusted with carborundum and inoculated with infected leaf extract, diluted (1:2 w/v) in 0.1 M phosphate buffer pH 7.0. The local lesion technique (Kuhn, 1964) was used for biological purification of the virus using *Chenopodium amaranticolar Costs&Reyn* as a local lesion host.

Host range and symptomatology

Thirteen different host plants belonging to 5 families (Table 1) were sap inoculated as previously mentioned. The inoculated seedlings were kept under greenhouse conditions and observed for symptoms development.

Virus Purification

Tobacco rattle virus (TRV) was purified according to the method described by Robinson (1983). One hundred grams of frozen systemically infected *Nicotiona rustica* leaves were used for virus purification. Virus concentration was estimated using an extinction coefficient (A_{260} nm.0.1 .cm) of 3.0 (Brunt *et al.*, 1996).

Electron microscopy

Purified virus preparation was examined with an electron microscope unit Model JEOL-JEM- 1200 EX II (Faculty of Science Ain Shams University) after staining with 1% phosphotungstic acid.

Antiserum production

Polyclonal antibodies were prepared by injecting Newzealed white rabbit subcutaneously six times at weekly intervals with 2mg of purified virus preparation in 0.5 ml of buffer emulsified with an equal volume of Freund's incomplete adjuvant. Serum was collected 10 days after the last injection. Antiserum was separated by centrifugation at 5,000 rpm for 3 min. Antiserum titer was measured with indirect ELISA. The antiserum was diluted to 1/500-1/10000 with antigen buffer (coating buffer pH 9.6). Anti-TRV immunoglobulin was purified using the method described by Steinbuch and Audran (1969) and it was conjugated with alkaline phosphatase, using the method described by Brattey & Burns (1998). Optimum concentration of IgG and IgG conjugated with alkaline phosphates was determined using a check board test (Converse & Martin, 1990) to optimize the concentration for ELISA test.

Detection of TRV using dot blot immunobindinding assay (DBIA)

Dot blot immunobindinding assay technique on nitrocellulose membrane was applied for TRV detection in infected and healthy *Nicotiona rustica* leaves according to the methods described by Hsu and Lawson (1991).

Detection of TRV- coat protein using polyacrylamide gels electrophoresis (PAGE)

Total proteins were extracted from *Nicotiona rustica* leaves infected with TRV Electrophoresis of the protein of TRV in 10% polyacrylamide gels in phosphate buffer, pH 7.2, containing 0.1% SDS and 0.1% mercaptoethanol according to Maniatis *et al.* (1982) was used for molecular weight determination.

RESULTS AND DISCUSSION

Host range and symptomatology

Results presented in Table (1) showed that tested plant species varied in their response to viral infection. Some plant species did not develop any symptoms (*Vicia faba* L cv Giza) and other showed reactions. In general

plant species, tested could be classified according to their reactions as follows:
1-Plants species reacted with necrotic local lesions only *Cucumis sativus* L.cv.Balady (Table 1).
2-Plants species reacted with necrotic local lesions followed by systemic infection: *Nicotiona rustica* (Fig 1A), *Chenopodium amaranticolar* Costs&Reyn (Fig 1B), *Chenopodium quinoa Wild*, *N. tabacum* cv. Samsun, *Nicotiona glutinosa L.* (Fig 1D)
3-Plants species reacted with systemic infection only *N. tabacum* L cv. White Burley.
4-Plants species reacted with ring spot only *Capsicum annuum* cv.California wonder
5-*Dospyros kaki Linn* cv.Tarablos reacted with up ward rolling of leaves (1E) and vein banding (1F). Sabek (1973) summarized his results about the symptoms of TRV isolated from infected gladiolus. These symptoms were necrotic local lesion on *Chenopodium amaranticolor*, Costs& Reyn *Gomphrena globosa* L., *Phaseolus vulgaris*, and *Vigna sinensis*. The virus produced local lesion followed by systemis necrosis on *Nicotiona tabacum* L cvs.White Burley, and *Samson* and systemic necrosis on *N. tabaum* L.cv Xanthia.

Virus purification

Using the purification method two distinct bands were observed, 3.5, 1.5 cm below the meniscus in the sucrose density gradient columns. The ultraviolet absorption spectrum of TRV was characteristic of a nucleoprotein, had a maximum absorbance at 260 nm, a minimum absorbance at 250 nm and A_{260}/A_{280} ratio of 1.17 (Fig. 2A) (uncorrected for light scattering). This ratio is similar to that estimated by Brunt *et al.* (1996). These results suggested that the purification method was quite successful in purifying

Table (1): Symptoms on different plant species inoculated with TRV

Test plant	The main symptoms
Chenopodiaceae:	
Chenopodium amaranticolar Costs& Reyn	NLL+SNL
Chenopodium quinoa Wild	NLL+SNL
Cucurbitaceae:	
Cucumis sativus L. (Balady)	NLL
Ebenaceae:	
Dospyros kaki Linn cv.Tarablos	VB,UWRL
Solanaceae:	
Nicotiona glutinosa L.	NLL+SNL
Nicotiona tabacum L.white Burely	SM
Nicotiona rustica	NLL+SNL
N. tabacum cv. Samsun	NLL+SNL
Capsicum annuum cv. California wonder	LRS
Lycopersicon esculentum	YB
Petunia hybrida Vilm	SM
Leguminosae:	
Phaseolus vulgaris L cv Giza1	PP
Vicia faba L cv Giza 3	-

NLL = Necrotic local lesion
SNL = Systemic necrotic lesion
YB = yellow banding
VB=Vein banding
SM = systemic mottle
LRS = Local ring spot
UWRL= up ward rolling of leaves
PP = Pin Piont
- = no symptoms

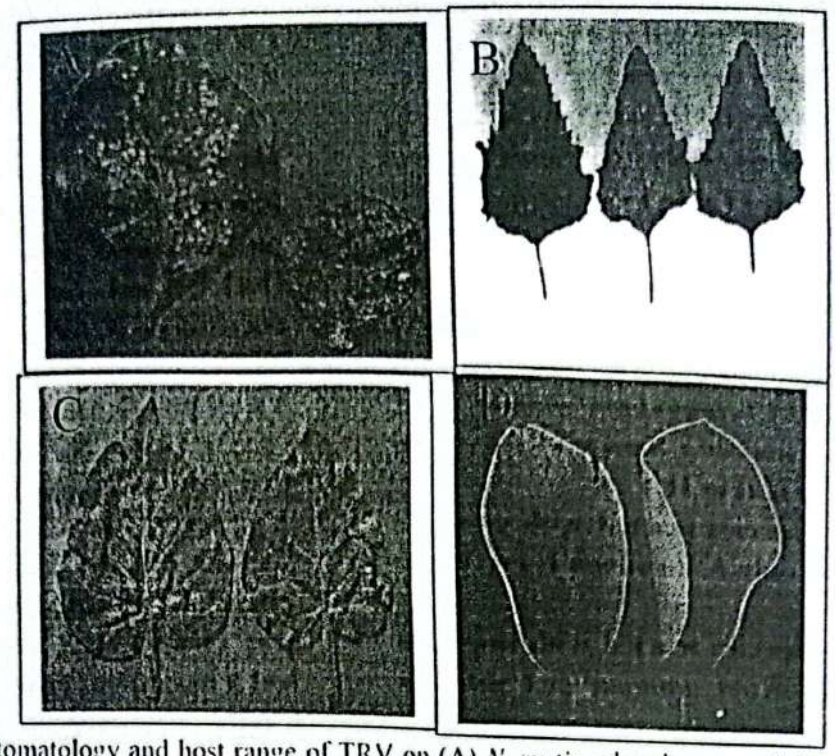

Fig (1): Symptomatology and host range of TRV on (A) *N. rustica* showing necrotic local lesion (A). (B) Necrotic local lesion on *C.amaranticolor*, (C) *N. glutinosa* exhibiting necrotic lesion. (D) *D.Kaki* naturally infected showing up- ward rolling of leaves.

the virus under study. Virus yields were 2.6-5.7 mg/100g of infected tissue. The similarity in UV absorbances as well as virus yield were agreed with results of Lister&Brącker (1969), Semancik&Kagjiyama (1967), Semancik&Odening (1969) and Brunt *et al.* (1996). Mathews (1991) mentioned that in the analytical centrifugation, purified preparation of TRV produced two sedimenting components (top and bottom).

Electron micrscop

Electron microscopic micrograph of the purified preparation revealed the presence of rod shape particles with average length of 48-114 nm and 22 widths (Fig.2B). The virus particles shwed end-to-end and side – to-side aggregations. These results are similar with Lister and Bracker (1969).

Sutic *et al.* (1999) mentioned that the purified preparation of this virus have two particle sizes, long (180 to 200×22nm) and short (50 to 110×22nm).

Production of TRV antiserum

Titer of the induced antiserum measured with indirect ELISA was

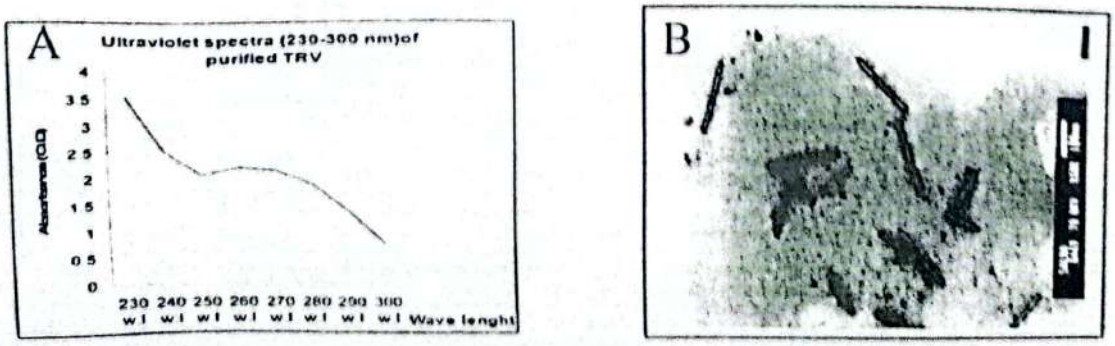

Fig (2). A:Ultraviolet spectra (220-320 nm) of purified TRV. B: Electron microscopy of TRV particles showed rod shape negatively stained with 1% phosphotungstic acid. Mag. 70000 X.

1/10000.That is an indication of the high immunogenecity of this antiserum. Data presented in Fig (3) showed that TRV was detected at 1:10000 dilution of antiserum when 1:10 dilution of TRV infected tobacco leaf extracts were used. Dilution 1/5000 was the best usable dilution for producing high absorption values at 405 nm. Positive reactions were obtained when purified IgG and IgG conjugated with alkaline phosphatase was 1:1000. One of the major goals in the present work is to produce ELISA reagents, which can be used as a rapid method for TRV when purified IgG and IgG conjugated with adetection in ornamentals, fruit trees, and vegetable crops.

Detection of TRV using dot blot immunobinding assay (DBIA)

In order to detect tissue infected with TRV, TRV-antiserum was used for detection in diseased and healthy plants. *N. rustica* leaves were used to check the presence of TRV by DBIA technique. Fig (4) showing strong positive reaction in dots applied with tissues of diseased *N. rustica* plants. Whereas remained green these of healthy tissues. The advantage of DBIA technique for detection of small amounts of antigen over standard ELISA and also provides simplicity, rapidity, sensitivity, and it .is convenience for large numbers of samples. This result had already been declared by many authors (Lin *et al.*, 1990; Fegla *et al.*, 2001and Ghanem *et al.*, 2002).

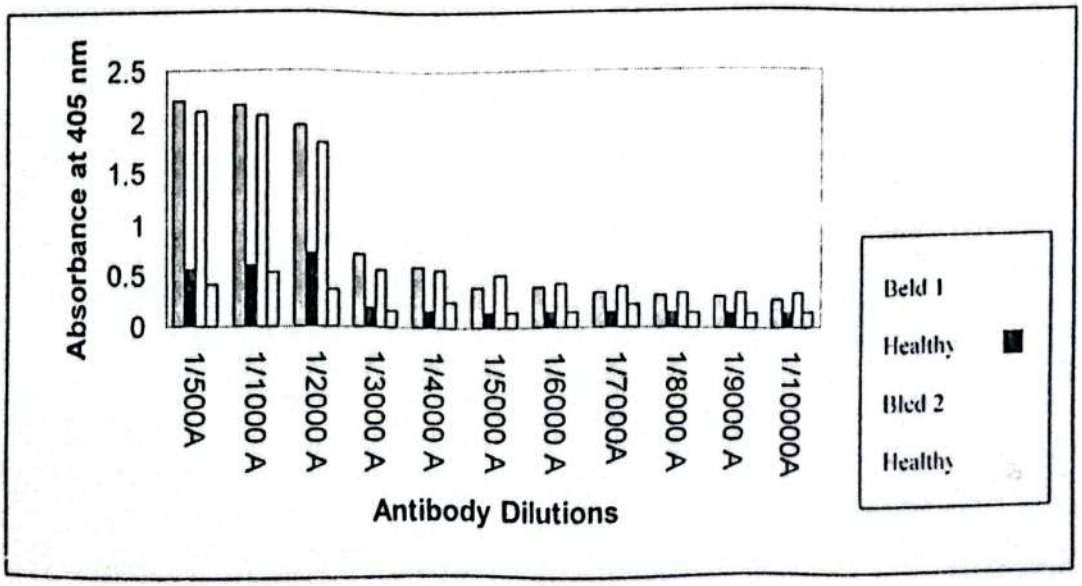

Fig (3): Titer of TRV measured with indirect ELISA for bled 1 and 2. antigens from infected *N. rustica* (D) and healthy *N. rustica* (H). Tobacco were extracted 1:10 in coating buffer.

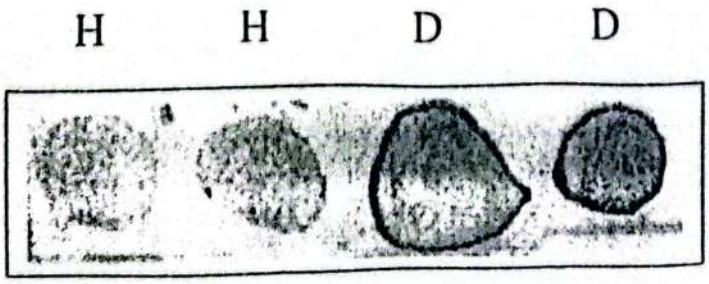

Fig (4): DBIA of TRV from *N. rustica* D= Diseased and H= Healthy

Detection of TRV-coat protein using polyacrylamide gels electrophoresis (PAGE)

Gels electrophoresis is a rapid method for detecting viral coat proteins. Provided they occur in high enough concentration in leaf extracts (Alper *et al.*, 1984). In the present study protein bands of molecular weight of 20 KDa were appeared (Fig 5). No similar protein bands were observed in samples of healthy plants. The same results were obtained by Ghabrial and Lister (1973).

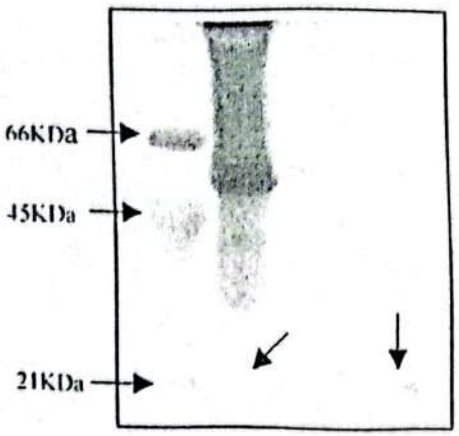

Fig (5): Electrophoresis of the protein of TRV. (1): Marker protein were: Bovine serm albumin 66,000 (BSV) egg albumin 45,000 (EG) trypsin inhibitor 21,000(TI) (2): protein from. (2): Protein from *N. rustica infected* with TRV. (3): *N. rustica* healthy, and (4): Purified virus of TRV.TRV protein upon staining with Commassie brilliant blue. Arrow indicates a coat protein of TRV.

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