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ISOLATION AND IDENTIFICATION OF *TOBACCO RATTLE TOBRAVIRUS* AFFECTING ONION (*ALLIUM CEPA* L.) PLANTS IN EGYPT

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ABSTRACT

Tobacco rattle virus (TRV) was isolated from naturally infected onion (*Allium cepa* L.) plants growing in the fields of onion plants during the survey carried out in two successive seasons (2007-2008 and 2008-2009) in seven Egyptian Governorates. Plants showing yellowing, malformation, yellow stripping, white necrotic stripes and stunting symptoms were collected and subjected to identification studies that based on host range, symptomatology, modes of transmission, serological tests, inclusion bodies and morphology of virus particles. The virus was transmitted by mechanical inoculation and by seeds. On the other hand, cytological changes accompanied with the infection were investigated. Different methods of serological detection of the virus were also tested. The obtained results indicated the host range of the virus was expanded to 7 different plant families. The mechanical transmission of the virus, which was also transmitted by seeds with percentages ranged between 8-13 %. Different serological methods were used successfully for detection of TRV e. i. DAS-ELISA, TBIA and DIBA. Infection with TRV resulted in the formation of amorphous inclusion bodies in the cytoplasm of infected tobacco leaves. By light microscopy of semi thin sections of both healthy and artificially infected onion leaves, several anatomical changes were observed reflecting the external symptoms on infected plants. By electron microscopy the virus particles were observed as tubular particles with two main dimensions (length 48-114 nm and 22 nm width). Investigation of ultrathin sections by transmission electron microscopy revealed changes in both the nucleus and the chloroplast. According to the available data this is the first report for isolation of TRV from onion plants under Egyptian conditions.

Keywords: *Tobacco rattle virus* (TRV), Host range, Inclusion bodies, Modes of transmission, Electron microscope, serological detection ELISA, DBIA and TBIA.

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INTRODUCTION

Onion (*Allium cepa* L.), have a world-wide importance, ranking second among all vegetables in economic importance after tomatoes **(Griffiths *et al.*, 2002).** In Egypt, onion is the third vegetable more consumed (15 kg, /capita/ year), after potatos and tomatoes and it is cultivated all over the country in Delta area and Upper Egypt (84.3% of total area) and new land areas (15.7%). The main onion cvs, white (Giza-6) and red (Behery) onions are the most produced varieties. The current production area is being around 147,490 feddan with total production 1.3 million ton, **(Annonymous, 2009).**

Tobacco rattle virus (TRV) is the type-virus of the genus *Tobravirus.* TRV was named after the rattle-like sound produced by the dried-out tobacco leaves, when the wind blows through an infected field **(Visser *et al.*, 1999).** TRV has probably one of the widest host range plant viruses, more than 100 plant species have been found to be infected by TRV, while the virus could be transmitted by sap-inoculation to about 400 species belonging to more than 50 families, including both mono- and dicotyledonous plants under greenhouse conditions **(Harrison and Robinson, 1978 and MacFarlane, 1999).**

TRV causes economic losses in bulbs production, such as tulip, narcissus, crocus and gladiolus. It was isolated from infected gladiolus in Egypt by **Sabek (1973),** from infected henbane (*Hyoscyamus muticus* L.), by **Shafie (1978)** and from Kaki (*Diospyros Kaki*) by **Zein (2004).**

The aim of the present study is to isolate and identify an isolate of TRV from the naturally infected onion plants.

MATERIALS & METHODS

1. Virus isolation:

Leaf samples of onion (*Allium cepa* L.) plants showing malformation, yellowing, yellow stripping, white necrotic stripes and stunting symptoms were collected from fields of onion plants during the survey carried out during the two successive seasons (2007-2008 and 2008-2009) among seven Egyptian Governorates.

The Collected leaf samples were homogenized with 0.1 M phosphate buffer- solution, pH 7(1:2 w/v) in a sterilized mortar. The infectious sap was passed through double layers of chees cloth. Ten seedlings

Burley 20 days after inoculation with TRV, epidermal strips were taken from the lower surface of leaves, and were treated with 5% Triton x-100 for 10 minutes to disrupt the plastids and facilitated the observation of the inclusions **(Edwardson *et al.*,1984).**

Morphology of virus particles:

Virus leaf dip preparation **(Noordam, 1973)** was negatively stained with 2% Phosphotungstic acid (PTA) and mounted on carbon coated cupper grids (400 mesh) and examined by transmission electron microscope (TEM Joel -1400 in the electron microscope unit Faculty of Agriculture Research Park (FARP). Images were captures by CCD camera (EMT) at magnifications of 40000X.

Ultrathin sections:

Generic Processing Protocol

Tissues of infected onion leaves were cut into small pieces about 1-2 mm., fixed in 2% glutraldhyde in 0.1 M Na-Cacodylate buffer, pH 7.2 and subjected to a vacuum for 1-4 minutes every 15 minutes for 2 hours on ice. Prior to vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2, for 45 minutes, with buffer changes at 15 and 30 minutes. Further fixation in 1% Osmium Tetraoxide in Na-Cacodylate buffer, under intermittent vacuum and poking, took place for 1.5 hours. Samples were then rinsed again in the Na-Cacodylate buffer. Dehydrated Samples were dehydrated through an Ethanol series in buffer: 35% - 50% - 70% - 80% - 95% - 100% - 100% for 60 minutes each. Then Infiltrate with res in Semi thin sections were prepared on glass slides through cutting at 1um using the ultramicrotome. Sections were stained with Toludine blue for 5 min and examined by light microscope model M-200M.

Ultra-thin sections were cut using ultramicrotome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Sections were stained with double stain (Uranyl acetate 2% 10 min followed by Lead citrate for 5 min and examined by transmission electronmicroscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632 x 1632 pixel formate as side mount configuration. **(Osmont and Freeling, 2001).**

RESULTS

1. Isolation of TRV

The virus was isolated from naturally infected onion (*Allium cepa* L.) plants collected from onion fields during the survey carried out during the two successive seasons (2007-2008 and 2008-2009) in seven Egyptian Governorates.

2. Identification of the virus isolate

The virus was identified according to host range, symptomatology, modes of transmission, serological reactions using (ELISA) techniques, inclusion bodies and morphology of virus particles.

A. Host range and symptomatology of TRV

As shown in **Table (1)** and **Figure (1)** the virus reacted positively with all plant species and cultivarietaes belonging to *Alliaceae*, *Amarnthaceae*, *Chenopodiceae*, *Cucurbitaceae*, *Fabaceae*, and *Solanaceae*, only *Zinnia elegauce* and *Vicia faba* L cv Giza 3 reacted negative with TRV inoculation.

The tested hosts could be classified according to their reactions as follows:

1. Plant species reacted with systemic symptoms

Systemic symptoms varied between mild and severe ones were observed on the tested, *A. ascalonicum* L., *A. kurrat* L, *Datura stramonium* and *N. tabacum* cv. White Burley plants 2-3 weeks after inoculation with TRV.

2. Plant species reacted only with localized symptoms:

TRV produced local lesions on the inoculated leaves of *C. album, C. amaranticolar, C. quina Cucurbit pepo. Cucucmis sativus* cv Baladi and *Gomphrena globosa,* while chlorotic and white necrotic stripes on onion *Allium cepa* cv. (Giza 6, 20 & Behery) and *A. sativum* L. *Phseolus vulgaris* L. cv Giza1 produced pin point.

3. Plant species reacted with local followed by systemic symptoms:

Inoculated leaves of *N. glutinosa* and *N. rustica* reacted with infection with local fallowed by systemic symptoms.

TRV was not detected either by ELISA or by back inoculation into the indicator test plant which gave no symptoms of first.

B. Modes of transmission

a. Mechanical transmission

The virus was transmitted by mechanical inoculation from infected source plants to the indicator test plants. Infection was confirmed by back inoculation and /or by ELISA.

b. Seed transmission

Results in **Table (2)** showed that, TRV could be transmitted through onion seeds of the three tested onion cvs. with different transmission percentages. It was noticed that TRV was transmitted through seeds of Behery cv with high percentage being 13%. This high percentage of seed transmission is very effective as a source of early infection in the field.

b. Serological diagnosis:

Direct and Indirect ELISA were used for confirm the identity of TRV. Positive reaction was obtained with the virus and its specific antiserum.

Tissue blot immunobinding assay (TBIA) and Dot blot immunobinding assay (DBIA):

The presence of TRV in diseased onion leaves was checked by both TBIA and DBIA and the results were compared with ELISA readings of the same samples. It's clear from **Figure (2)** that strong positive reaction was indicated by development of purplish-blue color, whereas those extracted from healthy ones remained green. 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.

3. Cytological effects of TRV infection

a. Light microscopy

Amorphous cytoplasmic inclusion bodies were observed with light microscopy in infected epidermal stripe of infected onion with TRV, taken from the lower surface of systemically infected *N. tabacum* cv. White Burley, 20 days after inoculation **(Figure 3).**

b. Semi thin sections:

Semi thin sections of both TRV infected and healthy onion plants were examined after staining with Tiludine blue by light microscope. The investigations revealed large differences between infected and healthy tissues **(Figure 4).** The mesophyll layer was reduced in infected plants **(Figure 4b)** if compared with healthy plants **(Figure 4a).**

The spongy tissue was more compact, characterized with reduced intracellular space **(Figure 4d)** in comparison with tissues of

healthy plants **(Figure 4c).** The number of chloroplasts was reduced in number in infected cells than in healthy ones **(Figure 4f and e, respectively).** This finding reflects the symptoms of chlorotic strips on TRV-infected leaves. On the other hand, the phloem tissues were also affected and necrosis was observed in infected **(Figure 4h)** comparing with healthy tissues **(Figure 4g).**

c. Electron microscopy

The morphology of TRV particles was studied by electron microscopy using virus preparations negatively stained with 2% phospotungestic acid. Tubular particles with average length of 48-114 nm and 22 widths were observed **(Figure 5).**

d. Ultrathin sections

Investigation of ultrathin sections by Transmission electron microscope revealed changes in the different tissues and cells organils as are illustrated in **(Figure 6.)** The number and organization of chloroplast was different in cells of infected onion tissues. The chloroplasts exhibited several degrees of deformation and lysis **(Figure 6 b,c and e)** in comparsion of healthy tissues **(Figure 6 a and d).** The nucleus of infected cells also affected as it was observed to be misshapen and the chromatin material was segmented **(Figure 6f).**

Table 1. Reaction of different hosts to infection with TRV

Host plant tested		symptoms
Family: *Alliaceae*		
Allium cepae	Giza 20	Ch + St +WN
	Giza 6	Ch + St+ WN
	red (Behery)	Ch+ WNS
Allium ascalonicum L.		Ys+ St
Allium kurrat L.		Ys+ St
Allium sativum L. Baladi		Ch+ WNS
Family: *Amaranthaceae*		
Gomphrena globosa L		CLL
Family : *Compsitae*		
Zinnia elegauce		-
Family: *Chenopodiacea*		
C. album		CLL
C. amaranticolor		NLL
Chenopdium quinoa L		CLL
Family :*Cucurbitaceae*		
Cucurbit pepo		CLL
Cucumis sativus L cv. Baladi		NLL
Family :*Fabaceae*		
Phseolus vulgaris L. cv Giza1		PP
Vicia faba L. cv Giza 3		-
Family: *Solanaceae*		
D. stramonium L.		SM
Nicotiana glutinosa L		NLL + SM
N. rustica L		NLL + SM
N.tabaccum L cv White Burley		SM
Solanum tuberosum L cv dimond		Chs+ YR

Ch	=	Chlorotic	St	=	Stunting
Chs	=	Chlorosis	YR	=	Yellow rings
CLL	=	Chlorotic local lesion	YS	=	Yellow stripping
NLL	=	Necrotic local lesion	WN	=	White necrotic
PP	=	Pin point	WNS	=	White necrotic stripes
SM	=	Systemic mosaic	-	=	No reaction

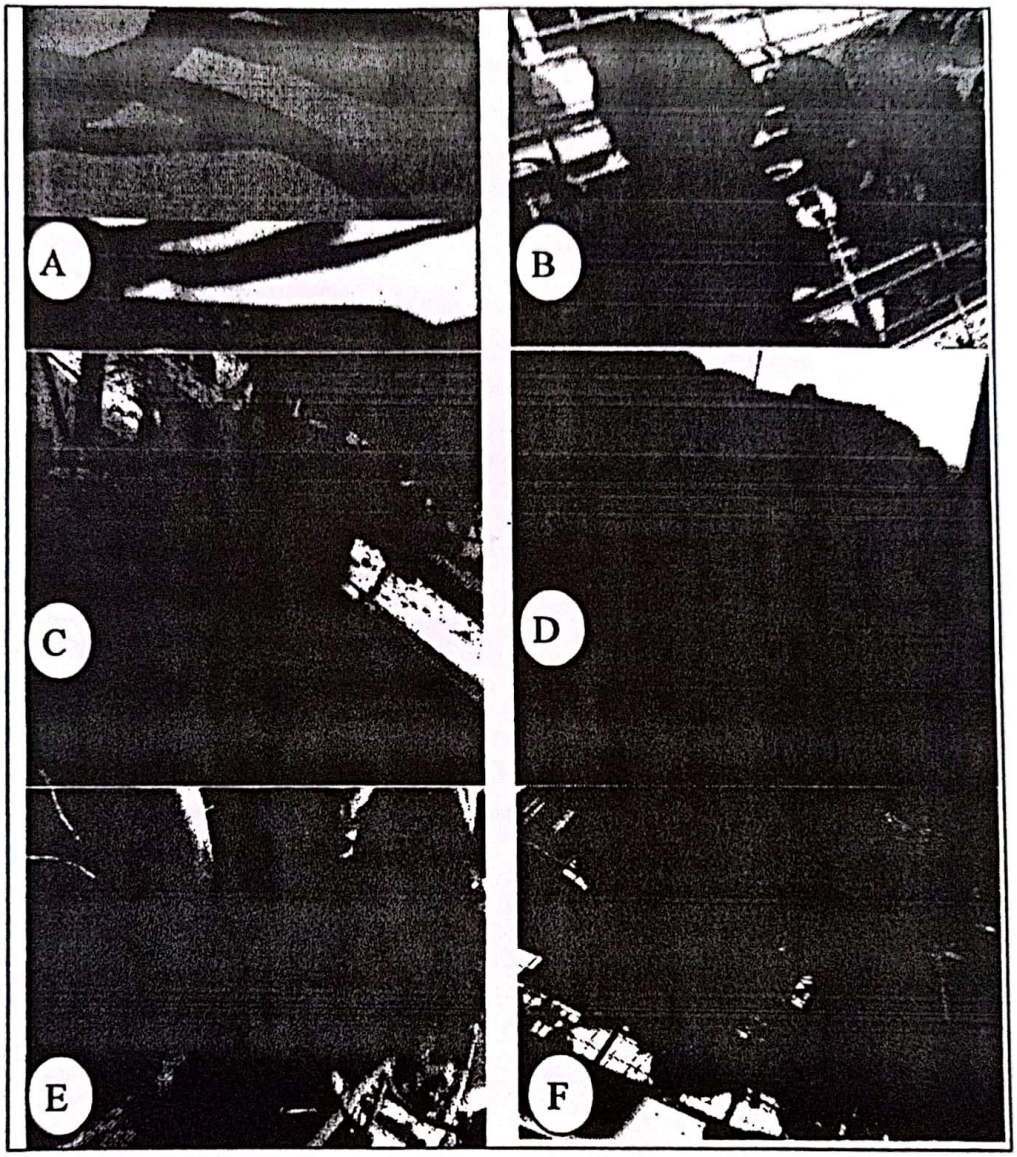

Figure 1. Reaction of some hosts with TRV infection: (A) white necrotic stripes on onion (*Allium cepa* L.cv) Behery, (B) pin point on *Phaseolus vulgaris*, (C) necrotic local lesion on *C. amaranticolor*, (D) necrotic local lesion on *Nicotiana glutinosa*, (E) chlorotic local lesion on *C. album*, (F) chlorosis on *Solanum tuberosum*.

Table 2. Seed transmission of TRV

varieties	No. of infected / No. of tested	% infection
Giza 6	50/500	10
Giza 20	40/500	8
Behery	65/500	13

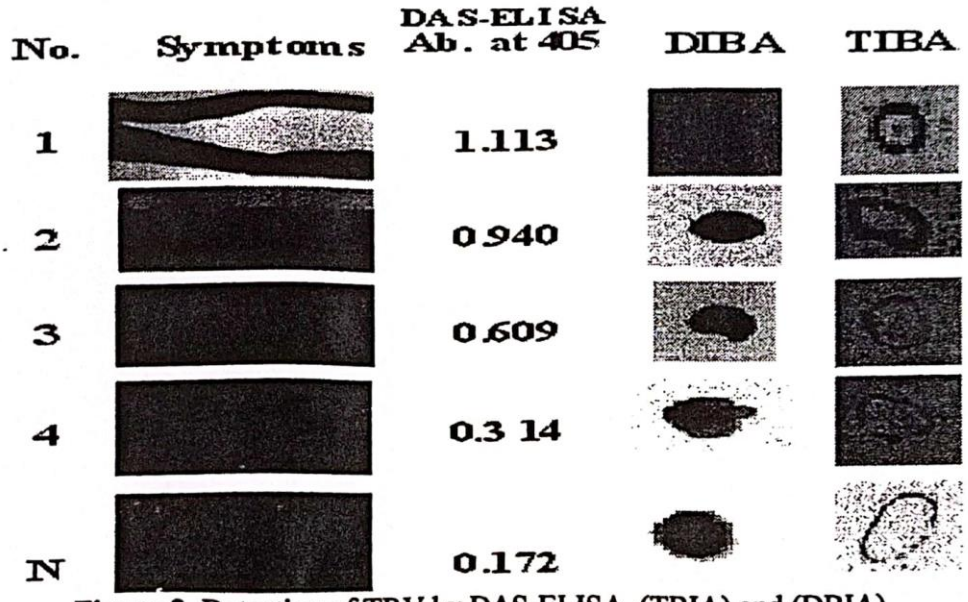

Figure 2. Detection of TRV by DAS-ELISA, (TBIA) and (DBIA).

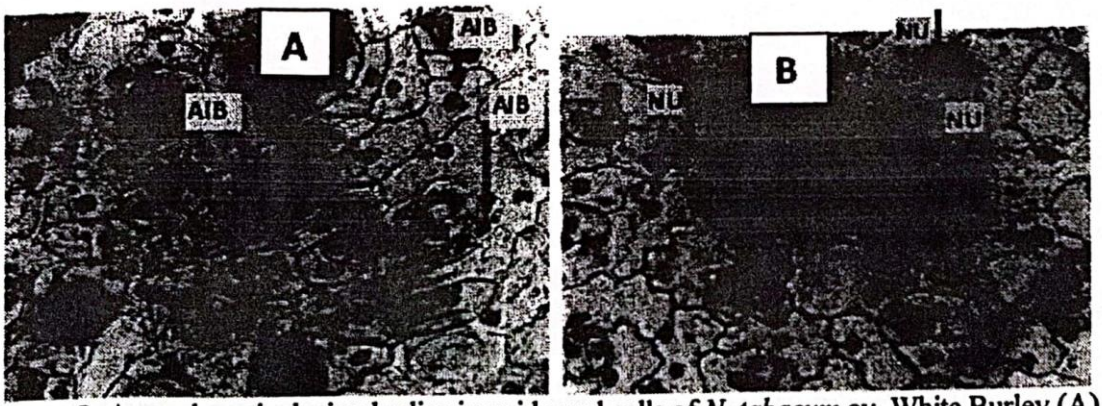

Figure 3. Amorphous inclusion bodies in epidermal cells of *N. tabacum* cv. White Burley (A) and Healthy (B). (X - 200) NU: Nucleus, AIB: Amorphous inclusion bodies.

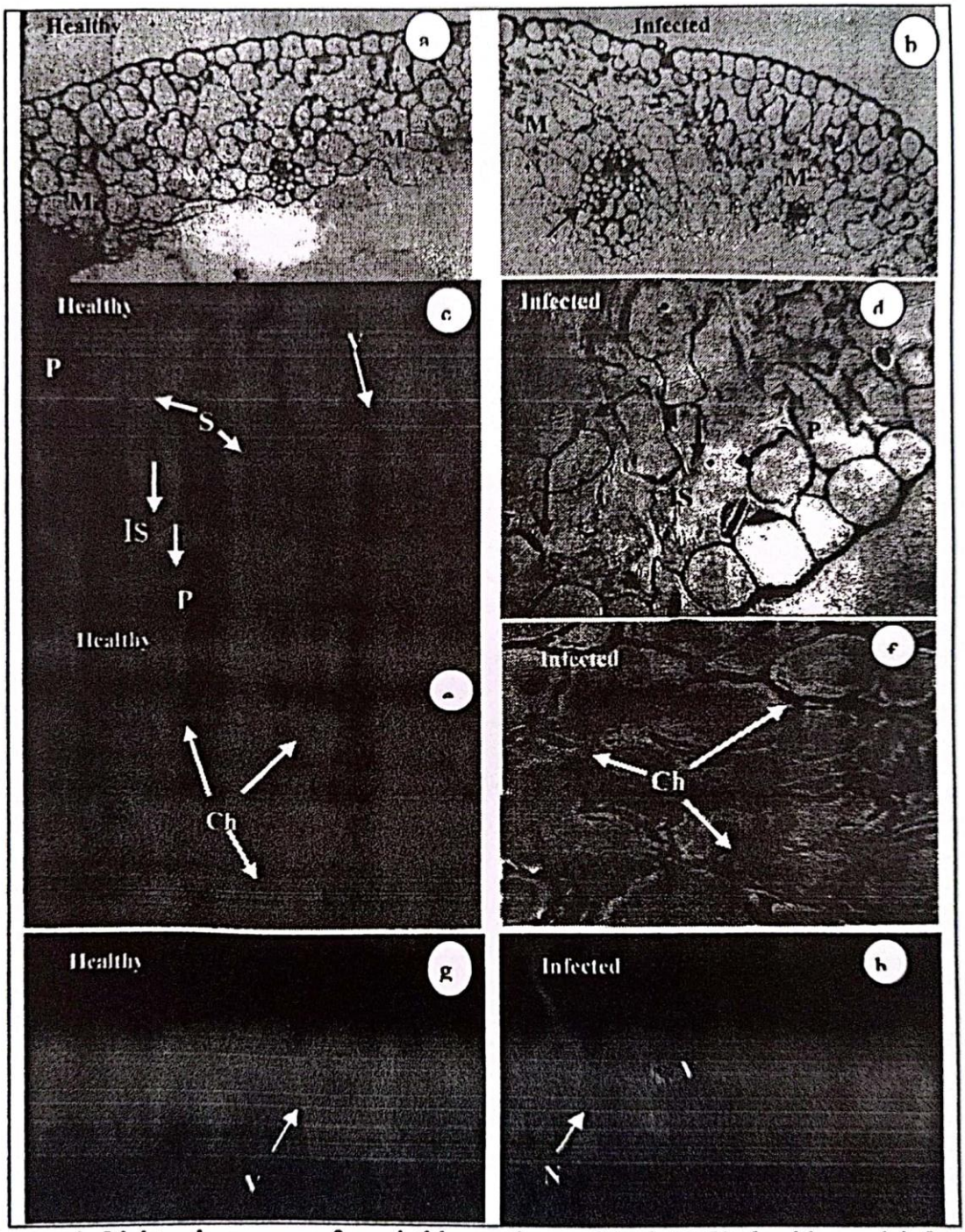

Figure 4. Light microscopy of semi thin sections of both TRV- healthy and infected Behery onion plants, a, b, mesophyll cells, c, d, spongy tissues, e, f, chloroplasts in parenchyma cells, and phloem tissues of healthy (g) and diseased leaves (h). Ch=chloroplast M=mesophyll N= necrosis S=spongy tissue IS=intercellular spaces PC=palisade cells V=vacuoles.

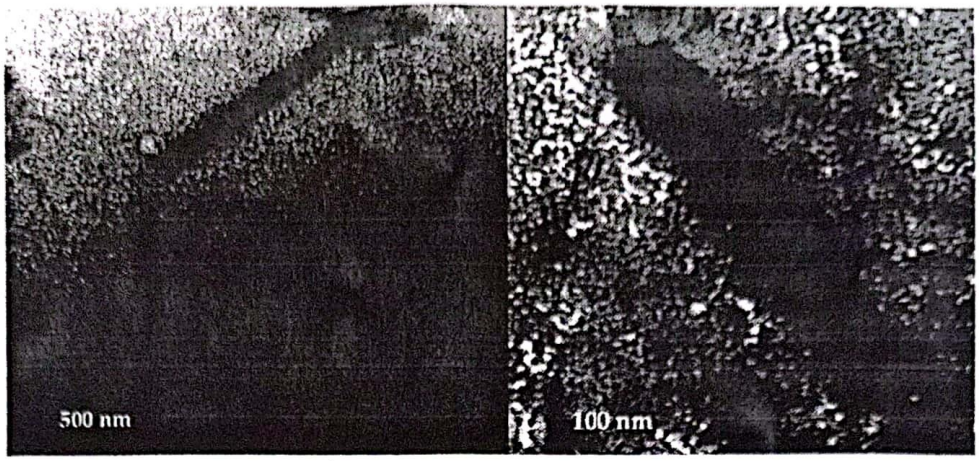

Figure 5. Electron micrograph of purified TRV negatively stained with 2% phosphotungestic acid.

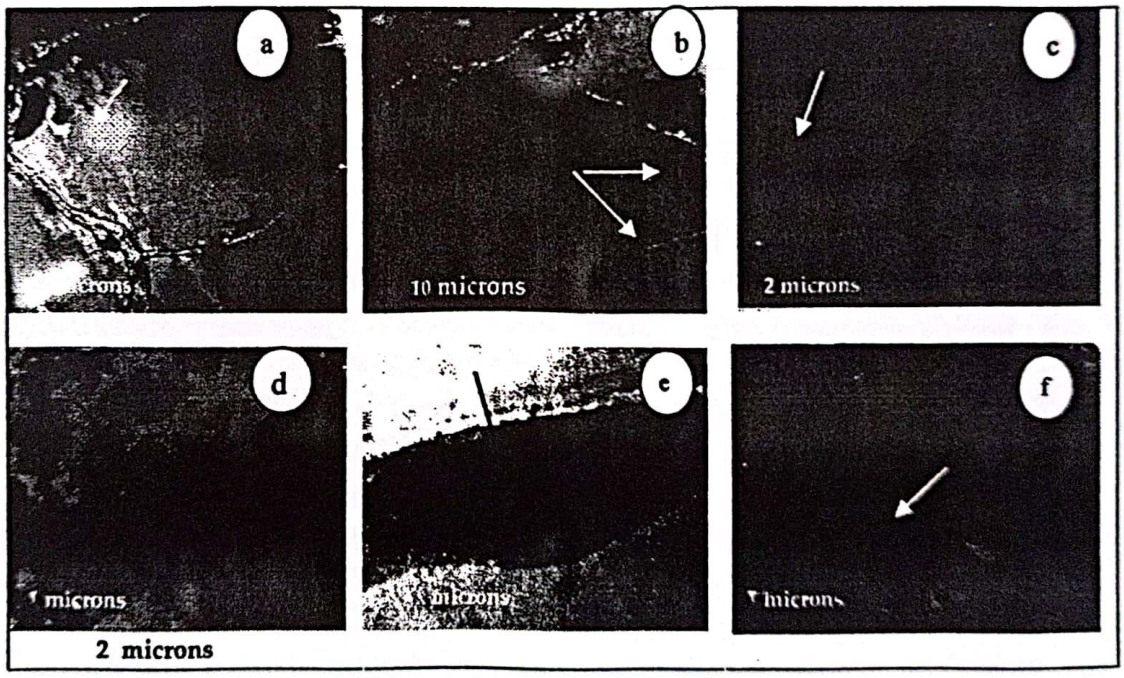

Figure 6. Electron micrographs representing the effect of TRV infection on onion cell organils compared with those of healthy cells. a: mesophll cell of healthy onion leaf with normal shape and number of chloroplast (arrows). b: cell of TRV infected leaf showing several degrees of chloroplast deformation and degrdation. c: Normal chloroplast at high magnification compared with affected one d. e: the nucleus of infected cell swollen and the chromatin is segmented (f) head arrow. Ch =chloroplast Chr= chromatin.

DISCUSSION

Crops of cultivated *Allium* species are commonly infected with one or more viruses, especially when propagated vegetatively **(Bos, 1983; Walkey, 1990).** Approximately 20 viruses infect *Allium*, of which about nine are considered to be the most important because they are widely distributed and often occur at a high incidence in *Allium* crops **(Barg *et al.*, 1997and Chen *et al.*, 2004).**

TRV had earlier been reported in garlic, onion, A. moly and A. ursinum in Europe **(Bos, 1983; Van Dijk, 1993 and Uhde *et al.* 1998).**

In this study, TRV was isolated from onion plants. The virus reacted positively with all plant species and cultivarietaes belonging to *Alliaceae*, *Amarnthaceae*, *Chenopodiceae*, *Cucurbitaceae*, *Fabaceae*, and *Solanaceae*, only *Zinia elegauce* (F: *Compsitae*) reacted negative with TRV inoculation. The obtained results are in agreement with those obtained by **(Shafie 1978).** On the contrary **Zein (2004)** recorded several differences in host range studies, such results could be attributed to virus strain plant species tested and physiological stage of host plants during inoculation process.

On the other hand **Van Dijk (1993)** reported that natural infection of onion and garlic with an isolate of TRV as plants showed cholorotic and white necrotic stripes on the leaves.

The virus reacted positively with all plant species and cultivarietaes belonging to *Alliaceae*, *Amarnthaceae*, *Chenopodiceae*, *Cucurbitaceae*, *Fabaceae*, and *Solanaceae*, only *Zinnia elegauce* and *Vicia faba* L cv Giza 3 reacted negative with TRV inoculation.

Systemic symptoms varied between mild and severe ones were observed on the tested, *A. ascalonicum* L., *A. kurrat* L, *Datura stramonium* and *N. tabacum* cv. White Burley plants 2-3 weeks after inoculation with TRV. Local lesions produced on the inoculated leaves of *C. album, C. amaranticolar, C. quina Cucurbit pepo. Cucucmis sativus* cv Baladi, *Gomphrena globosa* and produced pin point on *Phseolus vulgaris* L. cv Giza 1.

Inoculated leaves of *N. glutinosa* and *N. rustica* reacted with infection with local fallowed by systemic symptoms, while chlorotic and white necrotic stripes on onion

Allium cepa cv. (Giza 6, 20 & Behery) and *A. sativum* L. and reacted with infection with local fallowed by systemic symptoms. TRV was not detected either by ELISA or by back inoculation into the indicator test plant which gave no symptoms of first. Investigator transmitted TRV mechanically such as Kirk ***et al.*** **(2008)** on potato cv. FL1879 tubers was which used to transmit the virus mechanically to tobacco cv. Samsun NN and **Koike *et al.* (2010)** used symptomatic spinach from the field to inoculated *Chenopodium quinoa, C. murale, C. capitatum,* spinach, sugar beet *(Beta vulgaris*).

It was noticed that TRV was transmitted through seeds of Behery cv with high percentage being 13%. This high percentage of seed transmission very effective as a source of early infection in the field. Seed transmission of TRV through onion seeds was not breviously indicated but its transmission through different host was indicated by **Lister and Murant (1967)** through some weeds such as *Capsella bursa-pastoris* and *Myosotis arvensis*.; through tomatoes **Visser *et al.*, (1999) and Dikova (2005)** through sugar beet seeds).

The results of DBIA and TBIA techniques with TRV already been declared by many authors **(Kamenova and Adkins, 2004), (Lin *et al.*, 1990; Fegla *et al.*, 2001 and Ghanem *et al.*, 2002).**

Amorphous cytoplasmic inclusions bodies were observed with light microscopy in infected epidermal stripe with *N. tabacum* cv. White Burely leaves. Similar results were reported by **Biljana and Polak (1968)** who found inclusions bodies contain mature virions in epidermal cells of tobacco infected by *Tobacco rattle virus.*

Mesophyll layer was reduced in infected plants if compared with healthy plants. The spongy tissue was more compact, characterized with reduced intracellular space in comparison with tissues of healthy plants. The number of chloroplasts was reduced in number in infected cells than in healthy ones, it might be due to the degradation of the chloroplasts. This finding reflects the symptoms of chlorotic strips on TRV-infected leaves. On the other hand, the phloem tissues were also affected and necrosis was observed in infected in comparison with healthy tissues.

The anatomical changes were previously reported and explained by several investigators who indicated that the chloroplasts and nucleoli are the most organelles affected by viral **infections (Kim *et al.*, 1989; Ahmed, 1996 and Sallam *et al.*, 1999). Ibrahem *et al.* (1997)** revealed that the necrosis in the phloem of vascular bundles of wheat leaves infected by Burley yellow dwarf virus.

The morphology of TRV particles was studied by electron microscopy. Tubular particles with average length of 48-114 nm and 22 widths were observed. These results are similar with those recorded by **Lister and Bracker (1969).**

These changes expected as chloroplasts and nucleus are involved in virus replication and virus particles might accumulate in these two organils **(Matthews, 1991).**

Concerning that it is the first report of natural infection of onion plants with TRV, and as the results indicated the high percentage of seed transmission of the virus, it is recommended not to use seeds collected from infected plants and isolate the areas of seed production of onion in the different regions of Egypt.

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