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Isolation and Identification of *Tobacco rattle tobnavirus* Affecting Onion (*Allium cepa* L.) Plants in Egypt

¹A.H. Hamed, ²Om-Hashim M. El-Banna, ²G.A.M. Ghanem, ³H. Elnagaar and ¹M.S. Shafie

¹Department of Virus and Phytoplasma Research, Plant Pathology Research Institute, Agricultural Research Center, Egypt

²Department of Plant Pathology, Faculty of Agriculture, Cairo University, Egypt

³Department of Zoology and Nematology, Faculty of Agriculture, Cairo University, Egypt

Corresponding Author: A.H. Hamed, Department of Virus and Phytoplasma Research, Plant Pathology Research Institute, Agricultural Research Center, Egypt

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 that the host range of the expanded to 7 different plant families. The virus was transmitted mechanically and by seeds with percentages ranged between 8-13%. Different serological methods were used successfully for detection of TRV i.e., DAS-ELISA, TBIA and DIBA. Infection of tobacco leaves with TRV resulted in the formation of amorphous inclusion bodies in the cytoplasm. Light microscopy examination of semi thin sections in both healthy and artificially infected onion leaves showed anatomical changes which reflecting on the external symptoms on infected plants. Also, electron microscopy observed tubular particles with two main dimensions (length 48-114 nm and 180-197 nm and 22 nm width). Investigation of ultrathin sections by transmission electron microscopy revealed changes in both nucleus and chloroplast. According to the available data, TRV was isolated and identified for the first time in Egypt from onion plants during the present study.

Key words: Tobacco rattle virus, host range, inclusion bodies, modes of transmission, electron microscope, serological detection ELISA, DBIA and TBIA

INTRODUCTION

Onion (*Allium cepa* L.) has a worldwide importance ranking second among all vegetables in economic importance after tomatoes (Griffiths *et al.*, 2002). In Egypt, onion is the third vegetable crop more consumed (15 kg/capita/year), after potatoes 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 cultivars white (Giza-6) and red (Beheri) onion are the most produced varieties. The current production area is being around 147,490 feddan with total production 1.3 million ton.

Tobacco Rattle Tobravirus (TRV). 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 which infect more than 100 plant species. Also, it 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; MacFarlane, 1999). TRV causes economic losses in bulbs production, such as tulip, narcissus, crocus and gladiolus. In Egypt, TRV was isolated from several plants i.e., gladiolus (Sabek, 1973), henbane (*Hyoscyamus muticus* L.), (Shafie, 1978) and from Kaki (*Diospyros Kaki*) (Zein, 2004). For this reason, the aim of the present was to isolate and identify an isolate of TRV from the naturally infected onion plants.

MATERIALS AND METHODS

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 cheese cloth. Ten seedlings of each of *C. amaranticolor* and *N. tabacum* cv. White Burley were dusted with carborandum (600 mesh) and inoculated with the sap. Seedlings of each host were inoculated with distilled water to serve as control. The plants were grown in the greenhouse (22-25°C) and observed daily for symptoms appearance. Single local lesion technique (Noordam *et al.*, 1973) was used for biological purification of TRV. The virus was submitted for two serial passage in *C. amaranticolor* as a local lesion host and propagated in *N. tabacum* cv. White Burley.

Identification of TRV: The virus was identified according to host range, symptomatology, modes of transmission, serological reactions using ELISA techniques and by light and electron microscopy.

Host range and symptomatology of TRV: Twenty seedlings of each species and varieties belonging to seven families i.e., Alliaceae, Amaranthaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Ebenaceae, Fabaceae and Solanaceae were inoculated with infectious sap. The seedlings were grown in the greenhouse and examined daily up to 30 days for symptoms development. The developed symptoms were recorded. Symptomless plants were checked by back inoculation into *C. amaranticolor* and/or using ELISA kit for TRV detection.

Modes of transmission

Mechanical transmission: Mechanical inoculation was carried out as mentioned before.

Seed transmission: TRV was tested in onion seed batches collected from mechanically inoculated plants. Seedlings of cvs. Giza 6, Giza 20 and red (Beheri) were inoculated with TRV under

greenhouse conditions. Virus free seedlings for each cultivar were sown in 20 cm diameter clay pots one seedling for pots and kept in an insect proof green-house, mechanically inoculated with virus isolate. Inoculated plants were kept until seed maturation. Five hundred seeds from each cultivar were sown after 5 month storage at lab conditions in paper bags (22-25°C). Emerged seedlings of onion plants were observed for virus symptoms development. Percentage of seed transmission was calculated. One hundred seeds of healthy plants of each cultivar were sown under the same conditions as control.

Serological diagnosis: Several serologic techniques i.e., indirect ELISA, DAS-ELISA, TBIA and DBIA were applied to test leaf samples randomly selected from onion plants. Samples consisted of leaf disks homogenized in buffer (DAS-ELISA or indirect ELISA) at a ratio of 1:2 (w/v). Control of healthy and infected sap were used. ELISA kit for detection of TRV was supplied by SANOFI, Sante, Animals, Paris and France. The method of Clark and Adams (1977) was applied as follows:

Direct ELISA: DAS-ELISA protocol was developed for detection of the virus, using plates which were coated with immunoglobulin G (IgG) 200 μL well⁻¹ diluted in coating buffer at 1:500 and incubated for 2 h at 37°C, then the wells were washed 3 times with Phosphate Buffer Saline Tween (PBST). The tested sample (extracted in sampling buffer), were added to duplicate wells. The plate was incubated at 4°C overnight. Wells rewashed 3 times as before. Two hundred microliters of IgG conjugate diluted (1:500) in conjugate buffer and incubated at 37°C for 2 h then washed. A hundred microliter of freshly prepared substrate was used.

Indirect ELISA: Indirect ELISA was applied according to the method described by Clark and Adams (1977) and Koenig (1981). The absorbance was measured at 405 nm using ELISA reader (Dynatech MR 7000). Samples with an absorbance of at least twice that of healthy control were considered positive for the virus.

Tissue blot immuno bindingassay (TBIA) and dot blot immuno bindingassay (DBIA): Dot blot and tissue blot immunobinding assays were carried out as described by Lin *et al.* (1990).

Cytological changes in TRV infected plants light microscopy of inclusion bodies: Cytological changes were observed in *N. tabacum* cv. White Burley 20 days after inoculation with TRV, epidermal stripe were taken from the lower surface of leaves and were treated with 5% Triton x-100 for 10 min to disrupt the plastids and facilitated the observation of the inclusions (Edwardson *et al.*, 1984).

Morphology of virus particles: Virus preparations were negatively stained with 2% Phosphotungstic (PTA) acid (Noordam, 1973) and mounted on carbon coated copper 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: Onion leaves tissue infected with either OYDV or TRV, 45 days after transplanting were cut into small pieces about 1-2 mm., fixed in 2% glutaraldehyde in 0.1 M Na-Cacodylate buffer, pH 7.2 and subjected to a vacuum for 1-4 min every 15 min for 2 h 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 min, with buffer changes at 15 and 30 min. Further fixation in 1% Osmium Tetraoxide in Na-Cacodylate buffer, under intermittent vacuum and poking, took place for 1.5 h (Osmont and Freeling, 2001). Samples were then rinsed in the Na-Cacodylate buffer. Samples were dehydrated through an Ethanol series in buffer: 35, 50, 70, 80, 95, 100 and 100% for 60 min. each, then infiltrate with res. Ultra-thin sections were cut using ultramicrotome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mesh). Sections were stained with double stain (Uranyl acetate 2% 10 min followed by Lead citrate for 5 min and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632×1632 pixel format as side mount configuration (Osmont and Freeling, 2001).

RESULTS

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.

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

Host range and symptomatology of TRV: As shown in Table 1 and Fig. 1 all plant species and cultivars belonging to Alliaceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae the virus were reacted positively with TRV inoculation. Only *Zinnia elegance* and *Vicia faba* L. cv. Giza 3 reacted negatively with TRV. The tested hosts could be classified according to their reactions as follows: firstly, systemic symptoms varied between mild and severe were observed on the tested, *A. ascalonicum*, *A. kurrat*, *D. stramonium*, *N. rustica*, *N. tabacum* "White Burley" plants, 2-3 weeks after inoculation with TRV. Secondly, TRV produced local lesions on the inoculated leaves of *Cucumis sativus* "Baladi", *Gomphrena globosa*, chlorotic and white necrotic stripes on onion, *Allium cepa* "Giza 6" and 20" and "Beheri"; *A. sativum*, *Phaseolus vulgaris* "Giza 1" and *Cucurbita pepo*. Thirdly, inoculated leaves of *C. album*, *C. amaranticolar* and *C. quina*, reacted with local infection followed by systemic symptoms. TRV was not detected either by ELISA or back inoculation into the indicator test plants which gave no symptoms.

Modes of transmission

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.

Table 1: Reaction of different hosts to infection with TRV

Host plant tested	Symptoms
Family: Alliaceae	
<i>Allium cepae</i> Giza 20	Ch + St +WN
Giza 6	Ch + St+ WN
red (Beheri)	Ch+ WNS
<i>Allium ascalonicum</i> L.	Ys+ St
<i>Allium kurrat</i> L.	Ys+ St
<i>Allium sativum</i> L. cv. Baladi	Ch+ WNS
Family: Amaranthaceae	
<i>Gomphrena globosa</i> L.	CLL
Family: Compsitae	
<i>Zinnia elegance</i>	-
Family: Chenopodiaceae	
<i>Chenopodium album</i>	CLL
<i>C. amaranticolor</i>	NLL
<i>C. quinoa</i> L.	CLL
Family: Cucurbitaceae	
<i>Cucurbit pepo</i>	CLL
<i>Cucumis sativus</i> L. cv. Baladi	NLL
Family: Fabaceae	
<i>Phaseolus vulgaris</i> L. cv. Giza1	PP
<i>Vicia faba</i> L. cv. Giza 3	-
Family: Solanaceae	
<i>D. stramonium</i> L.	SM
<i>Nicotiana glutinosa</i> L.	NLL + SM
<i>N. rustica</i> L.	NLL + SM
<i>N. tabaccum</i> L. cv. White Burley	SM
<i>Solanum tuberosum</i> L. cv. Diamond	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



Fig. 1(a-f): Reaction of some hosts with TRV infection: (a) White necrotic stripe on onion (*Allium cepa* L. cv. Beheri), (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* and (f) Chlorosis on *Solanum tuberosum*

Table 2: Seed transmission of TRV

Variety	No. of infected/tested	Infection (%)
Giza 6	50/500	10
Giza 20	40/500	8
Beheri	65/500	13

Serological diagnosis: Direct and indirect ELISA were used to confirm the identity of TRV, positive reaction was obtained with the virus and its specific antiserum

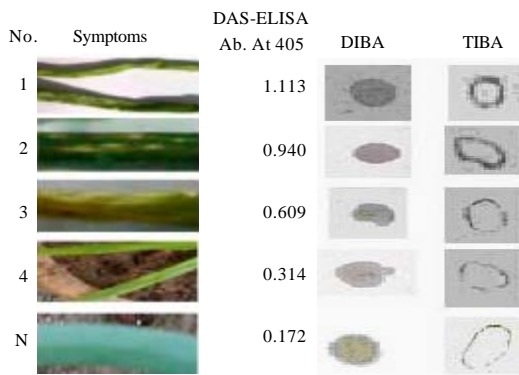


Fig. 2: Detection of TRV by DAS-ELISA, TBIA and DBIA

Seed transmission: Results in Table 2 showed that TRV could be transmitted through onion seeds of the three tested onion cultivars with different transmission percentages. The percentages of transmission ranged from 8,10 and 13% in Giza 20, Giza 6 and Beheri, respectively. This high percentage of seed transmission is very effective as a source of early infection in the field.

Tissue blot immunobinding assay (TBIA) and dot blot immunobinding assay (DBIA): The antiserum of TRV successfully detected infected samples as shown in Fig. 2. DBIA and TBIA were able to detect TRV in onion leaves. Infected samples changed to purple color while 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.

Cytological effects of TRV infection: Light microscopy: Amorphous cytoplasmic inclusion bodies were observed with light microscopy in epidermal stripe of TRV-infected onion leaf, taken from the lower surface of systemically infected *N. tabacum* cv. and White Burley, 20 days after inoculation (Fig. 3).

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 (Fig. 4). Mesophyll layer was reduced in infected plants (Fig. 4b) compared with healthy ones (Fig. 4a). The spongy tissue was more compacted, characterized with reduced intracellular space (Fig. 4d) in comparison with tissues of healthy plants (Fig. 4c). The number of chloroplasts was reduced in the infected cells than in healthy ones

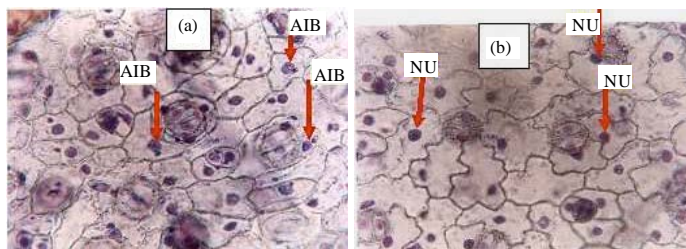


Fig. 3(a-b): Amorphous inclusion bodies in epidermal cells of *N. tabacum* cv. White Burley (a) and Healthy (b). (X-200) NU: Nucleus, AIB: Amorphous inclusion bodies

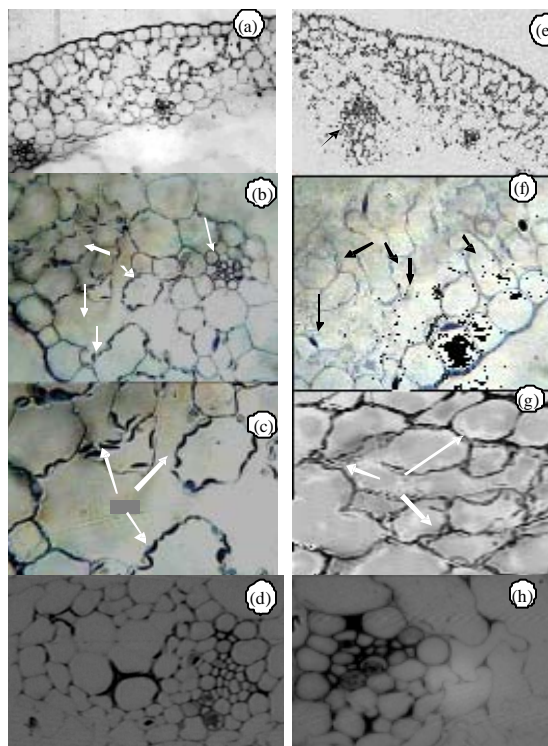


Fig. 4(a-h): Light microscopy of semi thin sections of both TRV- healthy and infected Beheri onion plants (a, b), mesophyll cells (c, d) spongy tissues (e, f) chloroplasts in parenchyma cells and phloem tissues of healthy (g) and diseased leaves (h). Ch: Chloroplast, M: Mesophyll, N: Necrosis, S: Spongy tissue, IS: Intercellular spaces, PC: Palisade cells, V: Vacuoles

(Fig. 4f, E, respectively). This finding reflects the symptoms of chlorotic stripe on TRV-infected leaves. On the other hand, the phloem tissues were also affected and necrosis was observed in the infected (Fig. 4h) comparing with healthy tissues (Fig. 4g).

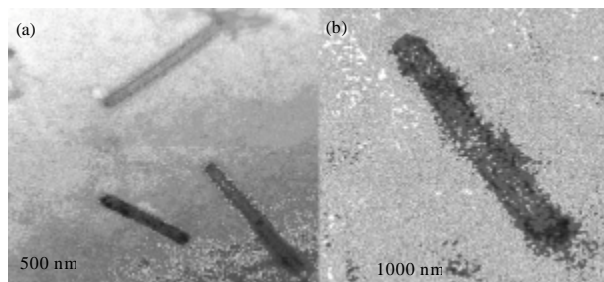


Fig. 5(a-b): Electron micrograph of purified TRV negatively stained with 2% phosphotungestic acid

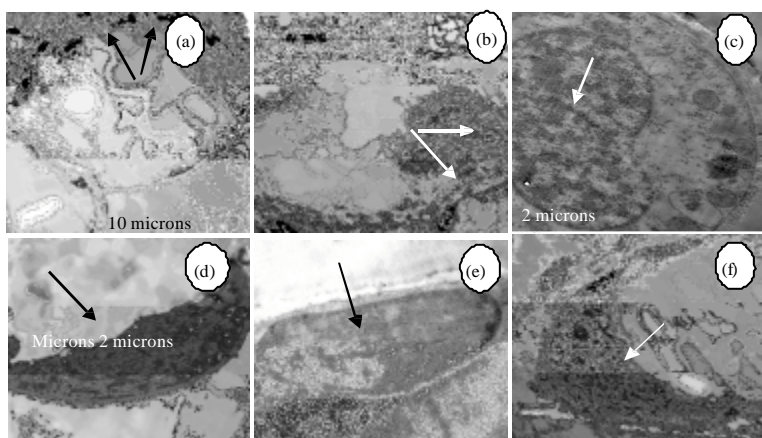


Fig. 6(a-f): Electron micrographs representing the effect of TRV-infection on onion cell organelles compared with those of healthy cells. (a): Mesophyll cell of healthy onion leaf with normal shape and number of chloroplasts (arrow), (b): Cell of TRV-infected leaf showing several degrees of chloroplast deformation and degradation, (c): Normal chloroplast at high magnification compared with affected, (d): One, (e): The nucleus of infected cell swollen and the chromatin is segmented and (f). Ch: Chloroplast and Chr: Chromati

Electron microscopy: The morphology of TRV particles was studied by electron microscopy using virus preparation negatively stained with 2% phosphotungestic acid. Tubular particles with two different lengths of 48-114 nm and 180-197 and 22 nm width were observed (Fig. 5).

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

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.*, 1997; Chen *et al.*, 2004). TRV had earlier been reported in garlic, onion, *A. moly* and *A. ursinum* in Europe (Bos, 1983; Van Dijk, 1993; Uhde *et al.*, 1998).

In this study, TRV was isolated from naturally infected onion plants. All tested plant species and cultivars belonging Alliaceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae reacted positively with TRV. Only *Zinia elegance* reacted negatively with TRV inoculation. The 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, tested plant species 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 showed chlorotic and white necrotic stripe on the leaves.

Systemic symptoms varied from mild to severe, 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. Whilst, local lesions produced on the inoculated leaves of *C. album*, *C. amaranticolar*, *C. quina*, *Cucurbit pepo*, *Cucumis sativus* cv. Baladi and *Gomphrena globosa*, but produced pin point on *Phaseolus vulgaris* L. cv. Giza 1.

Inoculated leaves of *N. glutinosa* and *N. rustica* reacted with infection as local lesions host followed by systemic symptoms while chlorotic and white necrotic stripes on onion *Allium cepa* cv. (Giza 6, 20 and Beheri). Also, *A. sativum* L. reacted with TRV-infection as local lesion followed by systemic symptoms. TRV was not detected either by ELISA or back inoculation into the indicator test plant which gave no symptoms. These results have already been recorded by Kirk *et al.* (2008) who transmitted TRV mechanically from potato cv. FL1879 tubers onto tobacco cv. Samsun NN. Also, Koike *et al.* (2010) isolated TRV from infected spinach in the fields to *Chenopodium quinoa*, *C. murale*, *C. capitatum*, spinach and sugar beet (*Beta vulgaris*).

It was noticeable that TRV was transmitted through seeds of Beheri cv with high percentage being 13%. This high percentage of seed transmission considered as a very effective source of early infection in the field. Seed transmission of TRV through onion seeds was not previously recorded, but its transmission through different hosts was reported by Lister and Murrant (1967) through some weeds i.e., *Capsella bursa-pastoris*, *Myosotis arvensis*, tomatoes (Visser *et al.*, 1999; Dikova, 2005) and sugar beet seeds.

Concerning serologic detection, DBIA and TBIA techniques proved to be able to detect TRV in infected plants. These results have already been declared by Kamenova and Adkins (2004), Lin *et al.* (1990), Fegla *et al.* (2001) and Ghanem *et al.* (2002).

Amorphous cytoplasmic inclusion bodies were observed with light microscopy in infected epidermal stripe with *N. tabacum* cv. White Burley leaves. Similar results were reported by Biljana and Polak (1968) who observed inclusion bodies contain mature virions in epidermal cells of tobacco infected by Tobacco rattle virus. Mesophyll layer was reduced in infected plants compared with healthy plants. The spongy tissue was more compacted, characterized with reduced intracellular space in comparison with healthy tissues. The number of chloroplasts was reduced in infected cells than in healthy ones, it might be due to the degradation of the chloroplasts. This finding reflects the symptoms of chlorotic stripe on TRV-infected leaves. On the other hand, the

phloem tissues were also affected and necrosis was observed in infected compared with healthy tissues. The anatomical changes were previously reported and explained by several investigators who indicated that the chloroplasts and nuclei are the most organelles affected by viral infections (Kim *et al.*, 1989; Ahmed, 1996; Sallam *et al.*, 1999). Also, Ibrahem *et al.* (1997) observed necrosis in the phloem of vascular bundles of wheat leaves infected by Barley yellow dwarf virus. Matthews (1991) found that these expected changes in chloroplasts and nucleus are involved in virus replication and virus particles might accumulate in these two organelles.

The morphology of TRV particles was studied by electron microscopy using virus preparation negatively stained with 2% phosphotungstic acid. Tubular particles with two different lengths of 48-114 and 180-197 and 22 nm width were observed. These results are similar with those recorded by Lister and Bracker (1969).

Finally, according to the present study, TRV was isolated and identified for the first time in Egypt from onion plants during the present study. We recommend prevention of seed collection from cultivated areas infected with TRV to be used for onion cultivation in the next plantations.

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