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RESEARCH

Detection of *Potato Virus M* in commercial local potato seed tubers in Egypt

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ABSTRACT

Background: *Potato virus M* (PVM) is found in potato cultivars worldwide including Egypt. Potato tubers are a common source of the virus during host cultivations. The yield loss can range from 15% to 45% and heavy losses can occur upon mixed infection with PVM and PVS or PVX.
Objective: The current work aims to characterize the PVM, study cytopathic effect of PVM on potato tissues, and detection of PVM in commercial potato seed tubers using RT-PCR.
Methods: The PVM was detected in symptomatic infected potato plants during growing seasons for the cultivated commercial local tuber seeds using Double Antibody Sandwich-Enzyme Linked Immuno-Sorbent Assay (DAS-ELISA). The PVM was mechanically isolated on potato cv. Diamant. Host range was carried out by mechanical inoculation of PVM on a set of different plant species. Electron Microscopy was used to examine the virus morphology. The cytopathic effect of the virus on tissue ultrathin section was examined by transmission electron microscope (TEM). Finally, the PVM isolate was detected using reverse transcription-polymerase chain reaction (RT-PCR) in the sprouts after dormancy breaking for the harvested potato tubers.
Results: The obtained results demonstrated that, PVM isolate causes severe symptoms on potato plants under the greenhouse conditions. The infected potato cv. Diamant gave positive DAS-ELISA result using polyclonal antibodies against PVM. Moreover, the RT-PCR confirmed PVM in tuber sprouts using coat protein gene specific primers with an expected amplicon of 520 bp. Electron Microscopy examination revealed that, the viral particles were slightly flexuous rod shape with about 680 x 13 nm in size. Ultrastructure analysis showed different degrees of chloroplast degradation in PVM-infected potato leaf tissues.
Conclusion: Occurrence of PVM was detected and confirmed in commercial potato seed tubers in Egypt using the RT-PCR technique.

Keywords: *Potato Virus M*; Potato seeds; DAS-ELISA; RT-PCR; Virus morphology; Cytopathology.

BACKGROUND

Potato is the world's most widely grown tuber crop. In Egypt, potato is an important crop produced under irrigation almost year-round, and virus infections represent a major problem on potato production and for seed certification (Elawady and Abdulkheir, 2015). There are several viruses can occur heavy yield losses individually or in mixed infections in potato cultivations, they include *Potato virus M* (PVM), *Potato virus S* (PVS), *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) (Nasr-Eldin *et al.*, 2018).

Potato virus M (PVM) is a member of the family *Betaflexiviridae*, genus Carlavirus. Virus particles are flexible rods (12-13 nm X 610-700 nm), containing a single-stranded, positive-sense RNA genome of approximately 8.5 kb in length, its coat protein composed of multiple copies of a 34-kDa (Tavantzis 1991; Adams *et al.*, 2012). On the open field PVM is transmitted non-persistently by various aphid species (Palukaitis, 2012), it can also be easly transmitted mechanically in experimental purposes. The main host of PVM is potato (*Solanum tuberosum* L.), and it has a narrow naturally host range. PVM causes severe symptoms include

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mottling, mosaic, crinkling, leaflet deformation, necrosis of petioles and stems in certain potato cultivars (Habib, 1980; Brunt, 2001; Palukaitis, 2012; Ali-Ali *et al.*, 2013).

In potato cultivations, infected tubers are a common source of the PVM and if potato tubers are serologically tested during the dormant period the results will typically underestimate (give false negative results) the presence of virus particles in the sample (de Bokx and Cuperus, 1987). The breaking of the potato tuber's dormancy stimulates transport mechanisms through the phloem of the young tissues of the growing sprouts, which are actively draining sap out of the mother tuber. In virus-infected tubers, this sap is loaded with virus particles that will concentrate at the rose end of the tuber where most of the sprouts are located (Gugerli and Gehriger, 1980; Basky and Almasi, 2005). The aim of this work is to characterize the PVM by serological, biological, morphological levels. Moreover, cytopathic effect of PVM was also explored and RT-PCR was used to detect PVM in commercial potato seed tubers.

MATERIALS AND METHODS

Source of samples

The commercial local potato seed tubers cv. Diamant were purchased and collected from local Egyptian marketing potato seed tubers and cultivated in the open field for winter season 2017 at Virology Lab., Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt. The vegetative potato leaves system was examined continuously during growing season for appearing viral like symptoms. Seven potato plants out of about 200 plant showed viral-like symptoms were sampled for serological detection, in order to, these plants were labeled and covered with insect-proof cages until tubers harvesting for the planning acceptably following experiment of virus transmitted by tuber.

Serological detection

The collected potato leave samples were serologically examined by DAS-ELISA at Serological Lab., Virus and Phytoplasma Dept., Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt using the protocols described by Clark and Adams (1977) against specific PVM, PVS, PVX, PVY and PLRV polyclonal antibodies.

Biological assays

The potato plants which gave +ve DAS-ELISA results with PVM only were selected to collect its tubers after yield harvesting (90 days after planting). The potato tubers were cultivated in the greenhouse after dormancy breaking by storing in refrigerator and examined after symptoms appearing that is PVM identical external systemic symptoms. The infectious PVM crud sap were mechanically transmitted by rubbing the leaves of potato cv. Diamant as a PVM propagative specific host, and the inoculated plants were maintained under greenhouse condition until viral symptoms development (Noordam, 1973).

The prepared leaf infectious tissue sap was inoculated onto 15 different potential host plants belonging to 3 families *Solanaceae*, *Chenopodiaceae* and *Cucurbitaceae* (Table 1), 3 replicates/each host. All inoculated plants were kept under greenhouse conditions (25±3 $^{\circ}$C) and daily observed for viral symptoms appearing and then checked for infection by PVM using DAS-ELISA.

Determination of viral cytopathological effects

To study the effect of PVM on potato cv. Diamant tissues, ultrathin sections from top leaves of infected potato plants were prepared and examined as done by Rocchetta *et al.*, (2007).

Samples were cut into pieces (1-2 mm²), fixed in 2.5 % (w/v) glutaraldehyde and 2% (w/v) para-formaldehyde in 0.1 M phosphate buffer, pH 7.2 for 1h, post fixed in 1% (w/v) OsO_4 for 2h. Dehydration was done in 70, 85, 95, and 100% ethanol for 15 min and 15 min in propylene oxide, and embedded in the agar 100 resins. The ultrathin sections were stained with double stain (2 % uranyl acetate for 10 min followed by lead citrate for 5 min) and examined with JOEL JM 100-C transmission electron microscope (TEM) at Electron Microscope Unit, AL Azhar University, Cairo, Egypt.

Detection of virus particles morphology

PVM-infected potato leaf samples, previously confirmed for the presence of the virus were used for determination of virus morphology using leaf dip method according to Abo-Senna *et al.*, (2014) by negative stain and examined under JOEL JM 100-C TEM (Electron Microscope Unit, AL Azhar University, Cairo, Egypt).

Detection of single infection of PVM by RT-PCR

The RT-PCR was used for confirmation of PVM single infection in potato tuber sprouts cv. Diamant after dormancy breaking. The sprout samples were directly used for total RNA extraction by using TRIzol™ Reagent (Invitrogen, USA).

For further extremely strong single PVM infection results, the RT-PCR technique used for checking the presence of PVS associated with PVM using RT-PCR. The RT-PCR was performed using PVM specific primer for coat protein region, forward primer PVM4: 5′-ACA TCT GAG GAC ATG ATG CGC-3′ and reverse primer PVM3: 5′ TGA GCT CGG GAC CAT TCA TAC-3′ (Xu *et al.*, 2010), in order to PVS by specific primer of the coat protein region (PVS-F: 5'-TGG CGA ACA CCG AGC AAA TG-3' and PVS-R: 5'-ATG ATC GAG TCC AAG GGC ACT G-3') (El-Saghir *et al.*, 2017). cDNA synthesis was carried out using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) using the antisense primer PVM3 and PVS-R. The RT-PCR amplification were carried as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of 94 °C for 60 seconds, 58 °C for 60 seconds, and 72 °C for 60 seconds. The final extension was at 72 °C for 7 min. using a Biometra T-Personal thermocycler (Biometra GmbH). PCR products were separated on a 1.2 % agarose gel, stained with ethidium bromide, and visualized under UV- transilluminator.

RESULTS

Field external disease symptoms

Seven potato plants cv. Diamant showed of external PVM-like symptoms (mottling, mosaic, and crinkling) (Fig.1-B) compared to healthy plants (Fig.1A).

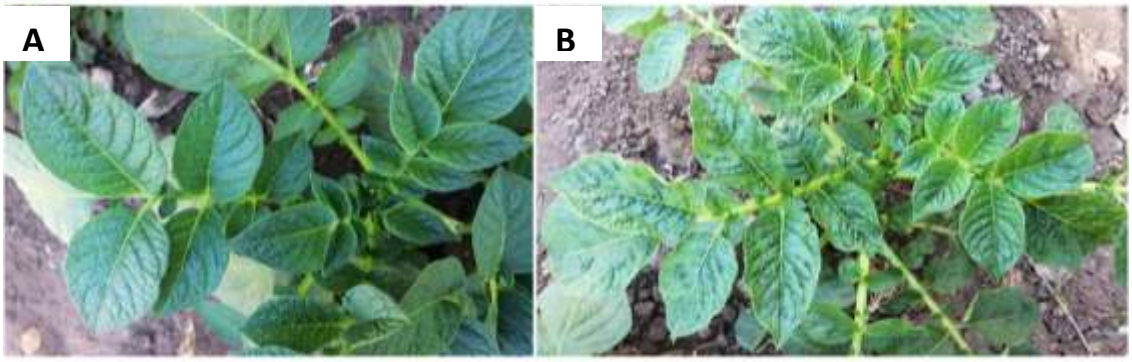

Fig. 1: Open field naturally systemic external viral symptoms observed in shoots of infected potato plants cv. Diamant (B) and healthy plants (A).

Serological detection of the virus

The infectious crude sap from collected symptomatic potato leave samples was checked serologically using polyclonal antibodies against 5 different viruses (PVM, PVX, PVS, PVY and PLRV) by DAS-ELISA. The results showed that, the sap of infected potato reacted positively only with an antiserum of PVM and negatively with other viruses.

Biological identification of the virus:

The obtained positive samples specific to PVM after DAS-ELISA detection were selected and their tubers were harvested and directly cultivated after dormancy breaking in the greenhouse conditions (25±3 °C). The observed systemic disease symptoms were recorded after 35 days from cultivation which were similar like that showed on the open field. The PVM symptoms were mosaic and increased after one week to leaf crinkling of examined young leaves of cultivated potato plants cv. Diamant. The same vegetative shoots were continuously observed for the external developing symptoms after another week that showed chlorosis with leaf and stem necrosis and all examined plants were detected by DAS-ELISA. These infected plants were used for mechanically inoculation to healthy cv. Diamant plants as PVM propagative host.

The infectious crude sap of PVM was mechanically inoculated onto 15 different plant hosts from 3 families *Solanaceae*, *Chenopodiaceae* and *Cucurbitaceae* (Table 1). The results showed that this virus have the potential to cause symptoms in some plant species, these symptoms ranged from moderate to severe symptoms. For main host potato plants, there are different reaction of symptoms as a result of PVM infection for 4 potato cultivars as severe mosaic, chlorosis for cv. Herms (Fig. 2-A), mosaic and crinkling for cv. selatar (Fig. 2-B), leaf petiole necrosis and crack of cv. Herms (Fig. 2-C). The most severe symptoms appeared on *Nicotiana tabacum* cv. White Burley (chlorosis, veinal necrosis, leaf deformation, stunting and different degrees of leaf necrosis), *Datura metel* L. (mosaic, crinkling and mottling) and *Datura stramonium* L. (mosaic, crinkling and mottling) for PVM infection, Table (1) and Figure (2-D, G, H). PVM-inoculated *Chenopodium amaranticolor* L., C. *album*, *C. quinoa* did not produce any external symptoms, in addition to *Lycopersicon. esculentum* was also symptomless systemic infection (Table 1). The hosts which did not show any external symptoms were tested by DAS-ELISA and gave negative results except *L. esculentum* gave positive result.

Table 1: Reaction of different plant species for inoculation with PVM.

Host plant tested		Symptoms
Family	**Plant species**	
Solanaceae	*S. tuberosum* cv. Herms	Mo, Ch
	S. tuberosum cv. Diamant	Mo, N, C
	S. tuberosum cv. Nicola	M, N
	S. tuberosum cv. Selatar	M, N, C
	N. tabacum cv. Samsun	M, Ch, N
	N. glutinosa L.	Ch, VN
	N. tabacum cv. White Burley	Ch, VN, St, LD
	N. tabacum cv. Turkish	Ch, NLL
	D. metel L.	M, C, Mo
	D. stramonium L.	M, C, Mo
	L. esculentum	NS
Chenopodiaceae	*C. quinoa* L.	NS
	C. *amaranticolor*	NS
	C. *album*	NS
Cucurbitaceae	*Cucurbita pepo* cv. Eskandarani	NS

M =Mosaic N = Necrosis C= Crinkling Ch= Chlorosis VN= Veinal necrosis St= Stunting
NLL= Necrotic local lesions LD= Leaf deformation Mo = Mottling NS= No syptom

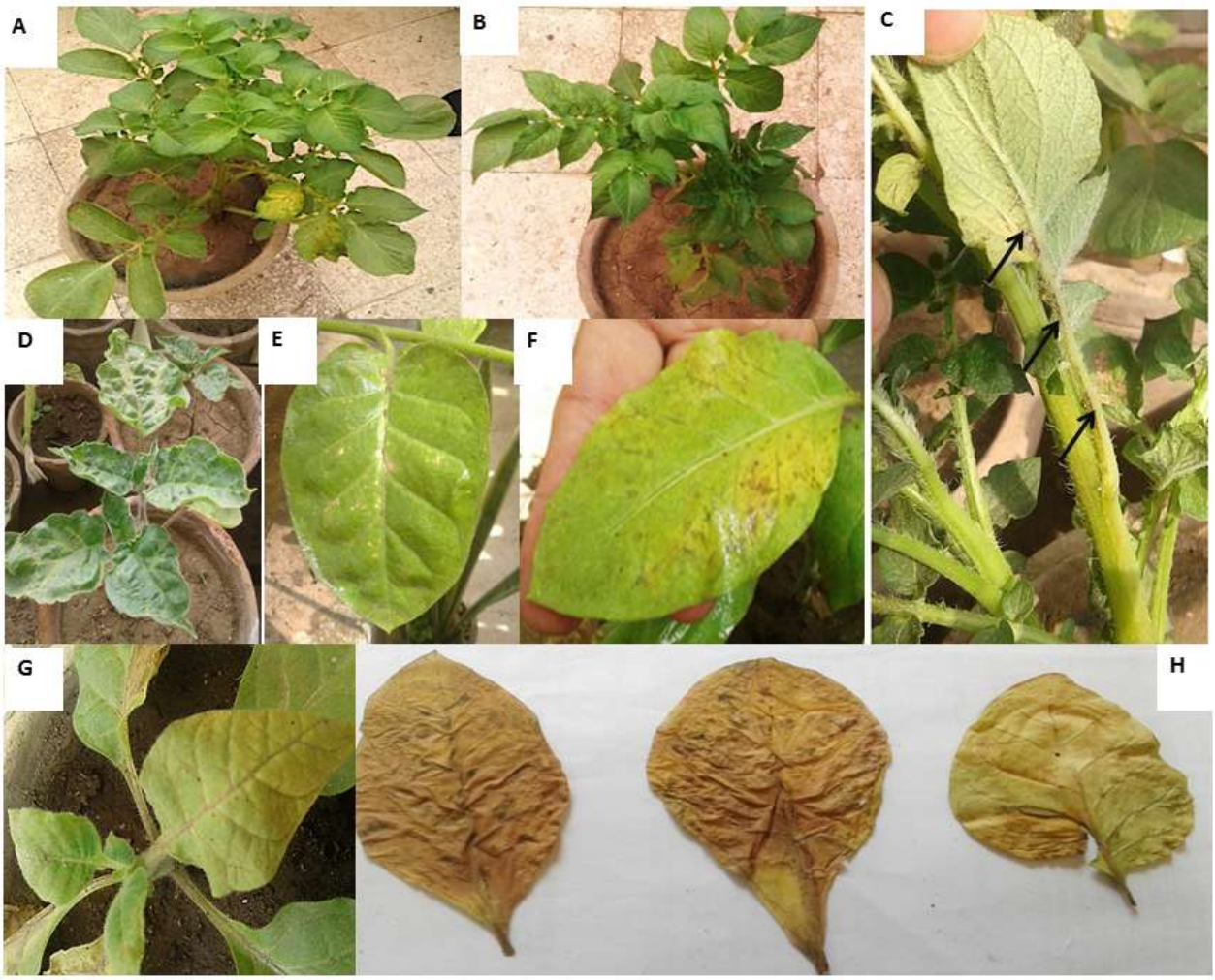

Fig. 2: Symptoms on different plant species inoculated with PVM: (A and C) potato cv. Herms; (B) potato cv. selatar; (D) *D. metel*; (E) *N. tabacum* cv. Turkish; (F) *N. tabacum* cv. Samsun; (G and H) *N. tabacum* cv. White Burley.

Cytopathic effect of PVM on potato plants:

The effect of PVM infection on potato histology structure was investigated by TEM in ultrathin sections. There is a significant alteration between the chloroplast structure of PVM-infected potato plants cells and the healthy ones including partial to complete destruction of the thylakoid structure and the stacking of grana was severely affected (Fig., 3). It was also observed that, in virus infected cell, the chloroplast membrane (chm) begins to dissolve and chloroplasts with fragmented stromatic lamellae (Fig. 3-B, C), the chloroplast contours are no longer discernible and chloroplast grana and lamellae are in different stages of degeneration (Fig. 3-C).

Fig. (3-D) showed that various deformation of the chloroplast grana, the chloroplast membranes completely dissolved until it almost disappears and whole chloroplast was complete destructed into oval or round bodies was detected in Fig. (3-E). Cytoplasmic inclusion bodies (CIB) also seen inside the cytoplasm of infected cells, this was consistent with a viral infection (Fig. 3-F).

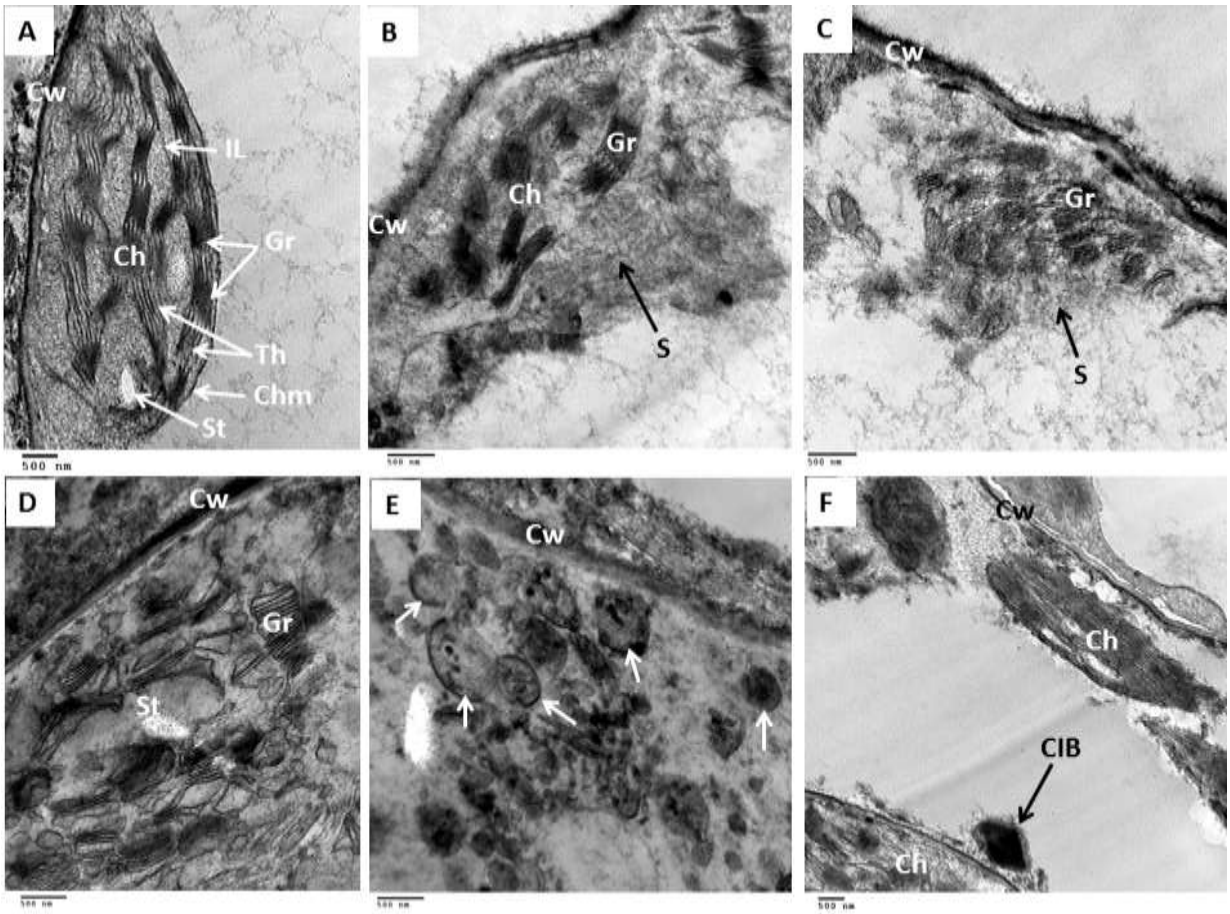

Fig. 3: Different degrees of chloroplast degradation in PVM-infected potato leaf tissues (B-F) and healthy one (A): Normal chloroplast (Ch) with grana (G), intergranal lamella (IL), thylakoid (Th), stroma (S), chloroplast membrane (Chm) and bright starch grains (St), cell wall (Cw). Cytoplasmic inclusion body (CIB).

Morphological characterization of virus particles:

Transmission electron microscopy of leaf dip preparation for infected potato leaves revealed flexible particles of PVM with 13 X 680 nm in size (Fig. 4).

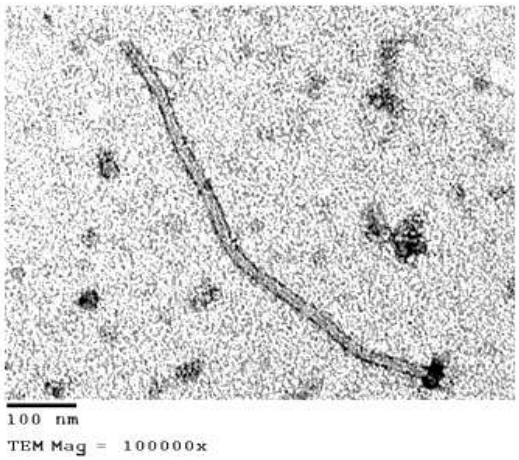

Fig. 4: Electron micrograph of PVM particles negatively stained with 2% uranyl acetate.

Molecular detection of PVM in potato tuber sprouts by RT-PCR:

RT-PCR technique was used to detect PVM in potato tuber sprouts resulting from infected plants using specific primers for PVM-cp gene, on the other hand for assaying of PVS present or not. The obtained results showed that, the DNA amplified product of PVM by specific primer was in the expected size (520 bp), Fig (5), lane (2), while negative results found for PVS (lane 3) compared with healthy samples (lane 4).

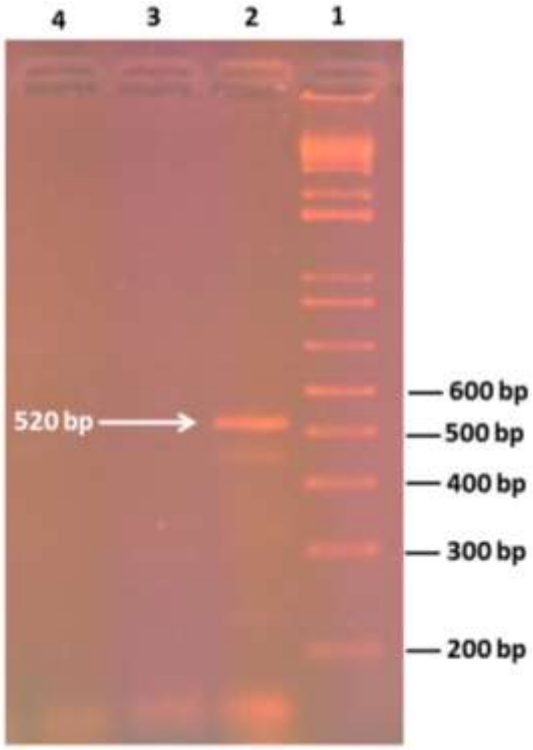

Fig. 5: Detection of PVM and PVS in potato tuber sprout samples by RT-PCR:
Lane (1): DNA marker. Lane (2): PCR amplicon (520 bp) for PVM.
Lane (3): PVS (negative result). Lane (4): Healthy sample (negative control).

DISCUSSION

Potato infected with different viruses, can cause severe economic losses. The early diagnosis is considered an important step for prevention of spreading of the viruses in potato. In our study for identification of viruses that infect potato plants, a combined biological, serological and a molecular based assay methods are required. The results of all the tests were positively identified the virus as *Potato Virus M* (PVM). Initially, potato plants with viral like symptoms were found to be naturally infected with PVM. The symptoms were mosaic, crinkling, mottling, and stunting on potato cv. Diamant growing in open field of Agric. Microbiol. Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt, these recorded symptoms are in according to Xu *et al.*, (2010) who noticed that, PVM induces mottle, mosaic, crinkling and stunting in susceptible potato cultivars. Severity of disease symptoms and tuber yield losses can differ depending on the virulence of PVM strain, the tolerance of potato cultivar and the environmental conditions (Cavileer *et al.*, 1998; Loebenstain, 2003).

ELISA is one of the most serological tests used for the detection of potato viruses, generally because of its rapidity, low cost, and reliability. In this study, sap of naturally infected potato plants was serologically tested in DAS-ELISA using 5 different antisera specific for

PVM, PVS, PVX, PVY and PLRV which showed that, the naturally infected plant reacted only to PVM-specific antibodies. PVM was detected based on the external symptoms and serologically by the DAS-ELISA test. All tested potato cultivars (Herms, Diamant, Nicola and Selatar) were infected with PVM and developed mosaic, crinkling and stem and leaf necrosis after mechanical inoculation with infectious sap like external symptoms which were recorded by Khan *et al.*, (2003). In current work, the virus was successfully transmitted mechanically and also through the tubers, and this agrees with that mentioned previously by Xu *et al.,* (2010) and Palukaitis (2012). The most severe symptoms appeared in PVM inoculated *N. tabacum* cv. White Burley, *D. metel* L. and *D. stramonium* L. plants as recorded by Pourrahim *et al.*, (2007). In the same context, PVM was reported on *D. metel* L. in Egypt by Habib (1980). Our results confirmed that PVM has a limited host range. Most susceptible species belong to the *Solanaceae*, of which the potato is the most important. PVM has not been mechanically transmitted to some species of *Chenopodiaceae* and *Cucurbitaceae*. In contrast, Loebenstein *et al.,* (2003) and Yin and Michalak (2017) reported that, PVM was experimentally transmitted to some species in *Amaranthaceace, Caryophyllaceae, Chenopodiaceae,Compositae, Cucurbitaceae* and *Rubiaceae*.

The obtained results of ultrastructural analysis for PVM infected potato leaf samples revealed various stages of transformation of the chloroplast grana structures into oval or round bodies and forming cytoplasmic inclusion bodies in accordance to El-Abhar *et al.,* (2018). Cytopathic effect of PVM reflected the external symptoms observed on infected potato leaves. The chlorosis on the leaves was a result of effects on the chloroplast grana which were completely or partially destructed. The same observation was made by El-Kammar *et al.,* (2016).

Flexuous rods shape structures (13 X 680 nm) of PVM particles were observed under transmission electron microscope after negative staining in the infected potato leaf sap, similar results have been reported by Singh (2006).

Currently, RT-PCR has become a valuable diagnostic tool for detection of potato viruses reliably (Xu *et al.,* 2010; Jeevalatha *et al.,* 2013; Kumar *et al.,* 2014 and 2017). RT-PCR was highly specific and sensitive for detection of PVM that infect potato tubers in the field (Kumar *et al.,* 2017). In this study RT-PCR assay was used for detection and confirmation of PVM occurrence using specific primer sets PVM3/PVM4 in the sprouts of infected potato tubers, because of PVM is accompanied in most potato varieties by the more widespread PVS, our results also investigated the potato tubers sprouts are infected by PVM alone as reported by Xu *et al.,* (2010), Kumar *et al.,* (2017), and Sharma *et al.,* (2018) who detect, PVM-RNA by RT-PCR, from total RNA preparations extracted from of infected potato tubers samples.

CONCLUSION

The biological and serological assays demonstrated that, PVM had transmitted by tubers; with a narrow host range, a dangerous cytopathic effect on potato plants and caused a dramatic ultrastructural change in chloroplasts of potato cells. Moreover, occurrence of PVM was detected and confirmed in commercial potato seed tubers in Egypt using the RT-PCR technique, therefore infected potato tubers acts as reservoir for presence and wide spread of the virus.

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