International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Volume 3, Issue 4 - 2016

Research Article

SOI: http://s-o-i.org/1.15/ijarbs-2016-3-4-28

Molecular Characterization and Cytopathological Studies of *Potato Virus Y* Isolated from Potato Sprout in Egypt

Sahar A.Youssef[1*], Eslam Arafa[2], Gehan Safwat[2] and A. A. Shalaby[1]

[1]Virus and Phytoplasma Res. Dept., Plant Pathol. Res. Inst., Agric. Res. Centre (ARC), Giza, Egypt.
[2]Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Giza, Egypt.
*Corresponding author: *saharyoussef@link.net*

Abstract

Potato Virus Y (PVY) is a plant pathogenic virus from *Potyviridae* and it belongs to *Potyvirus* genus. PVY is an aphid-borne virus that causes yield losses and tuber quality defects in commercial potato crops in Egypt. Infected seed potato tubers are the principal source of PVY spread to other potato plants. PVY was readily detected by RT-PCR of tuber sprouts. Molecular characterization of PVY isolated from infected potato sprout was investigated by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing analysis in addition to cytopathological study to detect the presence of virus infection in infected sprout tissue. Analysis of RT-PCR amplified cDNA product from infected tissue using primers designed to amplify the coat protein gene of the PVY genome verified that the *Potyvirus* infecting potato sprout is an isolate of PVY. These results were confirmed by nucleotide sequence analysis. Nucleotide sequencing of the PVY CP gene showed about 99%identitiy to that of other PVY^{NTN} isolate. Transmission electron microscopy (TEM) negative staining methods were used in order to detect the presence of viral cytoplasmic inclusion bodies. Typical pinwheels, scrolls, laminated aggregates and disruption of normal cells was observed in PVY infected potato sprout. RT-PCR technique has primarily been developed for large-scale screening of many samples for determining viral incidence in commercial fields or seed lots, and also amenable to use in smaller-scale research applications.

Keywords: Potato sprout, Potato Virus Y, RT-PCR, Nucleotide Sequence Analysis, Transmission Electron Microscope.

1. Introduction

Potato Virus Y (PVY) is virus that infects only plant not animal and causes serious disease in numerous solanaceae crops including potato, tomato, pepper and tobacco. In potato (*Solanum tuberosum* L.), PVY one of the most common and destructive viruses, it is a yield-restricting pathogen that can bring about as much as 50 to 80 percent yield misfortune in intensely contaminated business parcels around the world. The virus might likewise bring about post-harvest misfortunes because of tuber corruption and decreases capacity quality **(Leila *et al*., 2014).**PVY is the type member of the genus *Potyvirus*, family *Potyviridae* (the largest plant virus family) **(Syller, 2006)**. The PVY genome is a single-strand, positive sense RNA of about 9.7kb, with a virus encoded protein attached covalently to its 5'end and a 3'poly (A) tail **(Fauquet *et al*., 2005)**. PVY is widely distributed through the world and transmitted in a non-persistent manner by several aphid species, with varied transmission efficiency **(Vaveri, 2000; Spetz, 2003)**. It can also be mechanically transmitted **(Kerlan, 2006)**. The symptom of PVY is when infected, a potato plant can express side effects in 10 days, contingent upon the assortment. Indications of PVY infection are variable and extent from mild (foliar mottling, streaking, and mosaic) to severe (leaf necrosis, leaf drop, and hindering). The seriousness of the side effects relies on upon the potato cultivar, natural conditions, and the

strain of PVY contaminating the plant **(Moury *et al*., 2002; Lorenzen *et al*., 2006)**. Classically, PVY has been grouped into four main clusters of parental strains, PVY°, PVY^{N}, PVY^{C} and PVY^{NTN} based on the host hypersensitive response, genome sequence and/or serological properties **(Singh *et al*., 2008)**.

Since the early 1990s, a new diagnostic technique has been developed, reverse transcription-polymerase chain reaction (RT-PCR) which is based on detection of a nucleic acid component of the virus particle. This method has a much higher theoretical sensitivity than ELISA, and has been extensively studied as a possible alternative or as a complement to immune detection. Like ELISA, RT-PCR can be adapted for large-scale testing, and there is a keen interest in this te**c**hnique as far as potato viruses are concerned **(Lorenzen *et al*., 2006)**. RT-PCR technique is a traditional two-step protocol with separate reverse transcription of viral RNA into cDNA, then PCR to amplify the presence of viral cDNA fragment. This technique has primarily been developed for large-scale screening of many samples for determining viral incidence in commercial fields or seed lots, they are also amenable to use in smaller-scale research applications **(MacKenzie *et al*., 2015)**.The transmission electron microscope is an exceptional instrument which provides basic information on the morphology of virions, making possible tentative placement of a virus in particular family or genus. One of the main advantages of using EM for viral diagnosis is thatit does not required organism specific reagents for recognizing the pathogenic agent and modern TEM with a connected digital camera enables high quality observations of subtle changes in the interior and/or exterior of healthy and infected plant cells **(Otulak *et al*., 2014)**. It has been known for over 80 years that potyviruses induce the formation *in vivo* of cytoplasmic and nuclear inclusions which are observed by light microscope **(Kunkel, 1922; Kassanis, 1939)**. Electron microscopy has shown that these inclusions are aggregates of virus particles with host cell components **(Edwardson, 1974; Martelli and Russo, 1977)**. Several reports have been mentioned that cytoplasmic cylindrical inclusions are one of the main characteristics of the *Potyuvirus* group **(Singh *et al*.,2003)**. All potyviruses produced pinwheel inclusions, although scroll and laminated aggregate inclusions are also produced by some *potyviru*l species **(Hammond, 1998; Shukla *et al*., 1994; Rouis *et al*., 2000)**. The main ultrastructural diagnostic criterion of the attendance of *Potato virus Y* in the host plant tissues is the presence of viral cytoplasmic inclusion. Literature data define these structures as a result of the expression of protein coded by virus genome. A number of 5–15 protein structures are connected to the central core of the inclusion, forming sheets bent around the axis of the inclusion. The transversal cross section of this type of inclusion presents a rosette shape, so it has been named “pinwheel”, while the longitudinal section that looks like a bundle is named, “bundles” **(Martelli and Russo, 1977**). Also an intermediate form between helical and plate inclusions was isolated, and it has been named “short curved laminated aggregates” (**Edwardson *et al*., 1984).** Therefore, the identification of virus free planting material for a vegetative propagated crop, like potato, is extremely important. In the present study, specific primers in one step reverse transcription-polymerase chain reaction (RT-PCR) based protocol has been standardized for detection of PVY in infected potato sprout in combination with nucleotide sequencing analysis. Our investigation also focus on ultrastructural analysis of PVY inclusion forms and its arrangement in potato sprout. Moreover, our observation concentrated on occurrence of cytoplasmic inclusions in potato sprout.

2. Materials and Methods

Source of plant materials:

Naturally infected potato plants showed mild mosaic; chlorotic foliage and crinkled leaves, in addition to appeared healthy plants were collected from El-Behera governorate during early 2015 growing season for processing and virus testing. PVY polyclonal antibody provided from Agritest Inc., was used for DAS-ELISA according to **(Clark and Adams, 1977).** Collected samples which tested PVY positive using ELISA and healthy plants were marked in the field till the end of the growing season. Tubers collected from infected and healthy plants, re tested by ELISA, then stored in the refrigerator waiting for sprouts differentiation. Later, 2-3 eyes were scooped out from apical and distal ends and combined for each sample and examined. Sprout from each infected and healthy tuber planted on MS medium **(Murashige and Skoog, 1962)** with macro and micronutrient salts and vitamins, waiting for sprout differentiation. Then samples were applied for RNA extraction,RT-PCR, cloning, nucleotide sequencing and TEM testing.

Total RNA Preparation:

Total plant RNA was extracted from 2 to 200 mg of plant material (depending on the amount of the material available) using the Plant Total RNA Mini Kit (RBC Labs) following the manufacturer recommendations.

One step reverse transcription polymerase chain reaction (RT-PCR):

The specific primers to PVY coat protein (CP) gene designed according to **Shalaby *et al*.(2007)** were used as fellows: the forward PVY primer [5’-TCA AGG ATC CGC AAA TGA CAC AAT TGA TGC AGG-3’] and the complementary PVY primer [5’- AGA

GAG AAT TCA TCA CAT GTT CTT GAC TCC -3'](Integrated DNA Technologies, Inc).The cDNA synthesis was based on the method given in Thermo Scientific Verso 1-Step RT-PCR Kit. PCR program consisted of cDNA synthesis at 50◦C for 15 min and 2 min at 95◦C for verso inactivation followed by 35 cycles at 94◦C denaturation, 1 min at 55◦C annealing and 1min at 72◦C extension, ending with a final extension for 7 min at 72°C.

Gel electrophoresis analysis:

Seven-micro liter of RT-PCR product were analyzed on 1% agarose gel in TBE buffer (89 mMTris-HCL, pH 8.5) at 120 volt.100 bp sharp DNA ladder marker (RBC) was used to determine the size of RT-PCR products. Gels were stained with ethidium bromide 10 µg/ml **(Sambrook *et al*., 1989)** and visualized using gel-documentation system (Bio-Rad, GelDoc XR).

Cloning and Nucleotide sequencing analysis:

PVY cDNA amplified product was cloned into pCRII-TOPO vector using TOPO-TA cloning kit (Invitrogen). Nucleotide sequence of PVY coat protein gene was carried out twice in both orientations using labeled primer cycle in the cDNA sequencing facility of the Macrogen Inc, Korea, by using M13forward and M13 reverses primer with the purified recombinant plasmid of PVY. The nucleotide sequence data were assembled using DNAMAN program (Lynnon Bio Soft. Quebec, Canada) and compared against databases using blast on the NCBI Web server [http://www.ncbi.nlm.nih.gov/BLAST]. Phylogenetic trees were constructed using mega 4·0 program (Tamura *et al*., 2007). PVY sequences were fetched from the GenBank and referred to as the isolate name (Table 1).

Transmission electron microscopy (TEM):

The TEM negative stain method was applied on healthy and infected potato sprouts according to **Rocchetta *et al.* (2007)**. Samples were cut into pieces (1mm²), fixed for 1h in 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) para-formaldehyde in 0.1 M phosphate buffer, pH 7.2, post fixed in 1% osmium tetroxide. Dehydration was done for 15 min in 70, 85, 95, and 100% ethanol and 15 min in propylene oxide, and embedded in the agar 100 resins. Sections were cut with ultra-microtome, stained with 5% uranyl acetate for 20 min, stained with Reynold's lead citrate for 10 min. Images were recorded by JEOL electron microscope (JEM-1400 TE Japan),at the Faculty of Agriculture, Cairo University. Research Park (FARP).

3. Results

Source of sample:

Potato tubers collected from healthy and PVY infected plants, which tested using ELISA and marked in the field. The tuber re tested using ELISA, then stored in the refrigerator prior to sprouts differentiation (Fig. 1). The sprouts were collected and planted on MS medium for ten days. The differentiated sprouts were applied for RNA and TEM.

Fig. (1). Sprouts differentiated from potato tubers A) healthy and B) infected.

One step RT-PCR amplification of the PVY cp gene from infected potato sprout.

RT-PCR was performed using Plant Total RNA Mini Kit from RBC and a pair of primer specific for the extreme ends of coat protein gene of PVY with an expected amplicon size of 801bp.

The one step RT-PCR reaction combining of cDNA synthesis at 50°C for 15 min, initial denaturation at 95°C for 2 min, followed by the modified three steps repeated cyclically for 35 times, after verification on 1% agarose gel in electrophoresis, the expected band of ~801bp size was amplified in infected samples. It was found to be highly efficient method of detection as shown in (Fig. 2).

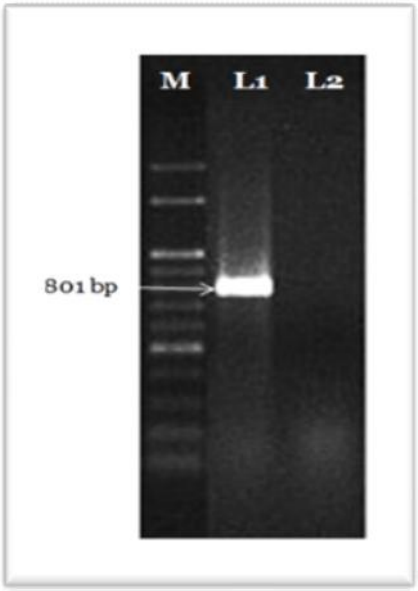

Fig. (2). Agarose gel electrophoresis analysis of amplified reverse-transcription polymerase chain reaction (RT-PCR) for potato virus Y (PVY) isolated from potato sprout when primer for PVY coat protein gene were used. Lane M, 100 bp sharp DNA ladder marker (RBC). L1, PVY infected potato sprout and L2, uninfected potato sprout.

Nucleotide sequencing analysis:

Nucleotide sequencing of the RT-PCR amplified product in the recombinant plasmid for the, PVY-CP was sequenced to determine if this RT-PCR fragments was from the *Potyvirus* group or not and to compare the sequences from our isolate with those of other sequences of potato-infecting *Potyvirus* group available in GenBank. The predicted PVY-CP gene is 801 nucleotides in size, starting from ATG start codon (methionine), as obtained by comparison with other PVY sequences and ending with a TGA stop codon from which the 3` NCR (non-coding region) ends. Translation of the CP gene codes for a 267 **amino acid** protein giving a molecular weight of 30 KDa. The amino acid sequence of 15 different PVY isolate were aligned and compared to the Egypt isolate (data not shown). PVY-Egypt showed amino acid similarity reached to 99% when compared to other isolates. The highest degree of similarity was found with (GU550076) and (GU980964) necrotic isolates from Egypt followed by (Eu073859) from Jordan and (JQ969041) necrotic strain from Belgium.

The similarity of the nucleotide sequences suggested that the architecture of the *Potyvirus*is highly conserved. Multiple sequence alignment of the nucleotide sequence of the coat protein gene of PVY [Egypt] with the corresponding sequence of 15different PVY isolates available in GenBank were analyzed using DNAMAN software. Phylogenetic tree was constructed based on alignment and displayed as a circular tree (Fig 3).A comparative analysis of the cp sequences of 15 isolates of PVY available in GenBank revealed that *Potato virus Y* (Egypt) is closely related to necrotic isolates PVY^{NTN} with percentage of similarity reached to 99 %. The highest degree of similarity with PVY- Egypt was found with the two PVY necrotic strains (GU550076) and (GU980964) from Egypt and PVY necrotic strain (EU 073859) from Jordan. The sequence results also observed the high similarity between PVY –Egypt isolate and the necrotic strains from Canada (AY745492), tobacco isolate from Poland (JF927753), and necrotic strain from South Africa (GQ853632) in addition to the isolates described in Table (1). The previous results from the sequence analysis observed that PVY-Egypt is belonging to PVY NTN strain.

Table (1). PVY sequences collected from the GenBank, used for nucleotide sequence comparison.

Accession number	Isolate	Host	References	Country
GU550076	PVYEG-NTN	Potato	Soliman *et al*.,(2006)	Egypt
GU980964	PVYEG2-NTN	Potato	Soliman *et al*.,(2006	Egypt
EU073859	aL-Ramtha	Potato	Odeh and Anfoka, unpublished data	Jordan
JQ969041	PVY34 N_Wi	Potato	Bahrami *et al*.,(2010)	Belgium
AY745492	N:O-L56	Potato	Nie *et al* .(2004)	Canada
JF927753	IUNG-5	Tobacco	Przyby *et al*.,(2013)	Poland
JN936435	-SS302_3"Wilga	Potato	Visser *et al*., (2012)	South Africa
GQ853632	N-W- NTN1	Potato	Visser and Bellstedt (2009)	South Africa
KR816236	Izh3-11 N-Wilga	Potato	Vologin *et al* ., unpublished data	Russia
KJ746455	HP5- N Wi-P	Tomato	Minicka,*et al*., unpublished data	Poland
EF027883	3290	Potato	Gow, *et al*.unpublished data	United Kingdom
JQ954313	German_65*2004	Potato	Cuevas *et al*.,(2012)	Germany
EF027900	19412b	Potato	Gow,*et al*., unpublished data	United Kingdom
JX432988	FI:275-1f:06	Potato	Tian and Valkonen,(2013)	Finland
HQ912868	N3-PVY -WN	Potato	Karasev *et al*., (2011)	USA

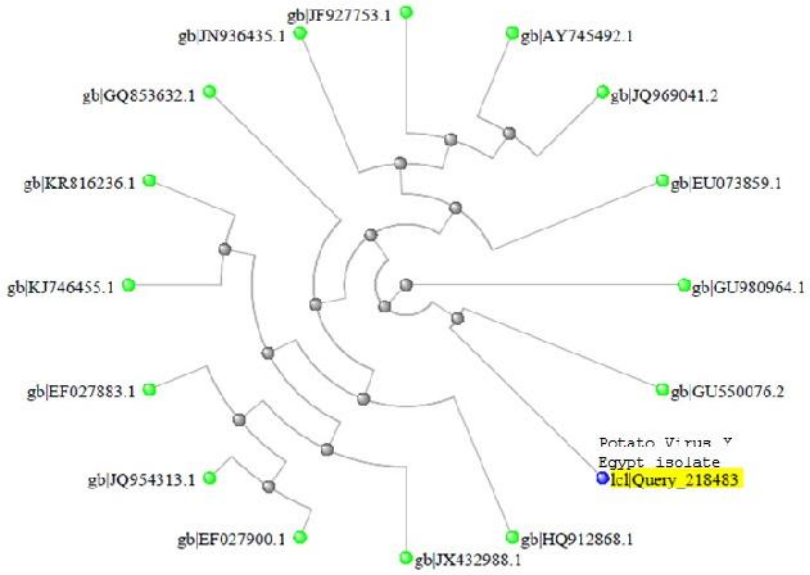

Fig.(3). Phylogenetic tree obtained from the alignment of nucleotide sequence analysis of coat protein gene (cp) of *Potato Virus Y* (Egypt) and 15 sequences for coat protein gene collected from the GenBank.

Transmission electron microscopy (TEM):

The ultrastructure analysis for the arrangement and form of cytoplasmic inclusions of PVY^{NTN} infected potato sprout was examined. All types of cytoplasmic inclusions were observed in the vascular tissue (Fig.4). Sprout tissues infected with PVY^{NTN} appeared the presence of cytoplasmic cylindrical inclusion consisting of pinwheels, scrolls and laminated aggregates. The cytoplasmic inclusion bodies concentrated in parenchyma cells in close vicinity to the epidermis in epidermal cells and in trichomes. Typical pinwheels, scrolls, laminated aggregates and endoplasmic reticulum were found in the cytoplasm of infected cells. (Fig.4).

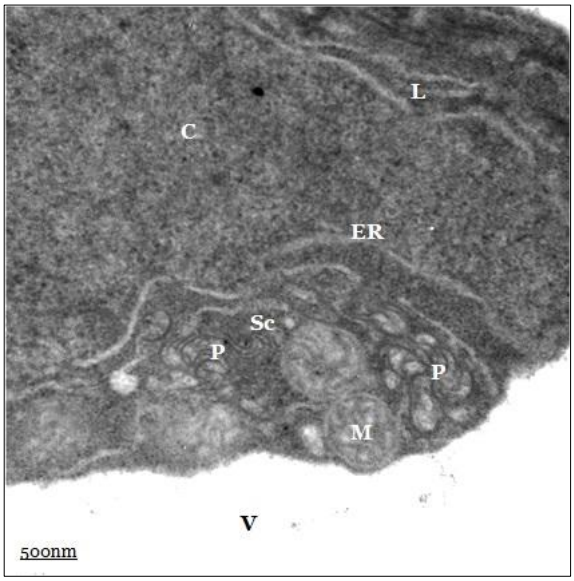

Fig. (4): Electron micrographs of different observation for potato sprout infected with PVY^{NTN} showed the cytoplasmic inclusions ultrastructure. P-pinwheels, Sc-scrolls, ER- endoplasmic reticulum, L-laminated aggregates, M-mitochondrion, V-vacuole; and C-cytoplasm in cytoplasmic invagination. Bar, 500 nm.

Large areas in cytoplasm of palisade and parenchyma cells were changed. Some other changes were observed in the ultrastructures of the mesophyll infected with PVY^{NTN} such as, changes in the outer cell wall, chloroplast structure and nuclei with unusual invaginations. Accumulation of the mitochondrion and laminated aggregates were noticed, with the presence of endoplasmic reticulum (Fig. 5). Some epidermal cells showed accumulation of peroxisomes with different shape and size located near the cytoplasmic inclusions (Fig.6 A & C). Tubular aggregates were also observed in the vicinity of pinwheels (Fig.6 B). Scrolls inclusions were observed in companion cells attached to cell wall (Fig. 6D). Neither pinwheels nor laminated aggregates were present in ultrathin sections prepared from healthy control potato sprout (Fig. 7).

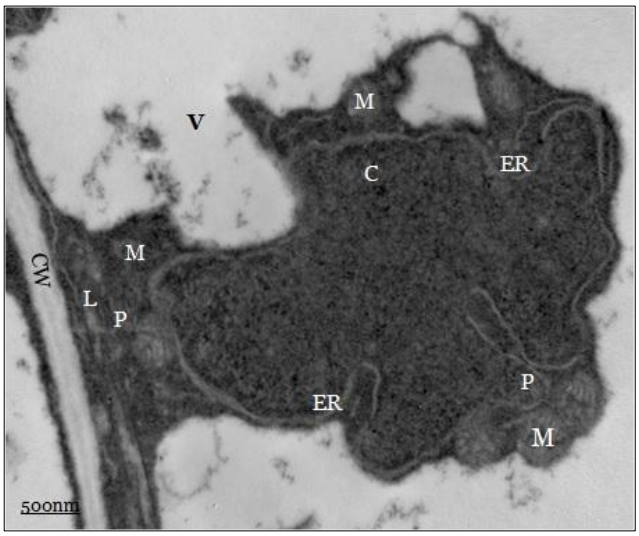

Fig. (5): Electron micrographs of different observation for potato sprout infected with PVY^{NTN} showed the cytoplasmic inclusions ultrastructure. P, pinwheels; ER, endoplasmic reticulum; L, laminated aggregates; M, mitochondrion; V, vacuole; CW, cell wall; and C, cytoplasm in cytoplasmic invagination. Bar, 500 nm.

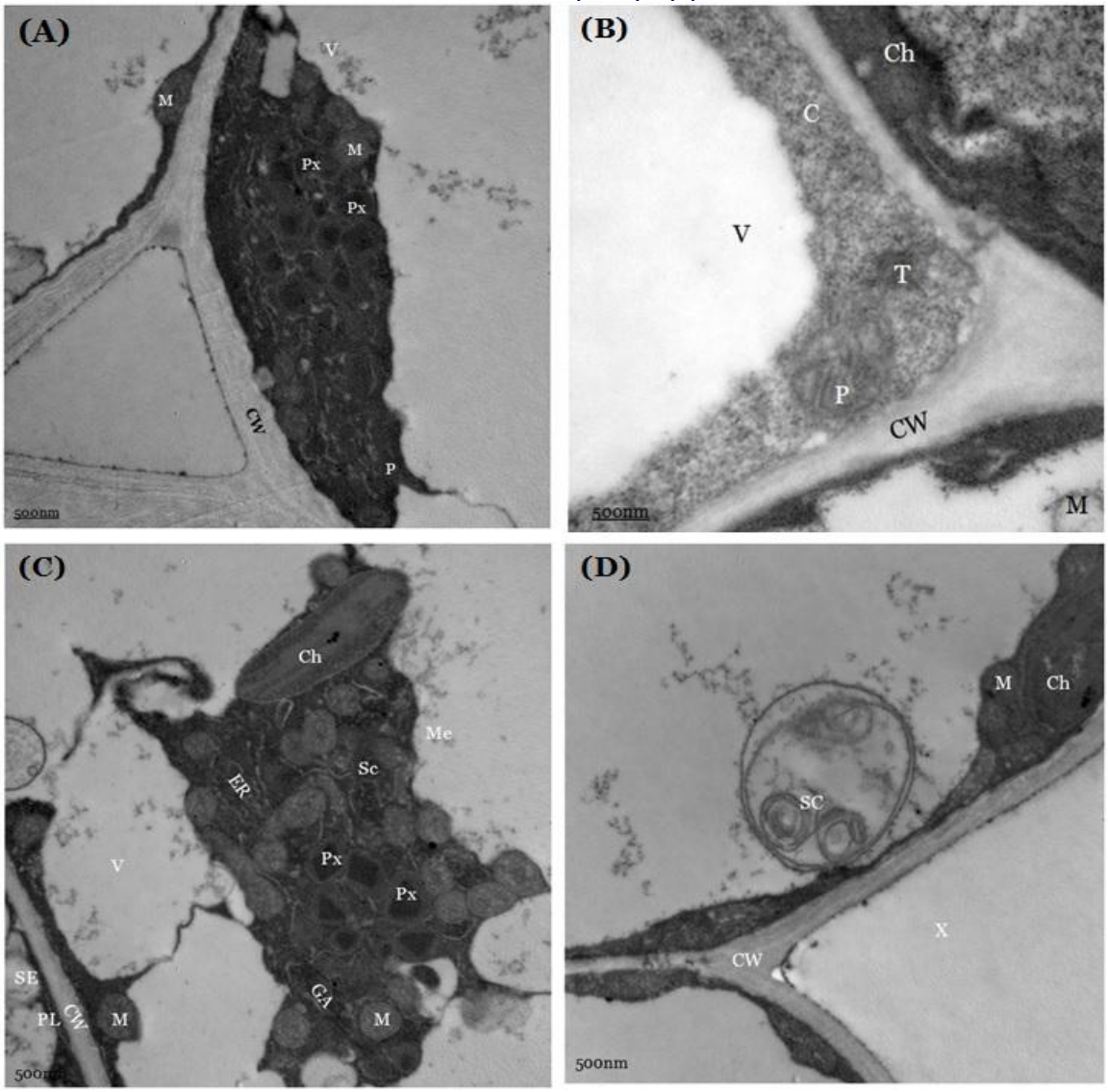

Fig. (6): Ultrastructure of potato sprout infected with PVY NTN. (A) P-pinwheels, Px-peroxisome, M-mitochondrion, V-vacuole, CW-cell wall. (B) Ch-chloroplast, CW-cell wall, C-cytoplasm in cytoplasmic invagination, M-mitochondrion, P-pinwheels, T-tubular structures, V-vacuole. (C) Ch-chloroplast, CW-cell wall, ER-endoplasmic reticulum, GA-Golgi apparatus, Me-mesophyll cell, M-mitochondrion, P-pinwheels, PL-plasmalemma, Px-peroxisome,Sc-scrolls, SE-sieve element, V-vacuole.(D) M-mitochondrion, Ch-chloroplast, CW-cell wall, Sc-scrolls, X-xylem tracheary element. Bar, 500 nm.

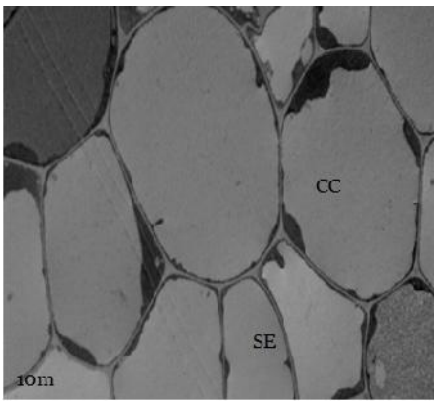

Fig. (7): Neither pinwheels nor laminated aggregates or any cylindrical inclusion appeared in ultrathin sections prepared from healthy control potato sprout.

4. Discussion

Potato is one of the important crops which vegetatively propagated using tubers after suitable storage period. Viruses present in the aerial plant parts are transmitted to the storage part, accumulated in them and passed on to the successive generation. One of the most important strategies in managing losses due to PVY in potato industry is to certify potato seed with low or no virus levels **(Mallik *et al*., 2012)**. No visual symptoms were observed in some potato cultivars infected with mild strains of PVY. Thus, making detection of such strains during inspection of seed potato fields more difficult **(Lorenzen *et al*., 2008; Nie *et al*., 2004).** An efficient method should be able to detect virus infection in those sub-optimal infected plants, which may not show any apparent symptoms. In the present study one step reverse transcription polymerase chain reaction (RT-PCR) based protocol of PVY detection from the potato sprout as a propagative material, was developed. The results clearly showed that one step RT-PCR based protocol, which greatly reduces material and labor costs for PVY detection, is sensitive and efficient in detecting PVY from plant samples including the sprout. As it had been reported, that RT-PCR is a versatile, sensitive and robust diagnostic technique **(Singh, 1998; Ptacek *et al*., 2002 and Vigano and Stevens, 2007)**.

Nucleotide sequence analysis was followed to confirm the results and to introduce the PVY strain. A comparison of the amino acid sequence of the CP with other PVY isolates showed that PVY-Egypt was closely related to PVYNTN strains **(El-absawy *et al*., 2012).** The results of the obtained DNA nucleotide sequences revealed that the coat protein sequences was closely related to PVY strains listed on Gene Bank with similarity reached to 99%. The phylogenetic tree represented in Fig.(3) revealed that the PVY-Egypt isolate was highly similar to the PVY Egypt necrotic isolates (GU550076) and (GU980964) with identity 99%. In addition to the similarity with necrotic strains aligned in the Genbank. This finding is in agreement with other studies reported the spread of PVY necrotic strains during the last decade in Belgium **(Bahrami *et al*., 2010)**, Canada **(Nie *et. al*., 2004)**, Poland **(Przyby *et al*., 2013)**, South Africa **(Visser *et al*., 2009)** and the representative countries in Table (1). The previous results confirmed that the nucleotide sequence determination of the CP is postulated as the basis for identification and classification in the *Potyvirus*genus **(Shukla and ward 1988).**

Cytopathological studies of cylindrical inclusions reported the presence of the cytoplasmic inclusions usually in epidermis and mesophyll cell. Literature indicated that several *potyviruses* induce inclusion, it become an important diagnostic tool to identify the infection similar to those shown in Fig. (4). It's clear to show the presence of cytoplasmic cylindrical inclusion consisting of pinwheels, scrolls and laminated aggregates. The cytoplasmic inclusion bodies concentrated in parenchyma cells in close vicinity to the epidermis in epidermal cells and in trichomes. Typical pinwheels, scrolls, laminated aggregates and endoplasmic reticulum were found in the cytoplasm of infected cells. Transmission electron microscopy is the most useful tool to investigate plant cell ultrastructural as well as direct virus particle observation and its influence on cell organelles, which are closely related to the type of plant-host response to viral infection **(Otulak *et al*., 2014).** The viral inclusion bodies are known to have a characteristic structures and composition according to the virus genus and consequently, constitute a diagnostic characteristic. The main ultrastructural diagnostic criterion of the presence of *potyvirus* like Potato virus Y in the host plant tissues is the presence of viral cytoplasmic inclusions **(Otulak and Garbaczewska, 2012)**. Literature data define these structures as the result of the expression of protein coded by virus genome.

Owing to the large diversity of these deposits, their classification has been attempted. It has been stated that the forms of cytoplasmic inclusion cannot serve as a cytological criterion to distinguish the *Potato virus Y* strains and do not depend on host resistance level. If *Potyvirus* infects a host–plant and is able to multiply itself, it deposits different proteins in tissues of such a plant. Since overproduction of these proteins is so high, which can be observed in ultrastructural analysis, these proteins have to perform a significant function for pathogen. Unfortunately, the knowledge on this issue is still incomplete. **Hari (1995)** suggested that polymerization of surplus *potyvirus* non-structural proteins in the form of cytoplasmic inclusions permits the process of merging particles of virus (linking the genome with protein of capsid), and also rendering virus spread easy in the plant. **Eagles *et al*. (1994), Klein *et al*. (1994) and Lain *et al*. (1990)** claimed that cytoplasmic inclusions protein, being a component of cytoplasmic inclusions, is not only as active as helices during replication but it also assists during inter-cellular transport.. **Wei *et al*. (2010)** show that newly identified protein P3N-PIPO as a plasmodesmata - located protein and directs the cytoplasmic inclusions protein to plasmodesmata, facilitating the deposition of the cone-shaped structures of cytoplasmic inclusions at plasmodesmata by interacting with cytoplasmic inclusions protein. **Wen and Hajimorad (2010)** reported that mutation of the putative SMV (Soybean mosaic virus) PIPO impedes cell-to-cell movement, providing genetic evidence that P3N-PIPO is a potyviral movement protein. The modern TEM with a connected digital camera enables high quality observations of subtle changes in the interior and/or exterior of infected plant cells comparing with healthy ones. Most crucial and invaluable for such

observations are high magnifications. Appropriate magnifications allow detailed assessment and identification of normal and altered cell structures, new elements in cells or even the discernment of different types of viral particles which are varied in size, diameter or length **(Otulak *et al* 2014)**. Finally, methods were used are being investigated through RT-PCR, nucleotide sequencing analysis in addition to ultrastructural studies can use especially RT-PCR technique in detection of large-scale screening of many samples for determining viral incidence in commercial fields or seed lots, and also amenable to use in smaller-scale research applications and the using sequencing analysis in addition to TEM can use to confirm and increase.

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How to cite this article:
Sahar A.Youssef, Eslam Arafa, Gehan Safwat and A.A. Shalaby. (2016). Molecular Characterization and Cytopathological Studies of *Potato Virus Y* Isolated from Potato Sprout in Egypt. Int. J. Adv. Res. Biol. Sci. 3(4): 194-204.