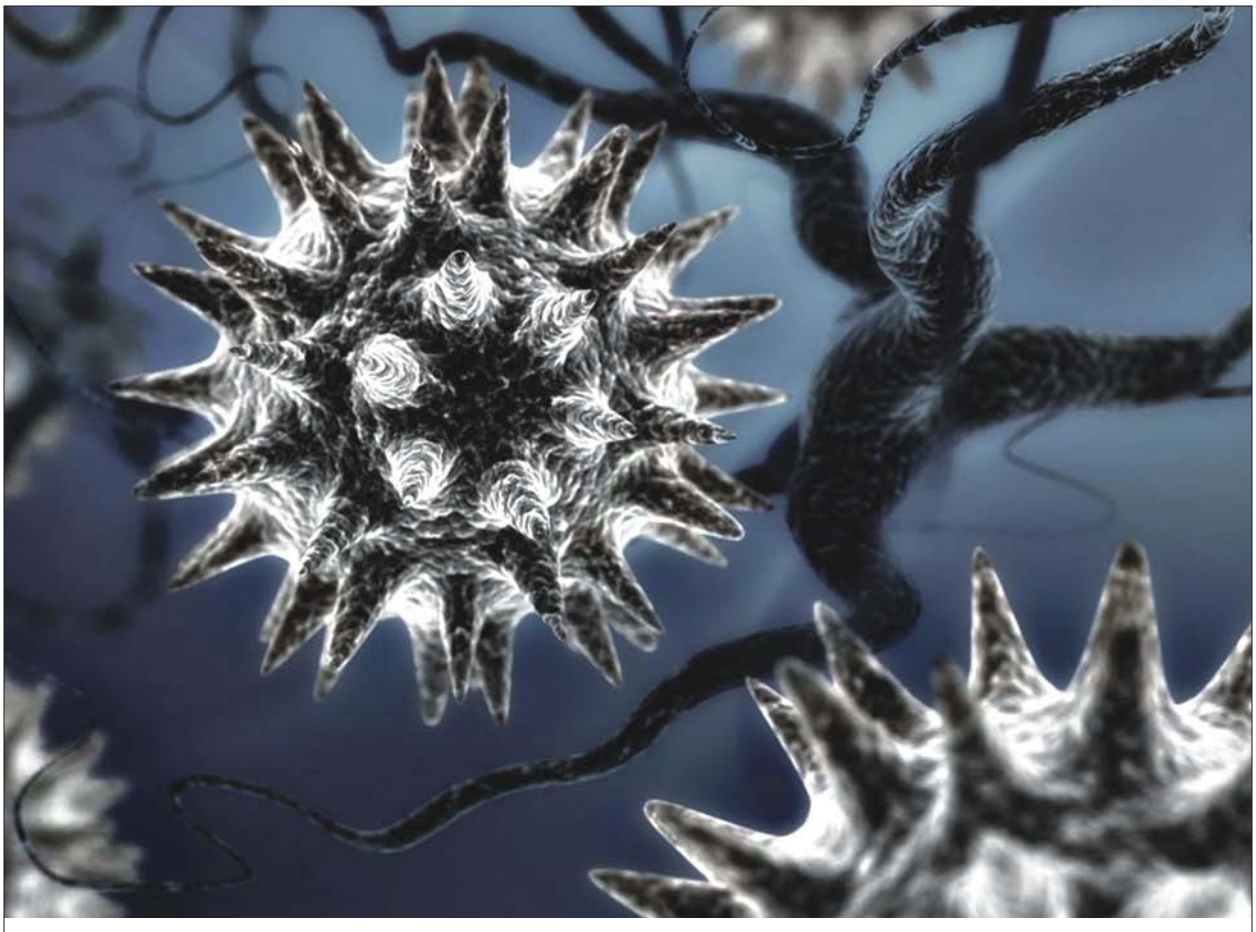


International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology, 5 (2): 77-88, 2009
ISSN 1816-4900

Biological, Serological and Molecular Diagnosis of Three Major Potato Viruses in Egypt

[1]W.S. El-Araby, [2]I.A. Ibrahim, [3]A.A. Hemeida, [2]Amal Mahmoud,
[1]A.M. Soliman, [1]A.K. El-Attar and [1]H.M. Mazyad
[1]Agriculture Research Center, Viruses and Phytoplasma Research Section,
Plant Pathology Research Institute, Egypt
[2]Department of Plant Biotechnology,
Genetic Engineering and Biotechnology Research Institute,
Munofia University, Sadat City, Munofia, Egypt
[3]Department of Bioinformatics,
Genetic Engineering and Biotechnology Research Institute Munofia University,
Sadat City, Munofia, Egypt

Abstract: Viruses are major diseases affecting potato plantations in Egypt. Different molecular and serological methods for virus detection were used to detect the Potato virus X (PVX), Potato virus Y (PVY) and Potato leaf roll virus (PLRV). ELISA, RT-PCR, immunocapture RT-PCR (IC-RT-PCR) and RT-PCR-ELISA were the detection methods used. Naturally virus-like infected potato samples were collected from different locations at El-Munofia Governorate in Egypt. PVX and PVY were isolated and identified biologically through host range and indicator plants. All detection methods mentioned above were sensitive enough to detect the three potato viruses in potato tissues. Using ELISA method, PVX, PVY and PLRV were detected in 4, 7 and 10 out of 26 samples (15.4, 26.9 and 38.5%), respectively. Mixed infection was found between PVX/PVY; PVY/PLRV and also among PVX/PVY/PLRV. According to the results, it is the use of molecular methods to confirm certain serological results i.e., the virus especially when low concentration in the infected samples. Using the advantage of IC with RT-PCR-ELISA will reduce the costs and avoid RNA degradation. Additionally, RT-PCR-ELISA could be the method of choice among the molecular methods.

Key words: Molecular diagnosis, serological methods, PVX, PVY, PLRV, ELISA, IC-RT-PCR, RT-PCR-ELISA

INTRODUCTION

Potato (*Solanum tuberosum* L.) is used worldwide for human and animal consumption and as raw material for starch and alcohol production. Nowadays, one of the most important aspects of potato production is tuber quality that includes biological traits (Carputo *et al*., 2005).

In Egypt, potato is one of the most important and economic vegetable crop. The total potato harvested area of Egypt reached about 100,000 ha; this area produced 2,500 tons ha^{-1} with an average of 25 tons ha^{-1} (FAO, 2006). Potato suffers from many diseases, which decrease the production of this crop. A wide range of diseases, including fungal, bacterial, viral and nematode diseases, impacts potato crop. More than 25 different viruses or virus diseases have been reported and all are pathogenic to potato (Salazar, 1977). Among several factors responsible for the low potato production, potato diseases like early and late blights, potato scab, black scurf and viral diseases i.e., PVX, PVY and PLRV are most important (Qamar and Khan, 2003).

Coresponding Author: W.S. El-Araby, Agriculture Research Center, Viruses and Phytoplasma Research Section, Plant Pathology Research Institute, Egypt

PLRV (genus *Polerovirus*, family Luteoviridae), like all other *luteoviruses* has a monopartite, single stranded RNA genome. PLRV is transmitted by aphids in a circulative non-propagative manner and is mainly restricted to phloem tissues of infected plants (Mayo and Ziegler-Graff, 1996). PLRV has genomic RNA of ~5.9 kb that contains six large Open Reading Frames (ORFs). A 5'-located gene cluster contains three ORFs that code for a polypeptide of 28 kDa with unknown functions (ORF0) and two proteins of 70 kDa (ORF1) and 108 kDa protein (ORF1/2) that appear to be replication-associated proteins (Mayo and Ziegler-Graff, 1996). Within the 3'-located gene cluster, ORF3 encodes the 23 kDa Coat Protein (CP). ORF4, which encodes a 17 kDa product (P4), is contained within the *CP* gene, but in a different reading frame. P4 is thought to be a movement protein (Tacke *et al*., 1993). PLRV ORF5 is separated from the upstream CP gene by a single amber termination codon.

PVY is an important pathogen in solanaceous crops. It belongs to the genus *Potyvirus*, of which it is the type species, in the plant virus family Potyviridae (Shukla *et al*., 1994). PVY has a single positive-sense genomic RNA ~10 kb long and forms flexuous virions. The genomic RNA contains a unique ORF encoding a polyprotein which is processed into functional viral proteins by virus-encoded proteases (P1, HC-Pro and NIa) (Riechmann *et al*., 1992). It is naturally transmitted by aphids in a non-persistent manner with great efficiency, causing epidemics in potato, tomato, pepper, tobacco and other solanaceous plants (De Bokx and Huttinga, 1981). The *CP* gene is the gene most frequently used for studies of genetic diversity in potyviruses (Shukla *et al*., 1994).

PVX, the prototype member of the potexvirus genus, is a rod-shaped virus containing single stranded RNA which is capped, polyadenylated and contains five ORFs (Huisman *et al*., 1988). Open Reading Frame (ORF) 1 encodes a 166 kDa protein which functions as a replicase. ORFs 2, 3 and 4 encode proteins of 25, 12 and 8 kDa, respectively and are known as the triple gene block. These gene products are thought to be involved in cell-to-cell movement of the virus. The fifth ORF encodes the CP, which is 25 kDa in size (Hefferon *et al*., 1997). PVX is readily transmitted by contact of plant parts in the field and by the cutting knife before planting (Bostan and Haliloglu, 2004).

Attempts to produce virus-free, high-yielding planting material are being made around the world by many laboratories, a prerequisite of which is the availability of accurate and rapid methods for detection of very low levels of virions and viral RNA molecules, for this reason, extensive research has been undertaken into potato viruses.

In Egypt, PVX, PVY and PLRV are common virus diseases of potato. Infections with these viruses may result in the loss of certification for potato seed and affect quality and yield in commercial production. In this study, we present biological, serological and molecular diagnostic tools to detect and differentiate potato viruses (PVX, PVY and PLRV).

MATERIALS AND METHODS

Source of Viruses

Field collected potato samples showed positive results against PVX, PVY and PLRV using serological method were used as source of viruses. *Datura stramonium* and *Datura metel* were mechanically inoculated with PVX and PVY crude sap, respectively.

Host Range and Symptomatology

Sixteen plant species belonging to four families (Amaranthace, Chenopodiacea, Fabaceae and Solanaceae) were mechanically inoculated with crude sap of PVX or PVY. Four seedlings of each host plant were inoculated and observed daily for symptoms development.

Serological Diagnosis of Potato Viruses

PVX, PVY and PLRV were tested in the field-collected samples using ELISA. PVX and PLRV were tested using direct ELISA methods (DAS-ELISA) (ELISA kit, Sanofi, Sante Animal, Paris France). PVY was tested using indirect ELISA method (DAC-ELISA) according to Mahmoud *et al*. (1996).

Molecular Diagnosis

RNA Extraction

Total RNA was extracted from naturally infected and non-infected potato tissues using two methods: CTAB method described by Gibbs and Mackenzie (1997) with some modifications and RNA Reagent kit (Biobasic, cat No. BS410) according to manufacturer's instructions.

Complementary DNA (cDNA) Synthesis

Complementary DNA was prepared by mixing 2 µg of total RNA, 1.5 µL of 10 µM of the complementary primers (pPVXc2, PVYCPcEcoRI and pPLRVc2, Table 1) and nuclease-free water to a final volume of 15 µL. The reaction tubes were heated at 70°C for 5 min and then the tubes were cooled on ice. The following components were added to the annealed primer/template: 5 µL of 5X M-MLV reaction buffer [250 mM of Tris-HCl (pH 8.3), 375 mM of KCl, 15 mM of $MgCl_2$ and 50 mM of DTT (dithiothretol)], 2 µL of 10 mM dNTPs, 25 units of RNasin® ribonuclease inhibitor and 200 units of Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) enzyme (Promega) and nuclease-free water to final volume of 25 µL. The tubes were mixed gently by flicking the tubes and incubated for 60 min at 37°C.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 µL of 2.5 µL of cDNA, 2.5 µL of 2.5 mM of dNTPs, 2.5 µL of 10X buffer, 2.5 µL of 25 mM $MgCl_2$, 1 µL of each forward and reverse primer at 10 µM, 0.2 µL Taq DNA polymerase and water. Mixtures were incubated for 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final incubation of 10 min at 72°C and then the reaction was held at 4°C. Five microliter aliquots were separated by electrophoresis in 1 and 1.5% agarose gel in 0.5X TBE buffer using 100 bp DNA ladder (Gibco BRL, cat No. 15628-019) and 100 bp+1.5 Kb DNA ladder (SibEnzyme, cat No. M23). The gel was visualized with UV illumination using Gel Documentation System (Gel Doc 2000, BioRad).

Immunocapture RT-PCR (IC/RT-PCR)

A volume of 100 µL of PVX, PVY and PLRV antisera was diluted to 1: 500 in coating buffer (0.035 M sodium, bicarbonate and 0.015 M sodium carbonate, pH. 9.6). Then was added to each PCR tubes and incubated at 4°C overnight. Polymerase Chain Reaction (PCR) tubes were washed with Phosphate-Buffered Saline-Tween (PBS-T) buffer, pH 7.4. Infected leaves were homogenized with grinding buffer (PBS-T, 2 % PVP).

The homogenate (100 µL) was added to the antiserum-precoated PCR tube and incubated at 37°C for 3 h. PCR tubes were washed 3 times then, dried and 10 µL of distilled water were added and boiled at 100°C for 5 min. Five microliter of immunocapture reaction was used directly into RT-PCR. Finally, 5 µL of the products were analyzed by electrophoresis as described before.

Table 1: Designed primers for RT-PCR and capture cDNA probes

Primers' name	Polarity	Nucleotides sequences	Fragm. size (bp)	Ref.
pPVXv1	S	5` GAYACNATGGCNCARGCNGCNTGG 3`	300	Soliman
pPVXc2	AS	5` YTGNGCNGCRTTCATYTCNGCYTC 3`		*et al.*(2000)
pPVXp3 Biotin	S	5` Bio GCN CCN GTN GTN TGG AAY TGG 3`		
PVYCPv*BamH*I	S	5` TCAAGGATCCGCAAATGACACAATTGATGCAGG 3`	801	Shalaby
PVYCPc*EcoR*I	AS	5` AGAGAGAATTCATCACATGTTCTTGACTCC 3`		*et al.*(2002)
pPLRVv1	S	5` GTNCARCCNGTNGTNATGGTNAC 3`	420	Shalaby
pPLRVc2	AS	5` RTGCCAYTCNACNCCRTTDATCAT 3`		*et al.*(2002)
pPLRVp4 Biotin	S	5` Bio GAYTGYCCNGCNTTYAARGAYGG 3`		

P: Primer; v1: Viral sense primer; c2: Complementary sense primer; p3 Biotin: Biotin labeled viral cDNA capture probe; p4 Biotin: Biotin labeled viral cDNA capture probe; S: Sense; AS: Antisense. Nucleotide at degenerate positions are represented by a single letter of the IUPAC ambiguity code, D = A, G, T; H = A, C, T; K = G, T; M = A, C; N = A, C, G, T; R = A, G; W = A, T; Y = C, T

RT-PCR-ELISA

Dig-Labeling

DIG-labeled DNA probe was prepared by PCR, using the primers pPVXv1 and pPVXc2. PCR was carried out in a 50 µL reaction mixture containing Tris-HCl (pH 8.4), 20 mM, KCl 50 mM, $MgCl_2$ 1.5 mM, dGTP, dATP, dCTP each 0.1 mM, dTTP 0.065 mM, DIG-dUTP 0.035 mM (Boehringer Mannheim Corp., Indianapolis, IN, USA, cat. No. 1636120), primers 1 µL each, Taq DNA polymerase 2 units (Gibco-BRL) and 2.5 µL template DNA.

Preparation of Biotin-Labeled cDNA Capture Probe

DNA oligonucleotides (21-23 nt in length; capture probe primers, Table 1) were synthesized and biotinylated at Life Technologies, Inc. The DNA sequences of capture probes were complementary to the internal nucleotide sequence of amplified DNA.

PCR-DIG Detection

Two hundred microliters of each mixture were pipetted into an ELISA microtiter plate well precoated with streptavidin and was covered with self adhesive tape (Scotch™, St. Paul, MN, USA). Microtiter plate was kept in a shaking water bath at 55°C for 3 h. The biotin-labeled capture probe was added to the hybridization buffer to a final concentration of capture probe of 7.5 nM. The hybridization solution was removed and the wells were washed six times with washing solution. Two hundred microliter of polyclonal anti-DIG Fab fragments conjugated to peroxidase diluted 1:100 in Tris-HCl (pH 7.5) were added to each well and the microtiter plates were shaken gently at 37°C for 30 min. Wells were washed six times with washing solution.Two hundred microliter of substrate solution were added to each well and microtiter plates were incubated for 30 min at 37°C in the dark. The absorbances of hybridized products were measured at 405 nm in an ELISA-reader (ELX 800, Biotek Instruments, Inc.).

RESULTS

Samples collected from naturally infected potato plants showed variable symptoms including stunting, vein clearing, severe and mild mosaic, yellowing, deeping veins, necrosis, leaf cup shape and crinkling (Fig. 1). Obtained samples were primary investigated using ELISA techniques to direct this research on the three major potato viruses (PVX, PVY and PLRV) some of positive plants were used in further studies (Table 2).

Mechanical Transmission

PVX and PVY symptoms appeared after 28 days of incubation period. PVX-inoculated plants (*N. tabaccum* and *D. stramonium*), showed sever mosaic, vein banding, mild mosaic, while PVY-inoculated plants (*N. tabaccum* and *D. metel*) showed veinal necrosis and severe mosaic.

Host Range and Symptomatology

Sixteen plant species belonging to four families were mechanically inoculated with PVX and PVY (Table 3). The tested plants showed different symptoms and serologically reacted against PVX and PVY antisera. Mild and severe mosaic, vein banding, leaf malformation and mottling symptoms appeared on different hosts after 28 days incubation period. External local lesions appeared on *Gompherena globosa* L. and *Chenopodium amaranticolor* for PVX and PVY, respectively. Severe mosaic was the most severe symptoms appeared on *Nicotiana tabaccum* cv. White burley for PVX and PVY. Variable symptoms (vein banding and mild mosaic) appeared on *D. stramonium* for PVX and *D. metel* (veins clearing, severe mosaic, malformation and leaf cup shape) for PVY. The following hosts were inoculated with PVX, they did not show any symptoms and no serological reaction was detected against PVX antiserum: Leguminaceae: *Vacia faba* cv. Giza 67,

Fig. 1: Naturally infected potato plants showing different potato viral symptoms. (a) Severe mosaic, (b) Yellowing, © Deeping veins, crinkle and necrosis, (d) Mild mosaic, (e) Leaf roll and (f) Healthy potato

Table 2: Detection of PVX, PVY and PLRV from naturally infected potato samples using DAS-ELISA and DAC-ELISA (A 405 nm)

No. of samples	Visual symptoms	PVX antiserum	PVY antiserum	PLRV antiserum
1	Veinal necrosis	0.095[1]	0.217	0.021
2	Severe mosaic	0.100	0.156	0.069
3	Mild mosaic	0.108	0.108	0.092
4	No symptoms	0.146[2]	0.134	0.027
5	Leaf curl and mosaic	0.102	0.126	0.051
6	Severe mosaic and leaf curl	0.126	0.125	0.141
7	Mosaic, yellowing	0.097	0.116	0.021
8	No symptoms	0.090	0.148	0.013
9	Mild mosaic, yellowing	0.087	0.212	0.049
10	Mild mosaic, leaf curl	0.048	0.139	0.165
11	Severe mosaic	0.413	0.153	0.017
12	Mosaic	0.096	0.244	0.058
13	Veinal necrosis	0.090	0.108	0.008
14	Mild mosaic, crinkle	0.085	0.112	0.127
15	No symptoms	0.098	0.103	0.019
16	Mosaic	0.094	0.103	0.021
17	Mild mosaic	0.074	0.104	0.033
18	Veinal necrosis, leaf curl and mosaic	0.074	0.569	0.011
19	Mild mosaic, yellowing and leaf curl	0.063	0.147	0.105
20	Severe mosaic	0.075	0.110	0.016
21	No symptoms	0.075	0.121	0.068
22	Mild mosaic	0.082	0.476	0.194
23	Mosaic, yellowing and leaf curl	0.098	0.175	0.023
24	Veinal necrosis	0.416	0.244	0.020
25	Veinal necrosis	0.084	0.094	0.014
26	Leaf curl	0.091	0.171	0.058
Positive control[3]	Severe mosaic	3.229	0.638	1.930
Negative control[4]	Negative samples	0.058	0.087	0.027

1: The mean A 405 nm value corresponds to 2 replicates, 2: Positive threshold = 2 times the mean A405 nm value of negative controls, 3: Potato leaves infected with PVX, PVY and PLRV reacted positively with PVX, PVY and PLRV antisera, respectively, 4: Healthy potato leaves

Phaseolus vulgaris cv. Potima; Solanaceae: *Capsicum annuum* L. cv. California wonder, *D. metel* L., *Lycopersicon esculentum* cv. Castle rock, *Physalis floridana* L., *Ch. amaranticolor*, *Ch. Quinoa* and

Table 3: Host range, symptoms and ELISA reaction of PVX and PVY after mechanical inoculation

Family	Species	Cultivars	PVX-observed symptoms	$A_{405\ nm}$-PVX antiserum	PVY-observed symptoms	$A_{405\ nm}$-PVY antiserum
Amaranthace	*Gomphrena globosa* L.		Local lesions	0.412[1] (0.061)[2]	No symptoms	0.049(0.051)
Chenopodiacea	*Chenopodium amaranticolor* L.		No symptoms	0.029 (0.031)	Local lesions	1.555(0.090)
	Chenopodiumalbum L.		No symptoms	0.034 (0.021)	No symptoms	0.018(0.011)
	Chenopodium quinoa Wild.		No symptoms	0.061 (0.040)	No symptoms	0.071(0.050)
Fabaceae	*Phaseolus vulgaris*	Potima	No symptoms	0.052 (0.034)	Vein banding, mosaic	0.199(0.042)
	Vacia faba	Giza 67	No symptoms	0.015 (0.053)	No symptoms	0.047(0.036)
Solanaceae	*Capsicum annuum* L.	California wonder	No symptoms	0.049 (0.042)	Mild mosaic	0.181(0.054)
	D. metel L.		No symptoms	0.090 (0.087)	Vein clearing, sever mosaic, malformation, leaf cup shape	2.101(0.062)
	Lycopersicon esculentum Mill	Castle rock	No symptoms	0.310 (0.092)	Mild mosaic	0.042(0.035)
	Physalis floridana L.		No symptoms	0.150 (0.079)	Necrotic local lesions, leaf falling, mosaic	0.190(0.073)
	Nicotiana benthamiana L.		Mild mosaic	0.069 (0.060)	No symptoms	0.218(0.051)
	N. glutinosa L.		Mosaic	0.299 (0.021)	Mild mosaic	0.809(0.079)
	N. rustica L.		Mosaic	0.199 (0.061)	Mosaic	0.989(0.024)
	N. tabacum. L.	White burley	Sever mosaic	0.155 (0.050)	Vein clearing, Mosaic	0.144(0.049)

1 and 2: Correspond to PVX-infected and healthy samples, respectively

ch. album (Table 3). Also, some hosts inoculated with PVY, did not show any symptoms or serological reaction against PVY antiserum. These hosts were as follow: *Vicia faba* L. cv. Giza 2, *N. benthamiana* L., *Ch. quinoa* Wild, *Ch. album* L., *Gompherna globosa* and *D. staramonium* L.

Serological Diagnosis of Potato Viruses

Twenty six samples of naturally infected potato were serologically tested against PVX and PLRV antisera using direct ELISA method (DAS-ELISA) while, PVY antiserum using indirect ELISA method (DAC-ELISA). PVX and PLRV were detected in 4 and 10 out of 26 samples (15.4 and 38.5%), respectively. However, PVY was detected in 7 out of 26 samples 26.9%, (Table 2).

Immunocapture RT-PCR (IC/RT-PCR)

Immunocapture RT-PCR was used for the detection of PVX, PVY and PLRV in potato plants. IC-RT-PCR amplified products were ~300, 801 and 420 bp for PVX, PVY and PLRV, respectively (Fig. 2).

Molecular Diagnosis

Some of the serologically screened samples were tested at the molecular level. Total RNA was successfully extracted from healthy and infected plants with PVX, PVY and PLRV.

RT-PCR

PCR products showed that the amplified fragments of ~300 and 420 bp were obtained for the partial coat protein genes of PVX and PLRV, respectively. While, 801 bp was obtained for the coat protein gene of PVY and no fragments were amplified from the RNA extracted from healthy plants (Fig. 3).

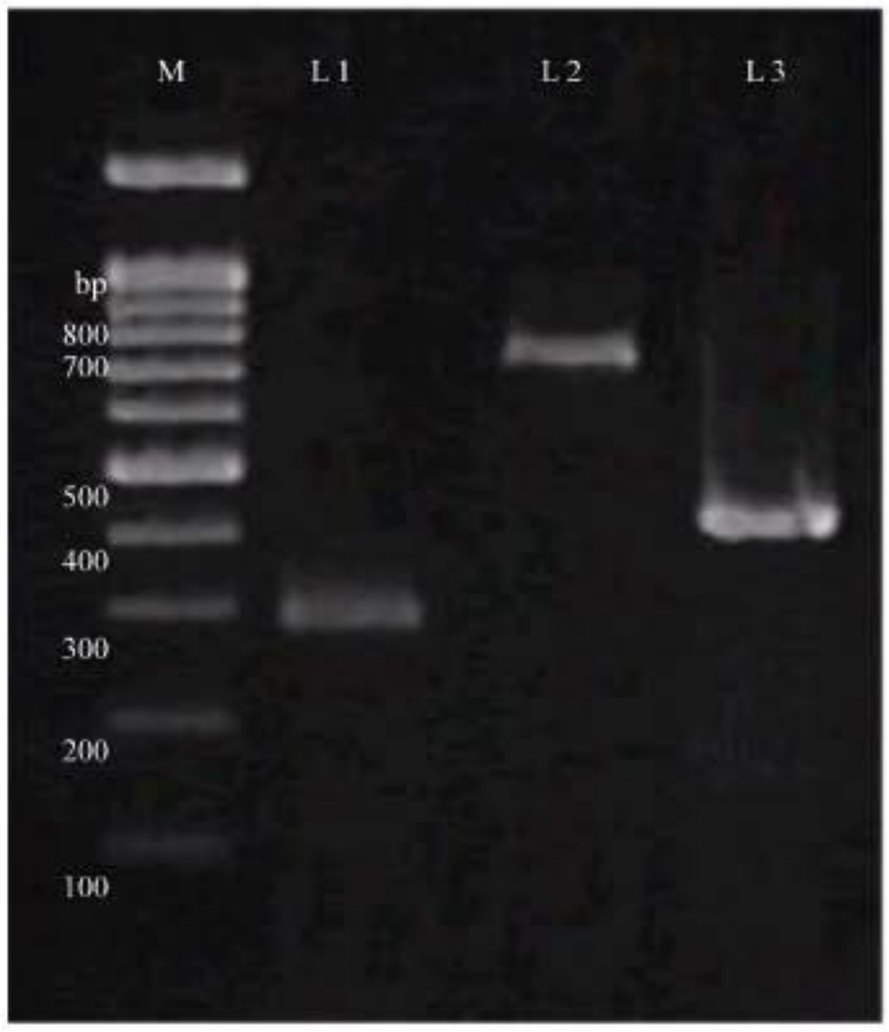


Fig. 2: Agarose gel electrophoresis analysis of IC-RT-PCR of PVX, PVY and PLRV. M: 100 bp DNA ladder; L1: IC-PCR of PVX infected sample with partial CP primer; L2: IC-PCR of PVY infected sample with CP primer and L3: IC-PCR of PLRV infected sample with CP primer

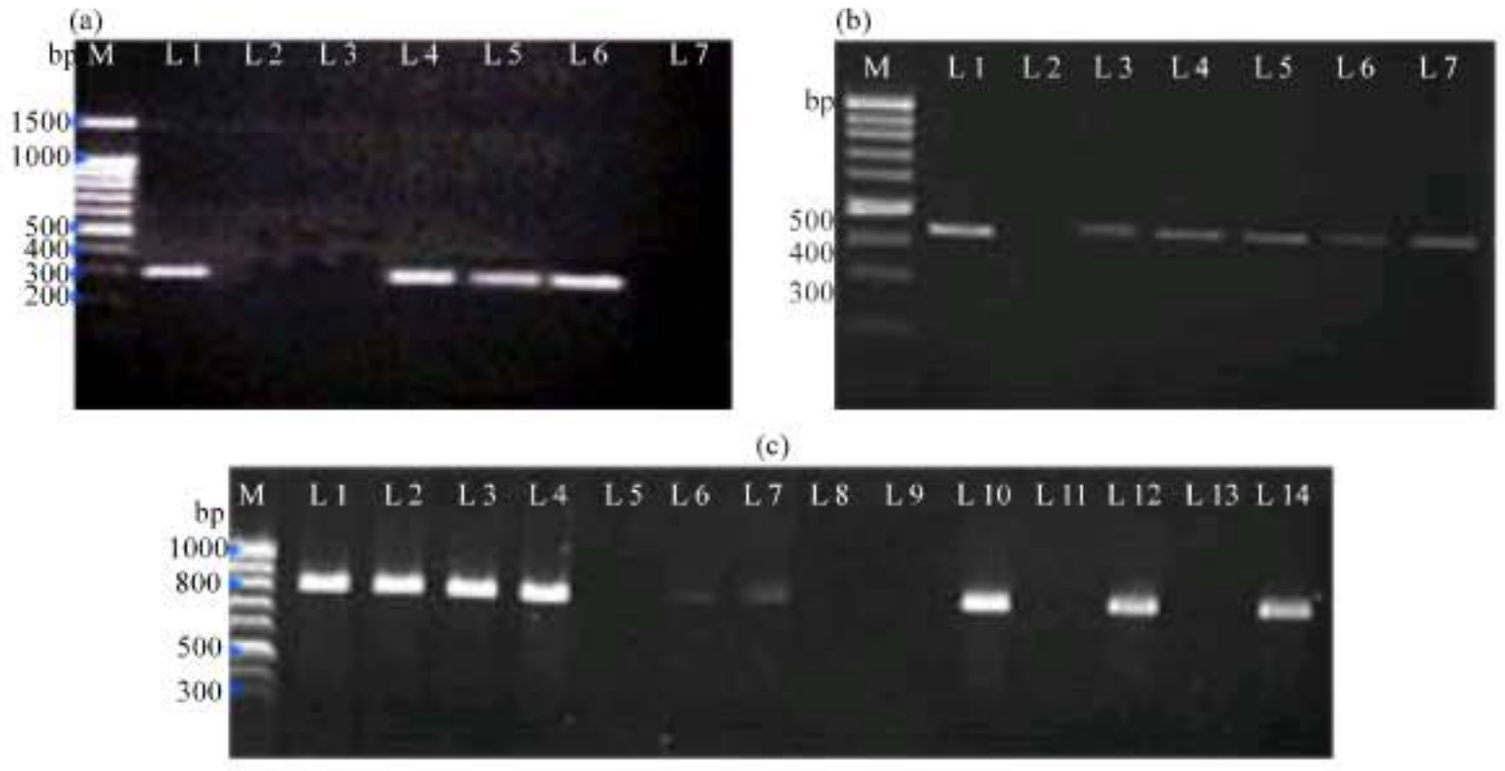


Fig. 3: Agarose gel electrophoresis analysis of RT-PCR amplified products of (a and b) PVX and PLRV, respectively. M: 100 bp DNA ladder; L1: Positive control; L2: Negative control; L 3 to L 7: Potato samples collected from field and © PVY. M: DNA ladder; L1: PVY-positive control; L2, L3 and L4: Three samples positive ELISA and PCR; L5: Negative control; L6 and L7: Two samples negative ELISA but positive PCR; from L8 to L14: Potato samples collected from field

RT-PCR-ELISA

DIG-labeled RT-PCR amplified cDNAs of PVX and PLRV from infected potato leaves were analyzed by agarose gel electrophoresis (Fig. 4). The DIG-labeled PVX-cDNA and PLRV-cDNA product was detected up to 10^{-5} dilution (Fig. 5). Color development was absent with products from healthy tissues, or buffer control samples.

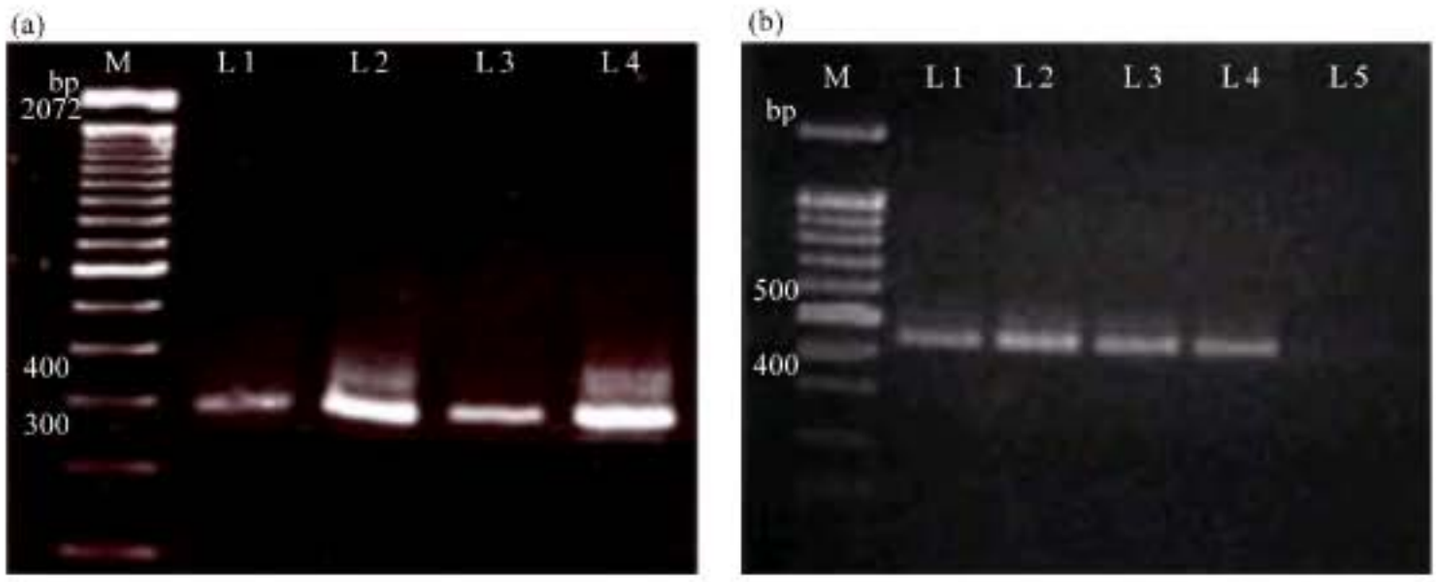
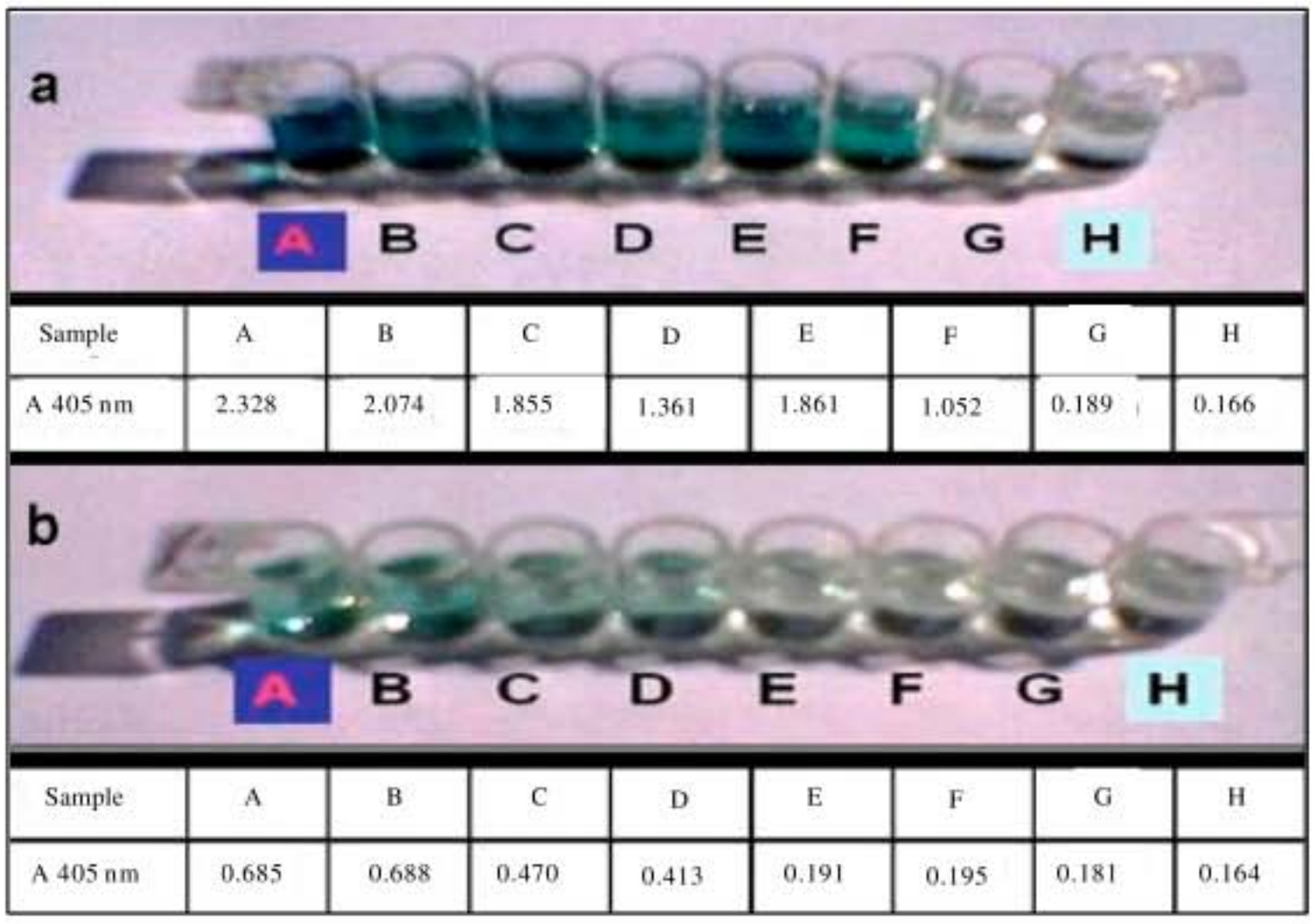


Fig. 4: Agarose gel electrophoresis analysis of (a) PVX and (b) PLRV DIG-labelled RT-PCR. M: 100 bp DNA ladder; L1 to L4: DIG-labelled RT-PCR amplified product from PVX or PLRV infected plants and L5 (b) Negative control



Sample	A	B	C	D	E	F	G	H
A 405 nm	2.328	2.074	1.855	1.361	1.861	1.052	0.189	0.166

Sample	A	B	C	D	E	F	G	H
A 405 nm	0.685	0.688	0.470	0.413	0.191	0.195	0.181	0.164

Fig. 5: Colorimetric detection of (a) DIG-labelled PLRV and (b) PVX-cDNA (b) products as shown by absorbance values of each assay. A: Positive control, B to G: Potato samples and H: Negative control

DISCUSSION

Mechanical Transmission

Mechanical transmission by sap inoculation to herbaceous indicator plants can be done with minimal facilities and characteristic symptoms produced by these plants allow both the detection and identification of many viruses (Horvath, 1993).

Host Range and Symptomatology

Visual inspection is relatively easy when symptoms clearly are characteristic of a specific disease. However, many factors such as virus strain, host plant cultivar/variety, time of infection and the environment can influence the symptoms exhibited (Matthews 1980). Obtained data are similar to those obtained by Salazar (1977) and Soliman *etal*. (2002). Systemic symptoms were appeared on *N. tabacum* L. cv. White Burley; *N. glutinosa* L.; *N. rustica* L. and (*Solanum tuberosum* L. cvs. Nicola and Spunta). On the other hand, *Phaseolus vulgaris* L., *Chenopodium quinoa* wild, *Chenopodium amaranticolor* L. and *Chenopodium album* L. are not reacted positively against PVX infection under greenhouse conditions. Similar results were also recorded by Yousef (1992) and Salazar (1977).

Serological Diagnosis

Many investigators have used ELISA test for serological diagnosis of PVX, PVY and PLRV, (Varveri, 2006; Li *et al*., 2006; Boukhris-Bouhachem *et al*., 2007).

Molecular Diagnosis

The most common method for the detection of plant viruses is ELISA because it is rapid and inexpensive. However, the serological methods have the drawbacks of limited availability of antisera and questions regarding the specificity of antisera produced from preparations containing virus mixtures. Raising antisera is also time-consuming. By contrast, a diagnostic technique using RT-PCR can be rapidly implemented in independent laboratories after the basic protocol and primer sequences are made available. However, RT-PCR requiring separate amplification of each virus of interest are potentially expensive and resource intensive (Peiman and Xie, 2006).

A technique that combines the technical advantages of PCR with the practical advantages of ELISA, called immunocapture (IC)-PCR, was developed for the detection of several different plant viruses (Nolasco *et al.*, 1993). The benefits of using IC in with RT-PCR to remove inhibitory plant compounds and to make nucleic acid extractions unnecessary have been well recognized (Harper *et al*., 1999).

Colorimetric detection of hybridized PCR products combine the specificity of DNA probe hybridization, the sensitivity of colorimetric or luminescent detection and the ease of sample handling in microplates (Marinho *et al*., 2003). Comparison of sensitivity among ELISA, PCR, IC-PCR and ELISA-PCR for PVX, PVY and PLRV is showing in Table 4. Using ELISA method, PVX, PVY and PLRV were detected in 4, 7 and 10 out of 26 samples (15.4, 26.9 and 38.5%), respectively. Mixed infection was detected between PVX, PVY and PLRV (e.g., samples 3-6 for double infection and 2, 9 and 12 for mixed infection with the three viruses. The serologically positive samples were confirmed as positive by the molecular methods. Except for samples no 1 and 22, PVY was detected in these 2 samples using ELISA and PCR-ELISA but not using RT-PCR. The negative results obtained by RT-PCR could be due to RNA degradation during extraction. Also, certain serologically negative samples (no virus was detected) were shown to be positive using the molecular methods. The absorbance at 405 nm for these samples was higher than the absorbance of the healthy control but less than the threshold (more than twice the healthy control). In a comparison between ELISA and the

Table 4: Comparison among ELISA, PCR, IC-PCR and RT-PCR-ELISA

	PVX				PVY			PLRV			
No.	ELISA	RT-PCR	IC-RT-PCR	RT-PCR-ELISA	ELISA	RT-PCR	IC-RT-PCR	ELISA	RT-PCR	IC-RT-PCR	RT-PCR-ELISA
1	0.095	-		-0.195[1]	0.217	-		0.021			
2	0.100	+			0.156	+faint		0.069	-		+2.074
3	0.108	+			0.108	-		0.092	+		
4	0.146[2]	-		+0.470	0.134	+		0.027			
5	0.102	-		-0.191	0.126	+		0.051	+		
6	0.126	+		+0.413	0.125			0.141			
7	0.097	+			0.116			0.021			
8	0.090				0.148	+		0.013			
9	0.087	+			0.212	+		0.049	+faint		+1.855
10	0.048				0.139			0.165	+	+	+2.328
11	0.413	+		+0.688	0.153	+		0.017			
12	0.096	+		-0.181	0.244	+		0.058			
13	0.090	+			0.108	-		0.008			
14	0.085	+			0.112	+		0.127			
15	0.098				0.103			0.019	-		+1.861
16	0.094				0.103			0.021			
17	0.074				0.104	+		0.033	+		+1.361
18	0.074				0.569			0.011	-		-0.189
19	0.063				0.147			0.105			
20	0.075				0.110			0.016	+		
21	0.075				0.121			0.068			
22	0.082	+			0.476	-		0.194			
23	0.098				0.175	+Sharp	+	0.023	+		+1.052
24	0.416	+	+	+0.685	0.244			0.020	+		
25	0.084	+			0.094			0.014			
26	0.091	+			0.171	+		0.058			
*	3.229				0.638			1.930			
**	0.058	-		-0.164	0.087			0.027	-		-0.1660

1: The mean $A_{405\ nm}$ value corresponds to 2 replicates, 2: Positive threshold = 2 times the mean $A_{405\ nm}$ value of negative controls, *: Potato leaves infected with PVX, PVY and PLRV reacted positively with PVX, PVY and PLRV antisera, respectively, (Positive control), **: Healthy potato leaves (Negative control)

other molecular methods, ELISA have the advantage of low coasts and less precautions, however, the molecular methods are more sensitive. According to present data the method works well when the virus is in a high enough concentration in the infected samples. Accordingly, we advice the use of molecular methods to confirm certain serological results i.e., the virus is in low concentration in the tested samples. According to the results in Table 4, RT-PCR-ELISA could be the method of choice among the molecular methods. Using the advantage of IC with RT-PCR-ELISA, this will reduce the coasts and avoid RNA degradation.

The results obtained in this study demonstrated that the successful use of RT-PCR-ELISA to detect, directly, plant viruses in sap extract from infected leaf tissue and indicated its feasibility as a rapid laboratory assay for detecting PVX and PLRV. No more than 10 h are required for positive identification of the virus from infected tissue. RT-PCR-ELISA using a microtiter plate has reported, recently, for the detection of the plant viruses and viroids: potato virus Y (Hataya *et al*., 1994), tomato spotted wilt (Weekes *et al*., 1996), plum pox (Olmos *et al*., 1997) and potato spindle tuber (Shamloul and Hadidi, 1999). Moreover, PCR-ELISA has been reported to be sensitive and specific for the detection of the targeted DNA (Kawai *et al*., 1994; Shamloul *et al*., 2001). Besides, the safety of non-radioisotopic detection, the method of microtiter well hybridization has advantages of speed, suitability for a large number of samples, visual examination and adaptation to automation (Shamloul and Hadidi, 1999). The detection of DIG-labelled RT-PCR products by microwell capture hybridization assay is more sensitive than the sensitivity of analysis of the amplified product by agarose gel electrophoresis. Consequently, the use of ethidium bromide is no longer needed.

Colorimetric PCR uses microtiter plates and ELISA plate reading equipment, thus conferring two additional advantages. First, as the PCR and hybridization steps become more standardized, it will be relatively simple to perform quantitative analysis of viral infection rates within plants. Second, many virus detection facilities already use microtiter plates and plate reading equipment, as a result, it should be fairly easy for old facilities to convert to this new assay. Existing equipment can be re-used and technicians already know some of the techniques used in colorimetric PCR (Rowhani *et al*., 1996).

REFERENCES

Bostan, H. and K. Haliloglu, 2004. Distribution of PLRV, PVS, PVX and PVY (PVY^N, PVY^o and PVY^c) in the seed potato tubers in Turkey. Pak. J. Biol. Sci., 7: 1140-1143.

Boukhris-Bouhachem, S., M. Hulle, J. Rouze-Jouan, L. Glais and C. Kerlan, 2007. Solanum elaeagnifolium, a potential source of *Potato virus* Y (PVY) propagation. Bull. EPPO, 7: 125-128.

Carputo, D., R. Aversano and L. Frusciante, 2005. Breeding potato for quality traits. Acta Horticul., 684: 55-64.

De-Bokx, J.A. and H. Huttinga, 1981. *Potato virus* Y. CMI/AAB DESCRIPTIONS of Plant Viruses, No. 242.

FAO, 2006. FAOSTAT. Food and Agriculture Organization, Rome, Italy.

Gibbs, A. and A. Mackenzie, 1997. A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. J. Virol. Methods, 63: 9-16.

Harper, G., G. Dahal, G. Thottappilly and R. Hull, 1999. Detection of episomal banana streak badnavirus by IC-PCR. J. Virol. Methods, 79: 1-8.

Hataya, T., A. Inoue and K.E. Shikata, 1994. A PCR-microplate hybridization method for plant virus detection. J. Virol. Methods, 46: 223-236.

Hefferon, K.L., H. Khalilian, H. Xu and M.G. AbouHaidar, 1997. Expression of the coat protein of *Potato virus* X from a dicistronic mRNA in transgenic potato plants. J. Gen. Virol., 78: 3051-3059.

Horvath, J., 1993. Host Plants in Diagnosis. In: Diagnosis of Plant Virus Diseases, Matthews, R.E.F. (Ed.). CRC Press, Boca Raton, Florida, USA., pp: 15-48.

Huisman, M.J., H.J.M. Linthorst, J.F. Bol and B.J.C. Cornelissen, 1988. The complete nucleotide sequence of *Potato virus* X and its homologies at the amino acid level with various plus-stranded RNA viruses. J. Gen. Virol., 69: 1789-1798.

Kawai, S., S. Maekawajiri, K. Tokunaga, T. Jujo and A. Yamane, 1994. A simple method of HLA-DRB typing using enzymatically amplified DNA and immobilized probes on microtiter plate. Hum. Immunol., 41: 121-126.

Li, N., X. Wang, G. Zhou and J. Dong, 2006. Molecular variability of the coat protein gene of *Potato virus* Y from tobacco in China. Acta Virologica, 50: 107-113.

Mahmoud, A., J.C. Thouvenel, S.E. Abol-Ela, G.H. Sewify and E.D. Ammar, 1996. Detection of maize yellow stripe tenui-like virus by ELISA and dot-blot tests in host plants and leafhopper vector in Egypt. Phytopathol. Mediterranean, 35: 19-23.

Marinho, V.L.A., J. Daniels, J. Kummert, A. Chandelier and P. Lepoivre, 2003. RT-PCR-ELISA for detection of apple stem grooving virus in apple trees. Fitopatologia Brasileira, 28: 374-379.

Matthews, R.E.F., 1980. Host Plant Responses to Virus Infection. In: Comprehensive Virology, Virus-host Interaction, Viral Invasion, Persistence and Diagnosis, Fraenkel-Conrat, H. and R.R. Wagner (Eds.). Plenum Press, New York, USA., pp: 297-359.

Mayo, M.A. and V. Ziegler-Graff, 1996. Molecular biology of luteoviruses. Adv. Virus Res., 46: 416-460.

Nolasco, G., C. Deblas, V. Torres and F. Ponz, 1993. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. J. Virol. Methods, 45: 201-218.

Olmos, A., M. Cambra, M.A. Dasi, T. Candresse, O. Esteban, M.T. Gorris and M. Asensio, 1997. Simultaneous detection and typing of *Plum pox potyvirus* (PPV) isolates by Heminested-PCR and PCR-ELISA. J. Virol. Methods, 67: 127-137.

Peiman, M. and C. Xie, 2006. Sensitive detection of potato viruses, PVX, PLRV and PVS, by RT-PCR in potato leaf and tuber. Aust. Plant Dis. Notes, 1: 41-46.

Qamar, N. and M.A. Khan, 2003. Relationship of environmental conditions conducive for *Potato virus* X (PVX) disease development on six varieties/advanced lines of potato. J. Biol. Sci., 3: 247-252.

Riechmann, J.L., S. Lain and J.A. Garcia, 1992. Highlights and prospects of potyvirus molecular biology. J. Gen. Virol., 73: 1-16.

Rowhani, A., L. Biardi, D.A. Golino and S.D. Daubert, 1996. Detection of viruses of woody host plants using colorimetric PCR. Acta Horticul., 1: 265-272.

Salazar, L.F., 1977. Studies on three potato viruses from South America. Ph.D. Thesis. University of Dundee, Scotland. pp: 242.

Shalaby, A.A., M.K. Nakhla, A.M. Soliman, H.M. Mazyad, A. Hadidi and D.P. Maxwell, 2002. Development of a highly sensitive multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) method for detection of three potato viruses in a single reaction and nested PCR. Arab J. Biotechnol., 5: 275-286.

Shamloul, A.M. and A. Hadidi, 1999. Sensitive detection of potato spindle tuber and temperate fruit tree viroids by reverse transcription-polymerase chain reaction-probe capture hybridization. J. Virol. Methods, 80: 145-155.

Shamloul, A.M., N.A. Abdallah, M.A. Madkour and A. Hadidi, 2001. Sensitive detection of the Egyptian species of sugarcane streak virus by PCR-probe capture hybridization (PCR-ELISA) and its complete nucleotide sequence. J. Virol. Methods, 92: 45-54.

Shukla, D.D., C.W. Ward and A.A. Brunt, 1994. The Potyviridae. CAB International, Wallingford.

Soliman, A.M., A.A. Shalaby, B.N. Barsoum, G.G. Mohamed, M.K. Nakhla, H.M. Mazyad and D.P. Maxwell, 2000. Molecular characterization and RT-PCR-ELISA detection of a *Potato virus* X (PVX) isolate from Egypt. Ann. Agric. Sci., 9: 1791-1804.

Tacke, E., J. Schmitz, D. Prüfer and W. Rohde, 1993. Mutational analysis of the nucleic acid-binding 17 kDa phosphoprotein of *Potato leafroll luteovirus* identifies an amphipatic a-helix as the domain for protein-protein interactions. Virology, 197: 274-282.

Varveri, C., 2006. Biological, serological and molecular characterization of *Potato virus* Y isolates in Greece. Ann. Benaki Phytopathol. Inst., 20: 67-81.

Weekes, R., I. Barker and K.R. Wood, 1996. An RT-PCR test for the detection of *Tomato spotted wilt tospovirus* incorporating immunocapture and colorimetric estimation. J. Phytopathol., 144: 575-580.

Yousef, A.A., 1992. Studies on some viral diseases of potato. M.Sc. Thesis, El-Mansoura Univ., Egypt.