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Full Length Research Paper

Identification of six potato virus Y isolates from Saudi Arabia

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Six potato virus Y (PVY) were isolated from 20 potato plants (*Solanum tuberosum* sp. *tuberosum* L.) from the Riyadh region of Saudi Arabia showing leaf systemic symptoms (necrotic spots and mild mosaicism). 16 virus-infected plants gave positive indirect enzyme-linked immunosorbent assay (ELISA) results with PVY commercial antiserum. Electron microscopy revealed the presence of rod-shaped particles (300 × 17 nm). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated the 34 kDa viral coat protein and agarose gel of the immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) products indicated the 800 bp *cp* gene. The sequences were aligned together, narrowed to six (one PVY-N and five PVY-O isolates) and then aligned with all published worldwide PVY *cp* sequences. The highest similarity index among the six isolates was shown between PVY-saudi-O1 and PVY-saudi-O4 (99.9%), while the least involved PVY-saudi-N and PVY-saudi-O3 (99.1%). The phylogenetic analysis of the *cp* gene nucleotide sequence revealed a cluster of PVY-saudi-N and the Egyptian strain GU980964. The results indicate the need for more sensitive detection of the virus in the imported seeds or tubers from countries, especially in the Middle East like Egypt, to avoid high threat to the Saudi potato trade.

Key words: Reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), coat protein (CP), sequence alignment, similarity index.

INTRODUCTION

Potatoes have become an important food crop in Saudi Arabia in recent years. More than 15 varieties are grown in both autumn and spring seasons. The seed for the spring crop is imported mainly from the Netherlands, then from Scotland, France, Ireland, the Middle East (example Egypt) and the US. The autumn crop is planted from seed tubers produced locally from the spring crop. Potato virus Y (PVY), the type member of the genus Potyvirus, family Potyviridae, is one of the most important pathogens of pepper, potato, tobacco and tomato crops, to which it is non- persistently transmitted by many aphid species (Shukla et al., 1994). It can also be spread mechanically by human activity. The virus host range also includes many solanaceous and non-solanaceous weeds (Kerlan, 2006). Earlier reports indicated the detection of this virus in central (Al-Shahwan et al., 1997) and northern (Al-Shahwan et al., 1998) regions of Saudi Arabia.

This work focused on detecting this virus using molecular tools [polymerase chain reaction (PCR) with the cp gene] in one cultivar (Baraka) and one region (Riyadh) to get a figure on the future of this trade in the country. The function of the coat protein is to enclose the newly formed genomes to give rise to new virions. It has been suggested that enclosure of the newly formed virions is initiated by the interaction of the coat proteins with the 5' terminus and that the coat protein is built up towards the 3' terminus of the viral RNA (Wu and Shaw, 1998).

MATERIALS AND METHODS

Characterization and collection of infected plants

20 potato (*Solanum tuberosum sp. tuberosum* L.) plants of Baraka cv. with virus-like symptoms were collected in spring 2010 from

Table 1. Accessions utilized for the design *cp*-specific forward and reverse primers.

Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)
M95491.1	CAAGGAAATGACACAATC	TCACATGTTCTTGACTCCAA
AB185833	CAAGGAAACGACACAATC	TCACATGTTCTTGACTCCAA
NC_001616	CAAGCAAATGACACAATT	TCACATGTTCTTGACTCCAA
D00441	CAAGCAAATGACACAATT	TCACATGTTCTTGACTCCAA
A08776	CAAGCAAATGACACAATT	TCACATGTTCTTGACTCCAA
AM113988	CAAATGACACAATCGATG	TCACATGTTCTTGACTCCAA

three field locations in the Riyadh region, KSA. No discrete source of imported seed was utilized in these locations. The symptoms were mild mosaic, malformation on leaves and necrotic spots on leaves. The virus was biologically confirmed in 16 isolates by mechanical inoculation on the host, *Nicotiana glutinosa*, and serologically by indirect enzyme-linked immunosorbent assay (I-ELISA, Reader Model 680, BioRad laboratories, USA) following the procedure of Koenig and Paul (1982) with polyclonal antibodies specific for PVY (Agdia, USA).

Virus isolation and purification

The virus isolates were maintained (from single local lesions produced on *N. glutinosa*) on *Nicotiana tabacum* cv. White Burley. Leaf strips or veinal necrosis were obtained from the inoculated *N. tabacum* (15-days post inoculation) and studied for viral inclusion bodies either without staining or after applying 0.5% methyl green and pyronine Y (MGP-Y) stain. PVY-infected leaves from the 16 plants of *N. tabacum* cv. White Burley were separately frozen and ground. Virus isolates were purified and preparations were negatively stained and the grids were sent to the Specialized Hospital, Ain Shams University, Cairo, Egypt, to be examined with a Philips 400T transmission electron microscope. Purified virus preparations were also evaluated spectrophotometry and viral yields were calculated according to the equation given by Noordam (1973).

Isolation and purification of immunoglobulin G (IgG)

Antisera against the different PVY isolates were prepared according to the method described by Makkouk and Gumpf (1976), then IgGs were isolated from the antisera of different PVY isolates.

SDS-PAGE of PVY coat protein

The molecular weight of PVY coat protein subunit(s) from infected tobacco leaves was determined through immunocapturing (IC) (Minafera and Hadidi, 1994) with cognate IgGs, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

IC-RT-PCR for PVY *cp* gene amplification

The immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) was performed using infected tobacco leaves with different isolates. IC and cDNA synthesis was carried out as described (Minafera and Hadidi, 1994). The primers (Invitrogen Corp., USA) used for the amplification of partial length *cp* gene were designed depending on PYV *cp* gene sequences (~8557/72 …….9357/72 nt) submitted to the PubMed (GeneBank) web site (http://www.ncbi.nlm.nih.gov) as follows: 5′CAAGCA-AATGACACAATT3′ (forward) and 5′TCACATGTTCTTGACT-CCAA3′ (reverse). A number of six accessions were aligned to determine sequences of the forward and reverse primers. They are M95491.1, AB185833, NC_001616, D00441, A08776 and AM113988 (Table 1).

RT-PCR was performed for RNAs of different virus isolates as described by Ghosh et al. (2002). Then, 1.5% agarose gel was used and electrophoresis was carried out at 80 V. Finally, the products were visualized and photographed.

PYV *cp* gene cloning, sequencing and sequence analysis

cDNA fragments representing viral *cp* gene were purified and ligated into Promega's pGEM®-T vector plasmid following the manufacturer's instructions. Sequencing of different gene fragments was carried out at Gene Analysis Unit (VACSERA, Cairo, Egypt) using ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA). Sequencing products were resolved in an automated sequencer model 310 (Applied Biosystems, USA). Sequence trimming was done using "Sequencher" program (http://www.genecodes.com), where low quality ends and primers got trimmed and then contigs were generated from forward and reverse sequence reads. For the clustered sequences combined with the PYV coat protein, multiple alignments were done using online MAFFT (Multiple Alignment using Fast Fourier trasnform) tool, version 6 (http://mafft.cbrc.jp/alignment/server/) with L-INS-i option (similar to that of T-Coffee). Jalview program (Waterhouse et al., 2009) was used in refinement of the alignment. Then, re-alignment using MAFFT online was performed following the same criterion. According to the results of the first alignment, the number of gene fragments was narrowed to six to represent six different isolates, two recovered from one field location and four repeatedly recovered from more than one field location.

Construction of phylogenetic tree

Phylogenetic tree was constructed using PhyML program v3.0 (http://www.atgc-montpellier.fr/phyml) against the six different gene fragments. Criterion used was sequential option, and random initial trees used are 3, tree topologies are NNI and SPRs and the model used is HKY85. A value for each internal node was estimated for statistical significance of branching by performing 1000 replications of the bootstrap resampling from the original data. The tree generated from PhyML program was displayed using iTOL/ interactive tree (http://itol.embl.de/index.shtml, Letunic and Bork, 2011).

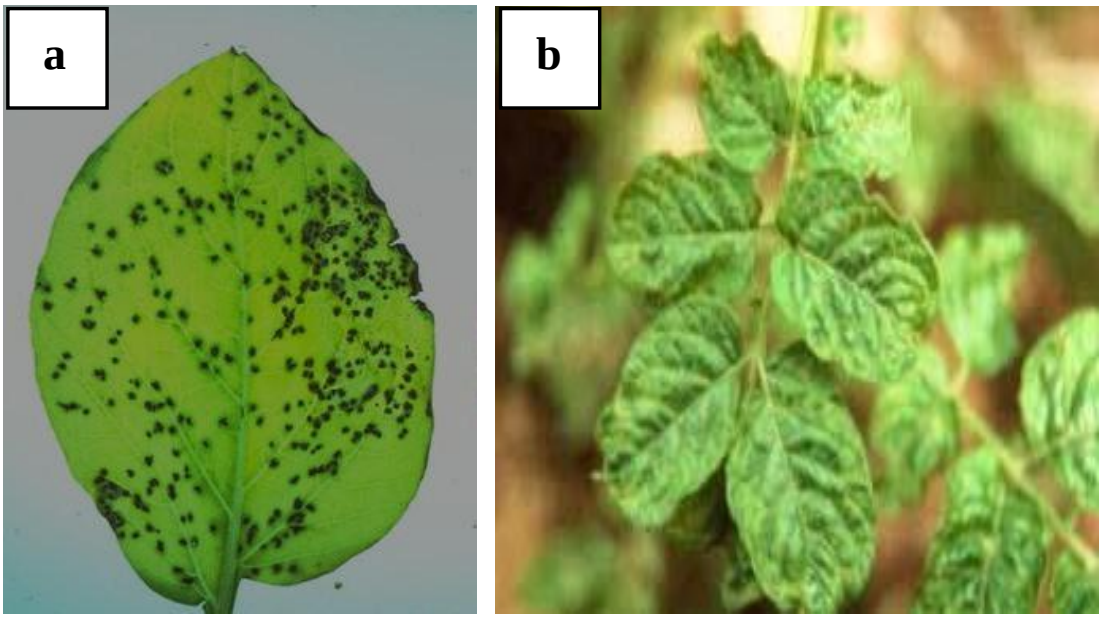

Figure 1. Symptoms observed on PVY infected potato plant (*Solanum tuberosum* sp. *tuberosum* L.). a, Necrotic spots; b, mild mosaicism.

as mild necrotic spots (3 PVY-N isolates) and mosaicism (17 PVY-O isolates) (Figure 1a and b, respectively). The virus characteristics were biologically confirmed by mechanical inoculation on *N. glutinosa*. Serologically, 16 virus-infected plants (3 PVY-N and 13 PVY-O isolates) gave positive indirect ELISA results with PVY specific antiserum (ranging from 0.855 to 1.324) compared with results obtained from healthy Jasmine plants (ranging from 0.034 to 0.177). The virus isolates were maintained (from single local lesions produced on *N. glutinosa*) on *N. tabacum* cv. White Burley. The purified virus preparations were negatively stained with 2% uranyl acetate and the results in Figure 2 reveal the presence of rod-shaped particles (300 × 17 nm). Results also show no virus particles in healthy tobacco plants.

Production of antiserum

IgGs were purified and their titers were determined using I-ELISA. The data in Table 2 shows that antisera reacted up to dilution of 1/2048 with clarified infectious sap and no positive results were obtained with the healthy sap.

Molecular detection of PVY *cp* in different isolates

SDS-PAGE analysis of the purified PVY preparations (Figure 3a) indicated that the viral coat protein appeared for different isolates as one band with a molecular weight of about 34 kDa and IC-RT-PCR analysis indicated a single band with size of about 800 bp (Figure 3b), which are the expected sizes for the coat protein and gene, respectively.

Sequence analysis of PVY *cp* gene for different isolates

The nucleotide sequence of PVY *cp* gene for different isolates started with the initial codon ATG and ended with the terminal nonsense codon TTG. The 16 sequences were first aligned together, narrowed to six (one PVY-N and five PVY-O isolates), two from each location - in which isolates with 100% similarity index were considered as one isolate - and then aligned with all published worldwide PVY *cp* sequences. The alignment was converted to phylogenetic tree (Figure 4) and similarity indices were scored (Table 3). The highest similarity index was shown between isolate PVY-saudi-O1 and PVY-saudi-O4 (99.9%) followed by that between isolate PVY-saudi-O1 and PVY-saudi-O2 or between PVY-saudi-O2 and PVY-saudi-O3 (99.8%). The least similarity indices involved isolates PVY-saudi-N when compared with isolate PVY-saudi-O3 (99.1%) followed by PVY-saudi-O4 or PVY-saudi-O5 (99.3%). The results show that PVY-saudi-N showed the highest similarity (100%) with the Egyptian strain GU980964. The five PVY-saudi-O isolates showed the highest similarities with four strains isolated from South Africa and one strain each from the United Kingdom and China. Two South African strains, namely GO853635 and GO853643 were shown to be 100% similar for their *cp* gene sequence and, hence, might represent the same strain. The least similarity index that involved any of the new isolates was between isolate PVY-saudi-N and strain GQ853631

Figure 2. As a model, electron microscopy of one purified virus preparation stained with 2% uranyl acetate (Mag. X60, 000).

Table 2. Determination of antisera titers using I-ELISA.

Dilution	ELISA value at 405 nm	Result
Undiluted	1.875	+
1/2	1.577	+
1/4	1.006	+
1/8	0.966	+
1/16	0.891	+
1/32	0.843	+
1/64	0.756	+
1/128	0.671	+
1/256	0.622	+
1/512	0.476	+
1/1024	0.398	+
1/2048	0.201	+
1/4096	0.060	-
Control*	0.087	-

***=** Clarified healthy sap with normal serum (1/2); + = positive; - = negative.

(98.9%) followed by that between isolate PVY-saudi-N and either strain GQ853658, EF027889 or EF027900 (99.0%). These strains were isolated from South Africa or the United Kingdom.

The phylogenetic analysis of the *cp* gene nucleotide sequence revealed two clusters, one involved the new isolate PVY-saudi-N and the Egyptian strain GU980964 and the other cluster involved the rest (Figure 4). The latter was divided into two subclusters, one involved the isolate PVY-saudi-O5 and two strains from China (namely HM036200 and HM036204) and the other involved the rest. The latter was subdivided into two groups, the first involved two new isolates, namely PVY-saudi-O2 and PVY-saudi-O3 and five South African strains. The second group involved two new isolates, namely PVY-saudi-O4 and PVY-saudi-N, four strains

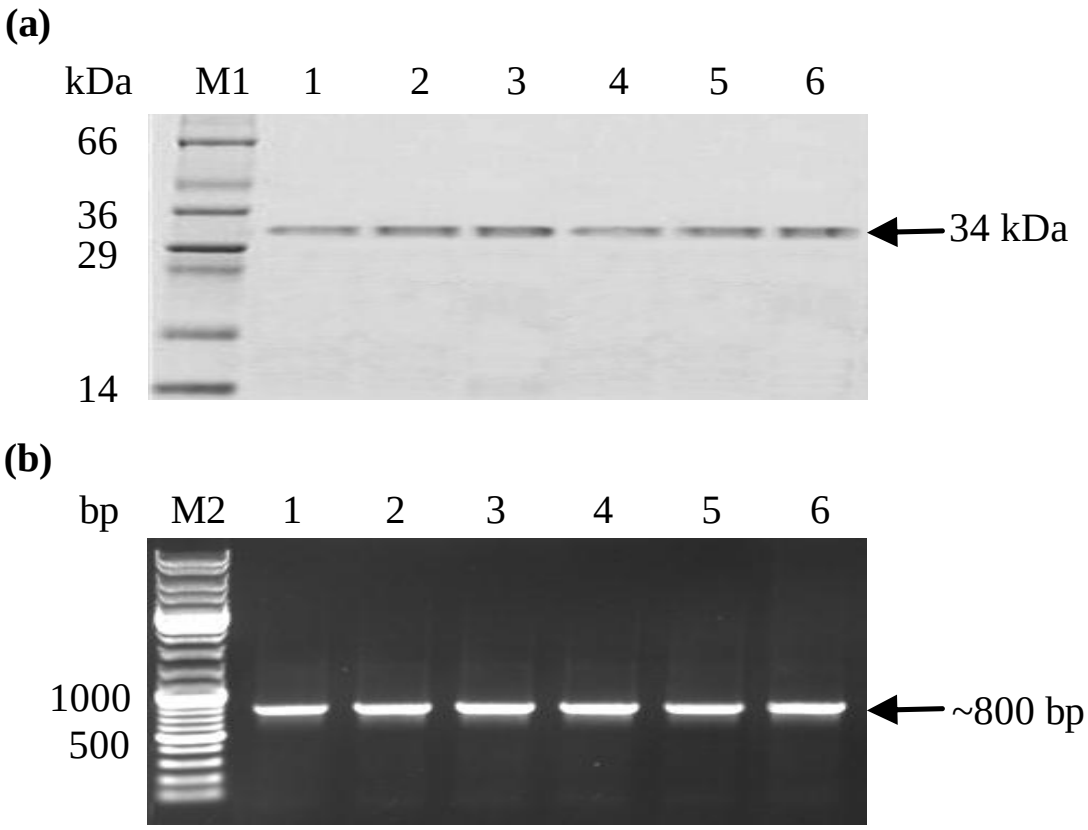

Figure 3. Detection of PVY through SDS-PAGE of purified virus coat protein preparations (a, lanes 1 to 6) and IC-RT-PCR of *cp* gene (b, lanes 1 to 6). M1, LMW protein standard (Promega, USA); M2, 100 bp/1 kb DNA ladder mix (Promega, USA).

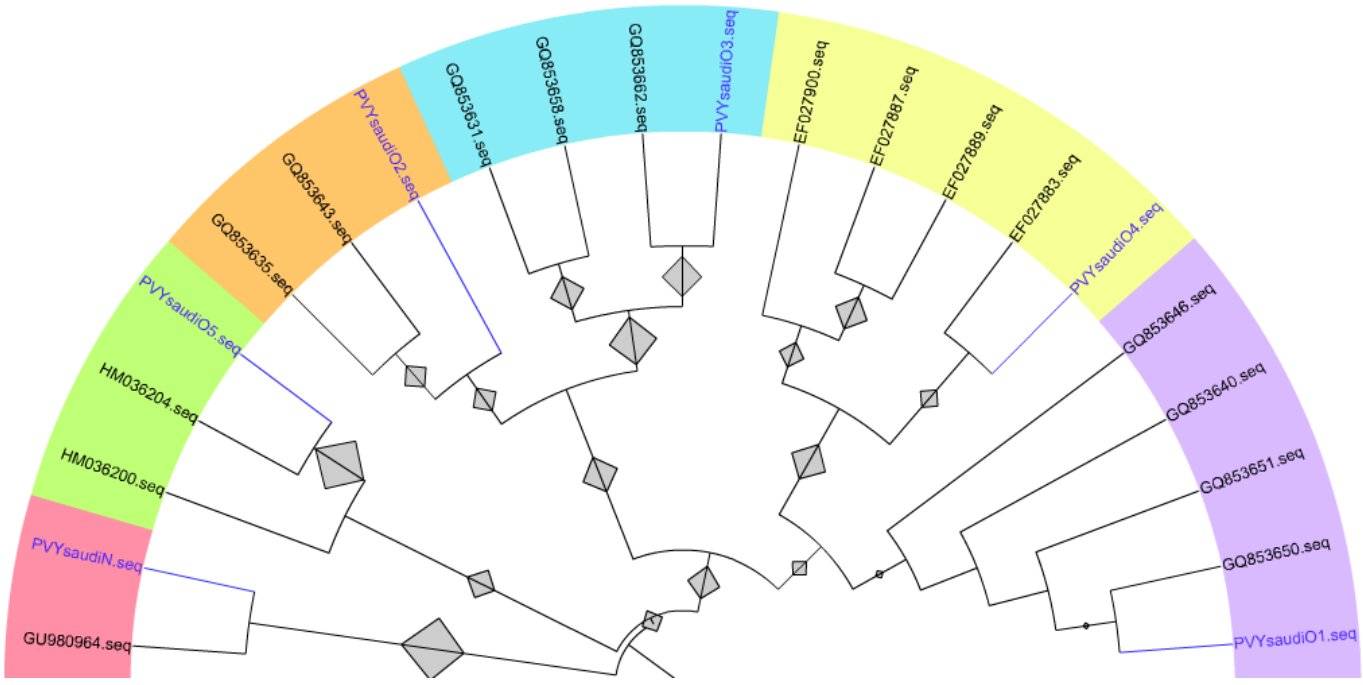

Figure 4. Phylogenetic tree between *cp* gene sequences of the six Saudi PVY isolates and the closest sequences for PVY isolates in the gene bank. Plot square marks on the branches refer to bootstrap. Square size is proportional to the bootstrap value.

Table 3. Similarity indices among different PVY isolates of the Riyadh region as well as those published worldwide based on *cp* gene sequences.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	No.	Accession no.
	99.9	99.8	99.8	99.1	99.6	99.8	99.6	99.9	99.6	99.8	99.3	99.4	99.3	99.5	99.5	99.3	99.9	99.6	99.4	**100.0**	99.5	1	EF027883
		99.9	99.6	99.0	99.5	99.6	99.5	99.8	99.5	99.6	99.1	99.3	99.1	99.4	99.4	99.1	99.8	99.5	99.3	99.9	99.4	2	EF027887
			99.8	99.1	99.4	99.5	99.4	99.6	99.4	99.5	99.3	99.4	99.0	99.3	99.3	99.0	99.6	99.4	99.4	99.8	99.3	3	EF027889
				99.1	99.4	99.5	99.4	99.6	99.4	99.5	99.3	99.4	99.0	99.3	99.3	99.0	99.6	99.4	99.4	99.8	99.3	4	EF027900
					99.5	99.1	99.5	99.3	99.0	99.1	99.4	99.5	98.9	99.1	99.1	98.9	99.3	99.5	99.5	99.1	99.1	5	GQ853631
						99.6	**100.0**	99.8	99.5	99.6	99.6	99.8	99.4	99.6	99.6	99.4	99.8	**100.0**	99.8	99.6	99.6	6	GQ853635
							99.6	99.9	99.6	99.8	99.3	99.4	99.3	99.5	99.5	99.3	99.9	99.6	99.4	99.8	99.5	7	GQ853640
								99.8	99.5	99.6	99.6	99.8	99.4	99.6	99.6	99.4	99.8	**100.0**	99.8	99.6	99.6	8	GQ853643
									99.8	99.9	99.4	99.5	99.4	99.6	99.6	99.4	**100.0**	99.8	99.5	99.9	99.6	9	GQ853646
										99.6	99.1	99.3	99.1	99.4	99.4	99.1	99.8	99.5	99.3	99.6	99.4	10	GQ853650
											99.3	99.4	99.3	99.5	99.5	99.3	99.9	99.6	99.4	99.8	99.5	11	GQ853651
												99.6	99.0	99.3	99.3	99.0	99.4	99.6	99.6	99.3	99.3	12	GQ853658
													99.1	99.4	99.4	99.1	99.5	99.8	**100.0**	99.4	99.4	13	GQ853662
														99.3	99.3	**100.0**	99.4	99.4	99.1	99.3	99.3	14	GU980964
															99.5	99.3	99.6	99.6	99.4	99.5	99.5	15	HM036200
																99.3	99.6	99.6	99.4	99.5	**100.0**	16	HM036204
																	99.4	99.4	99.1	99.3	99.3	17	PVY-saudi-N
																		99.8	99.5	99.9	99.6	18	PVY-saudi-O1
																			99.8	99.6	99.6	19	PVY-saudi-O2
																				99.4	99.4	20	PVY-saudi-O3
																					99.5	21	PVY-saudi-O4
																						22	PVY-saudi-O5

NO, number.

from the United Kingdom and four from South Africa.

DISCUSSION

The nucleotide sequencing analysis showed that the *cp* gene fragment is 807 nucleotides (nt). It is well-known that the PVY genome encodes one polyprotein, which undertakes proteolysis to form various mature proteins including the coat protein. Therefore, it is irrelevant to imply that the *cp* gene *per se* can be utilized for survey of this virus. It is recommended that other viral genes, like replicase, must be included in subsequent study. El-Afifi et al. (2004) characterized PVY in potato depending on molecular characteristics. They sequenced the virus full genome, and aligned the nucleotide and amino acids sequences with other worldwide PVY isolates. However, van der Vlugt et al. (1993) used the *cp* cistron in order to distinguish between different isolates of one virus species as well as between different virus species.

PVY causes serious problems in countries in the Middle East, like Egypt and Syria, but potato is a relatively new crop in Saudi Arabia and hence, no comprehensive reports on the virus infection severity (roughly estimated in the present work as 3%) and related yield loss is recently available. However, it is highly recommended to make survey for this virus across the six different potato-grown regions (Tabuk, Hail, Gassim, Riyadh, Hofof and Najran) in the kingdom in both spring and autumn seasons for different imported seed cultivars. The latter will give an idea on the spread

and diversity of the virus.

There is no discrete source of imported seed in Riyadh region as the kingdom does not have the biosafety guidelines of imported seed segregation. The whole importation process is regulated through the Decree No 4/3/49943, dated 9/7/1415 H (12/8/1994), for the Regulations of Imported Seed Potatoes in the kingdom. It is mandatory that country-of-origin, radiation-free, genetically modified (GMO)-free and seed certificate is added to the phytosanitary certificate before the seed importation process is complete. The crops from which the potatoes were drawn have to be examined during growth by expert inspectors, and conform to the well-known required standards. At the time of the inspection, the potatoes in the consignment should contain no more than 0.5% infection by PVY for Class SE and 2% for Class E.

The results of this study confirm that seed potato importation might constitute an avenue of new viruses or viral isolates into the country, which may quickly spread throughout potato production areas. The results alsohighlight the possible need for more sensitive ways of virus detection of the imported seeds or tubers from countries, especially in the Middle East like Egypt, taking into consideration that an isolate like PVY-saudi-N might represent a threat to the Saudi potato trade.

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