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The Occurrence of Potato Viruses in Syria and the Molecular Detection and Characterization of Syrian *Potato virus S* Isolates

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Abstract *Potato virus Y* (PVY) is the main potato virus in Syria but no information is available on the prevalence of other potato viruses. A survey was conducted by ELISA using specific antibodies to nine potato viruses. PVY, *Potato virus S* (PVS), *Cucumber mosaic virus* and *Potato leafroll virus* were detected with infection rates of 54.2, 8.4, 3.7 and 0.9%, respectively. The overall virus infection rate was as high as 72.9% in ware potato fields. A preliminary characterization was carried out on PVS isolates. Syrian PVS isolates infected *Chenopodium amaranticolor* only locally and therefore were classified as PVS^{O}. Phylogenetic analysis of the coat protein gene showed that PVS comprised two main clusters, cluster-O and cluster-A, which contained the ordinary and Andean strains respectively. Cluster-O was separated into two subclusters, O1 and O2. Two Syrian PVS isolates, PVS3-5 and PVS6-2, fell into the O1 subcluster. PVS3-5 coat protein, however, shared the highest nucleotide identity with European isolates of the O1 subcluster, whereas PVS6-2 was closely related to Asian isolates of the same subcluster. Owing to the high incidence of PVY and PVS in Syria, a duplex reverse-transcription PCR was developed to detect these two viruses in a single PCR.

Keywords ELISA · General Organization for Seed Multiplication · Ordinary strain · PCR · *Potato virus Y*

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Introduction

As many as 37 viruses have been recorded as naturally infecting potato (*Solanum tuberosum* L.), some of which are restricted to a certain geographical region, while others occur worldwide (Jeffries 1998). Owing to their ability to be transmitted via infected seed tubers and the heavy yield losses they cause, potato viruses are a serious threat to potato production (Valkonen 2007). Moreover, potato viruses may directly affect potato quality since infected plants usually produce smaller tubers and some viruses such as the *Potato virus Y* (PVY) variant PVY^{NTN} induce necrotic symptoms on the tubers, rending them unsuitable for marketing (Beczner et al. 1984; Le Romancer et al. 1994; Van den Heuvel et al. 1994). Such potato viruses cause degeneration which requires the regular replacement of the seed to maintain quality and productivity (Lawson and Stace-Smith 2001). Detection and identification of potato viruses is a critical part of the management of seed potato production.

Potato virus S (PVS) is one of the main potato viruses infecting potatoes worldwide. PVS belongs to the genus *Carlavirus*, family *Flexiviridae* (Adams et al. 2004; Fauquet et al. 2005), with a capped single-stranded positive-sense genomic RNA of about 8.5 kb with a poly A tail at the 3′ terminus (Mackenzie et al. 1989; Matousek et al. 2005). The genome is encapsidated in a 34-kDa coat protein (CP) (Monis et al. 1987; Mackenzie et al. 1989; Foster 1992).

Two strains of PVS have been recognized, PVS^{O} (ordinary) and PVS^{A} (Andean), according to their ability to induce non-systemic and systemic infection in *Chenopodium* spp., respectively (Slack 1983; Foster and Mills 1992). However, the *Chenopodium*-systemic PVS isolates (PVS-CS) reported by Matousek et al. (2005) caused systemic infection in *Chenopodium quinoa* but were closer to PVS^{O} than to PVS^{A} on the basis of the genome identity (Matousek et al. 2005). PVS mainly causes symptomless infection in most potato cultivars, although severe symptoms can occur (Foster and Mills 1992; Jeffries 1998). PVS reduces the yield by 10–20% and mixed infections with other viruses dramatically increase the severity of symptoms which result in greater yield reductions (Jeffries 1998).

In Syria, potato is one of the main crops and is grown in 24,789 ha with a total production of 486,605 Mg (Anonymous 2003). Seed and ware potato production is mainly carried out in three provinces in northern Syria, Aleppo, Idleb and Hama, which produce 72% of the national ware potato production (Anonymous 2003). Although seed potatoes are mainly imported from Europe (5,000–6,000 Mg per year), local production is being carried out by the General Organization for Seed Multiplication, the Syrian Ministry of Agriculture and Agrarian Reform. Local seed potato production starts with the tissue culture multiplication of potato plantlets and is followed by greenhouse and net house multiplication and finally by at least two open field multiplication cycles.

PVY is the main potato virus infecting potatoes in Syria (Chikh Ali et al. 2006). To study the occurrence of potato viruses in Syria, particularly of potato viruses other than PVY, a survey of nine potato viruses was conducted during the 2006 autumn growing season. In addition, Syrian PVS isolates were characterized and a sensitive simultaneous detection method for PVS and PVY was developed.

Materials and Methods

Potato Samples

A total of 107 potato leaf samples were collected from 12 potato fields (four seed and eight ware potato fields) in the three main potato production provinces, Aleppo, Idleb and Hama, located in the northern part of Syria during the 2006 autumn growing season (Fig. 1). In general, the potato plants were sampled at random regardless of symptoms. Samples were kept as silica gel dried leaves at 4 °C. All positive controls except for PVY were provided by the National Agricultural Research Center for Hokkaido Region, Japan. Two Syrian PVY isolates, PVY-12 and SYR-NB-16, were used as PVY positive controls (Chikh Ali et al. 2007a, b).

Bioassay

For strain identification, seven PVS isolates were inoculated into *Chenopodium amaranticolor* to investigate the ability to induce systemic infection. Original potato leaf samples were ground in 10 mM sodium phosphate buffer, pH 7.0, at a dilution rate of 1:10 (w/v) and inoculated into leaves previously dusted with Carborundum. Inoculated plants were maintained at 20 °C and observed daily for 4−6 weeks. To

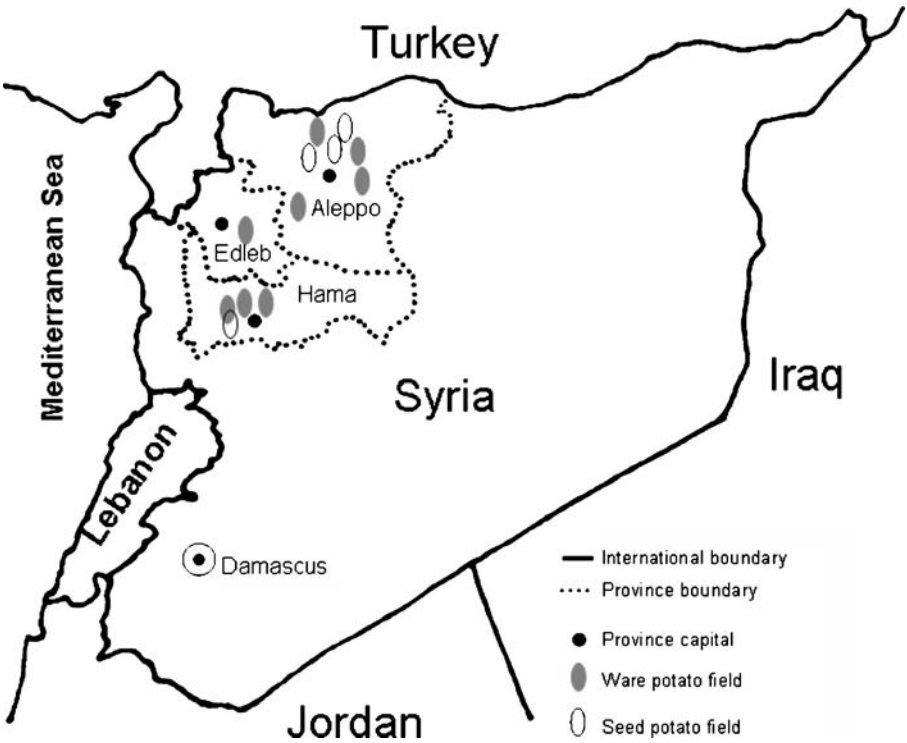

Fig. 1 Map of Syria showing the locations of ware and seed potato fields sampled

confirm virus infection, inoculated and non-inoculated leaves of *C. amaranticolor* were tested about 3 weeks after inoculation by reverse-transcription (RT) polymerase chain reaction (PCR) using PVS-specific primers and double antibody sandwich (DAS) ELISA using PVS and PVY antisera since some of the original potato samples were infected with both PVS and PVY (Table 2). A PVS isolate, PVS3-5 isolated from potato sample 3–5 (Table 2), was maintained by inoculation into *Nicotiana debneyi*.

ELISA

All potato samples were tested using antisera against nine viruses: *Cucumber mosaic virus* (CMV) (Agdia, USA), *Potato leafroll virus* (PLRV) (the National Center for Seeds and Seedlings, NCSS, Japan), *Potato mop-top virus* (Neogen Europe, UK), *Potato virus A* (PVA) (Agdia, USA), *Potato virus M* (PVM) (Agdia, USA), PVS (NCSS, Japan), *Potato virus X* (PVX) (NCSS, Japan), PVY (Agdia, USA) and *Tobacco mosaic virus* (PMV) (Agdia, USA). DAS-ELISA was carried out according to the manufacturer's instructions. Samples were considered positive when the absorbance (405 nm) was higher than 3 times that of the corresponding negative control and higher than 0.1 after incubation for 1 h at room temperature.

RNA Extraction

Total RNA was extracted from dried (20 mg) potato or tobacco leaves using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Resultant pellets were dissolved in 125 μl RNase-free water.

Reverse-transcription PCR

Seven samples were tested by RT-PCR using the PVS-specific primers S7P (5′-TTCCCAACAGGCGCAGTG-3′) and S2M (5′-CTAAACGGTCTGCCTTCAT-3′) (Sato 2001). The first-strand complementary DNA (cDNA) was synthesized using ReverTra Ace kit (Toyobo, Japan) according to the manufacturer's instructions with a 0.5 μM final concentration of the reverse primer S2M. PCR was carried out using the TaKaRa Ex Taq™ kit (Takara Bio, Japan) according to the manufacturer's instruction. The primers S7P and S2M were used at a final concentration of 0.5 μM. The PCR programme consisted of 5 min at 94 °C, 29 cycles of 1 min at 94 °C, 1 min at 57 °C and 2 min at 72 °C followed by the final extension for 10 min at 72 °C.

Duplex RT-PCR

For the simultaneous detection of PVY and PVS, two specific primer pairs were designed to be used in duplex PCR. PVY-specific primers PVY8139 and PVY8735 (Table 1) amplify a cDNA segment of 619 bp and PVS-specific primers PVSCPF and PVSCPR (Table 1) produce a band of 1,289 bp, including the full CP and 11K genes.

First-strand cDNA was synthesized using the ReverTra Ace kit using an oligo-dT primer at 0.5 μM final concentration as a reverse primer. The RT mixture consisted

Table 1 *Potato virus Y* (PVY) and *Potato virus S* (PVS) specific primers used in the duplex reverse-transcription polymerase chain reaction (RT-PCR)

Primer	Sequence (5′→3′)	Polarity	Nucleotide position[a]
PVSCPF	CTAGTCAATTGCGAGCTCAC	Sense	7,098–7,117
PVSCPR	TGGTATCACCTCAGTTACTCC	Antisense	8,366–8,386
PVY8139	TCACAACATTTCTCAGATCTTGG	Sense	8,117–8,139
PVY8735	GCATTCTCATTTTGGACGTGATAG	Antisense	8,712–8,735

[a] According to isolate Leona (NC-007289) for PVS and isolate PVY-12 (AB185833) for PVY

of 5 μl of total RNA, 4 μl 5XRT buffer, 2 μl dNTPs (10 mM each), 1 μl RNase inhibitor (10 U/μl), 1 μl oligo (dT)20 primer (10 μM) and 1 μl ReverTraAce (10 U/μl), and the total volume was adjusted to 20 μl using RNase-free water. The RT mixture was incubated at 42 °C for 20 min, transcriptase inactivated by heating at 99 °C for 5 min and the mixture cooled to 4 °C for 5 min.

PCR was carried out using the TaKaRa Ex Taq™ kit. PVY and PVS primers were used at final concentrations of 0.2 μM each. cDNA solution (1 μl) was added to a 0.2-ml PCR tube containing 2.5 μl 10× Ex Taq buffer, 2 μl dNTPs (2.5 mM each), 0.2 μM of each primer and 0.1 μl TaKaRa Ex Taq™ (5 U/μl). The final volume was adjusted to 25 μl using double-distilled H_2O. PCR was carried out in a PTC-100TM programmable thermal controller (MJ Research, USA) using the following programme: one cycle of denaturation at 96 °C for 2 min, 30 cycles at annealing temperatures of 62 °C for the first ten cycles, 60 °C for the second ten cycles and 58 °C for the last ten cycles. Each cycle consisted of denaturation at 96 °C for 30 s, primer annealing for 30 s, primer extension at 72 °C for 90 s followed by a final extension at 72 °C for 10 min. PCR products were separated in a 1.5% agarose gel, stained in a solution of ethidium bromide and photographed under UV illumination.

Table 2 Comparison of ELISA, uniplex and duplex RT-PCR assays for Syrian PVY- and PVS-infected samples

Sample/PVY isolate	Location	Cultivar	Original symptoms	RT-PCR and/or ELISA[a]		Duplex RT-PCR (bp)	Diagnosis
				PVY	PVS		
3-5	Aleppo	Unknown	Symptomless	−	+	1,289	PVS
3-7	Aleppo	Unknown	NR	−	+	1,289	PVS
3-9	Aleppo	Unknown	NR	+	+	619/1,289	PVY/PVS
3-10	Aleppo	Unknown	NR	−	+	1,289	PVS
6-2	Hama	Marfona	Yellowing	+	+	619/1,289	PVY/PVS
7-1	Hama	Unknown	Mosaic	+	+	NT	NT
10-1	Aleppo	Binella	Symptomless	+	+	619/1,289	PVY/PVS
10-2	Aleppo	Binella	Yellowing	+	+	619/1,289	PVY/PVS
10-3	Aleppo	Binella	Yellowing	+	+	619/1,289	PVY/PVS
PVY-12				+	−	619	PVY
SYR-NB-16				+	−	619	PVY

NR not recorded, *NT* not tested

[a] PVS-infected samples were tested by both ELISA and RT-PCR, whereas PVY infection was confirmed by ELISA only.

Nucleic Acid Sequencing and Sequence Analysis

The CP genes of PVS3-5 and another PVS isolate, PVS6-2 isolated from potato sample 6-2 (Table 2), were sequenced. The whole CP gene was amplified using PVSCPF and PVSCPR primers by RT-PCR. Purification of the amplified fragment, cloning and sequencing were performed according to the method of Chikh Ali et al. (2007a). Sequence analysis was conducted using DNASIS (Hitachi Software Engineering., Japan). The CP homology was searched for using the BLAST program provided by NCBI. For multiple alignment, CLUSTAL-X version 1.81 (Thompson et al. 1997) was used with default parameters. Phylogeny inference was conducted using the neighbour-joining (NJ) and maximum-parsimony methods. The neighbour-joining trees were calculated with 1,000 bootstrap replicates using the neighbour-joining option in CLUSTAL-X. Maximum-parsimony analysis was performed with PAUP* version 4.0 beta (Swofford 1998) using the HSearch option and 100 bootstrap resamplings. Phylogeny was displayed using the NJPLOT provided with CLUSTAL-X. The phylogenetic tree was constructed on the basis of the nucleotide sequences of the CP gene of PVS isolates available in GenBank. The PVX CP gene was used as an outlier. Isolate names and GenBank accession numbers are shown in Fig. 3.

Results

Virus Detection by ELISA

Of the 107 samples tested using DAS-ELISA, 58 (54.2%), nine (8.4%), four (3.7%) and one (0.9%) were infected by PVY, PVS, CMV and PLRV, respectively. Five samples were infected with PVY and PVS, four samples with PVY and CMV and one sample was infected with PVY, PVS and PLRV. The overall virus infection rate in ware potato fields was as high as 72.9%, whereas it was 4% in the superelite seed potato fields which are cultivated with the products of the net houses.

PVS Detection by RT-PCR

PVS-specific primers produced bands of 426 bp with the PVS-positive control and seven potato samples. All these samples tested positive to PVS antiserum in DAS-ELISA.

PVS Strain Identification by Bioassay

On *C. amaranticolor*, 10 days after inoculation, PVS isolates PVS3-5, 3-9, 7-1, 10-1 and 10-3 induced chlorotic local lesions which became necrotic. No obvious symptoms were seen on the upper non-inoculated leaves. PVS but not PVY was detected in inoculated leaves, whereas no infection was detected in non-inoculated leaves by DAS-ELISA. Bands of 426 bp indicating PVS infection were produced only for leaves of *C. amaranticolor* inoculated with PVS3-5, 3-9, 7-1, 10-1 and 10-3 isolates (Fig. 2). Neither PVS nor PVY was detected in *C. amaranticolor* plants inoculated with PVS6-2 and 3-10 isolates (Fig. 2).

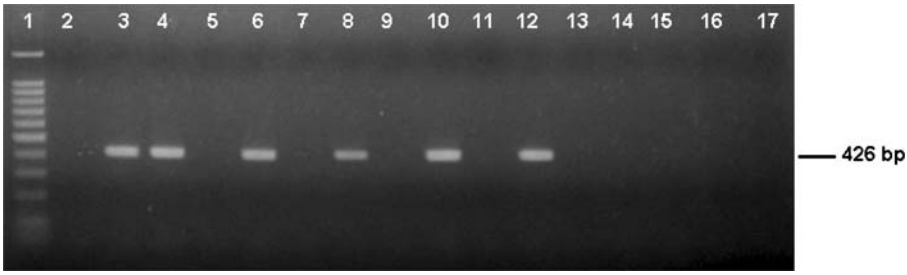

Fig. 2 Reverse-transcription polymerase chain reaction (RT-PCR) for the detection of *Potato virus S* (PVS) in inoculated (In) and upper non-inoculated (Up) leaves of *Chenopodium amaranticolor* with Syrian PVS isolates. *1* 100-bp DNA ladder, *2* healthy *C. amaranticolor*, *3* PVS-positive control, *4* PVS3-5 (In), *5* PVS3-5 (Up), *6* sample 3-9 (In), *7* sample 3-9 (Up), *8* sample 7-1 (In), *9* sample 7-1 (Up), *10* sample 10-1 (In), *11* sample 10-1 (Up), *12* sample 10-3 (In), *13* sample 10-3 (Up), *14* PVS6-2 (In), *15* PVS6-2 (Up), *16* sample 3-10 (In), *17* sample 3-10 (Up)

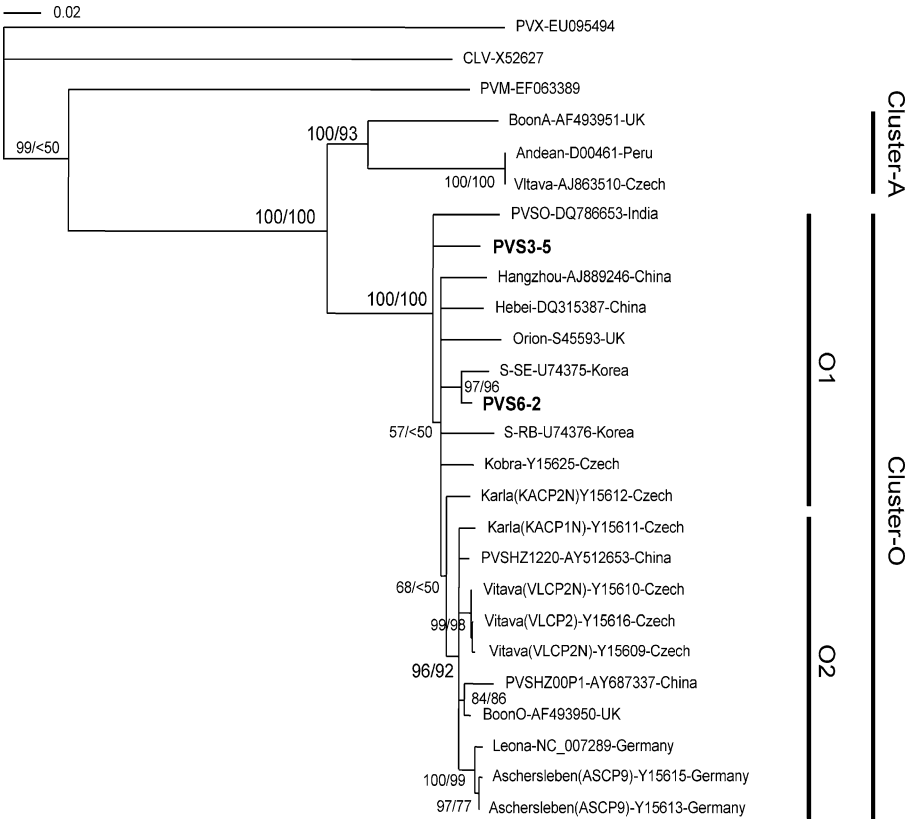

Fig. 3 Phylogenetic tree based on the nucleotide sequence of coat protein genes of PVS isolates. A set of numbers at internal nodes indicate the bootstrap values in the neighbour joining and maximum parsimony, respectively. Only values higher than 50% are shown. Data at external nodes indicate isolate name, GenBank accession number and geographic origin. Names between *brackets* indicate the available clone name. The phylogenetic tree was edited to collapse nodes with bootstrap value less than 50%

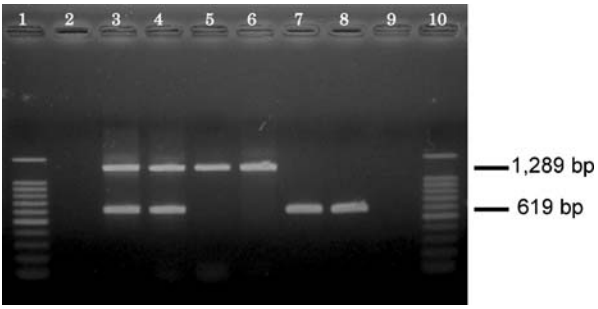

Fig. 4 Duplex RT-PCR for the simultaneous detection of *Potato virus Y* (PVY) and PVS. *1*, *10* 100-bp DNA ladder, *2* healthy potato leaf, *3*, *4* PVY and PVS naturally mixed infected samples, *5*, *6* PVS infected samples, *7*, *8* PVY-infected samples, *9 Potato virus M* positive control

Nucleic Acid Sequencing and Sequence Analysis

The CP genes of PVS3-5 and PVS6-2 consisted of 882 nucleotides encoding a putative CP of 294 amino acids. Sequence data for PVS3-5 and PVS6-2 appear in the DDBJ/EMBL/GenBank as accession numbers AB364945 and AB364946, respectively. PVS3-5 CP gene was 94.6% identical to that of PVS6-2, with 47 nucleotide substitutions. PVS3-5 CP was 97.6% identical to that of PVS6-2, with seven amino acid substitutions located in the N terminus of the CP. Compared with other isolates of PVS, the PVS3-5 CP nucleotide sequence shared the highest similarity (95.7%) with isolate Karla (Y15612). PVS3-5 CP had the highest amino acid identity (98%) with isolate Vitava (Y15610). PVS6-2 shared the highest identity with isolate S-SE (U74375) of PVS for both the nucleotide (98%) and the amino acid (99%) sequences of the CP. In the phylogenetic analysis, PVS strains fell into two main clusters. Isolates belonging to the PVS^{A} strain clustered in cluster-A, while isolates of the PVS^{O} strain group clustered in cluster-O. Moreover, two subclusters, O1 and O2, were identified within cluster-O. The Syrian PVS isolates PVS3-5 and PVS6-2 grouped in the O1 subcluster of the cluster-O (Fig. 3).

Development of Duplex RT-PCR

Using duplex RT-PCR, PVS singly infected samples produced expected bands of 1,289 bp (Fig. 4, Table 2). PVY isolates SYR-NB-16 and PVY-12 produced expected bands of 619 bp (Fig. 4, Table 2). Samples infected with both PVY and PVS produced two bands of 1,289 and 619 bp (Fig. 4, Table 2). The potato healthy control and the PVM-positive control did not produce any bands (Fig. 4). All PVS-infected samples (verified by ELISA and RT-PCR) that were examined by this assay produced bands of 1,289 bp (Table 2). The results of the duplex RT-PCR were in agreement with those of ELISA for all PVY and PVS samples tested (Table 2).

Discussion

In our previous survey of potato viruses in Syria, PVY was the main potato virus infecting potato (Chikh Ali et al. 2006) and the same holds true for the present

survey. This study showed that potato viruses other than PVY occur in Syria, with PVS having the second highest incidence, followed by CMV and PLRV, whereas in the former survey, using PVY, PLRV, PVS and PVX antibodies, only PVY was detected (Chikh Ali et al. 2006). In the previous study, since only samples with clear viruslike symptoms were collected, viruses that do not generate symptoms were not detected (Chikh Ali et al. 2006), whereas in the present study samples were collected at random, implying the possibility of detection of mild or non-symptomatic viruses such as PVS. The overall virus infection rate was very high, particularly in ware potato fields (72.9%), and ware potato fields with 100% infection rates were found (data not shown).

Out of nine PVS-infected samples, three showed mild yellowing, two were asymptomatic, one showed mosaic, while the symptoms were not recorded for the rest. In addition, seven PVS-infected samples were collected from two ware potato fields of unknown cultivars in Aleppo, which indicated that PVS infection is concentrated in certain areas and/or in certain potato cultivars. PVS was not found in seed potato production fields.

To increase our understanding of PVS in Syria, Syrian PVS isolates were characterized for the first time. Of seven PVS isolates, five including PVS3-5 infected *C. amaranticolor* locally gave rise to local lesions but were not able to evoke systemic infection and therefore they have been classified as PVS^{O} strains. On the other hand, two PVS isolates, PVS6-2 and PVS3-10, were not able to infect *C. amaranticolor* neither locally nor systemically, probably owing to sample degradation.

The PVS3-5 phenotype in *C. amaranticolor* is in agreement with the phylogenetic analysis of the CP gene (Fig. 3). The PVS3-5 CP gene shared the highest similarity with those of the ordinary isolates from Europe, Karla (Y15612) and Kobra. However, PVS6-2 was closely related to isolate S-SE from Korea on the basis of the CP gene identity and phylogenetic relation (Fig. 3). This indicates the heterogeneous nature and route of invasion of the PVS population in Syria.

In the phylogenetic tree, PVS isolates made two main clusters, A and O. Cluster-A consisted of PVS^{A} isolates, whereas cluster-O comprised the PVS^{O} isolates. In cluster-O two subclusters were identified, O1 and O2. The younger subcluster, O2, comprised mainly PVS^{O} isolates from Europe, with some isolates from Asia which grouped tightly, suggesting a common origin (Fig. 3). Isolates PVS3-5 and PVS6-2 grouped in the O1 subcluster, which was older than the O2 subcluster (Fig. 3).

Since PVS causes mild symptoms in general, field inspection is not expected to decrease PVS incidence in potato. Thus, the control system relies on PVS eradication from seed stocks and the continuous supply of healthy seed potatoes. This requires effective PVS detection and identification methods during the first steps of seed potato production. RT-PCR is a sensitive and reliable detection and identification method for potato viruses (Singh et al. 1999; Nie and Singh 2001). Taking into account the high rate of PVS and PVY mixed infection, the simultaneous detection of these viruses would save money, labour and time. We therefore developed a duplex RT-PCR that can detect PVY and PVS in a single test. This assay could detect PVY and PVS in both mixed and single infections. The results of this PCR assay were identical to those obtained by ELISA, which confirms its suitability to replace ELISA.

CMV is rarely reported in potatoes owing to the high resistance of potato cultivars to this virus (Celebi-Toprak et al. 2003; Chrzanowska et al. 2004). It has, however, been found in some regions with warm climates, such as Egypt (Jeffries 1998) and Saudi Arabia (Al-Shahwan et al. 1997). CMV has recently been detected in potato samples from Syria by serology (Haj Kassem et al. 2006). Further studies are required to address the possible effects of this virus in Syria.

PLRV is considered as one of the main potato viruses in the world (Jeffries 1998). Under the Syrian local conditions the leafroll symptoms related to PLRV are not common in the fields. The results of the present and the previous surveys suggest that PLRV is a minor virus in Syria.

In conclusion, PVS was found to be the second main potato virus after PVY in Syria. The PVS^{O} strain is prevalent in Syria, although the molecular analysis of two Syrian PVS^{O} strains indicated heterogeneous origins. The ordinary isolates of PVS form two lineages according to the CP nucleotide sequences. RT-PCR assay was developed to detect and differentiate PVS and PVY in a single test.

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