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ORIGINAL ARTICLE

Serological and molecular detection of *Bean leaf roll* and *Chickpea chlorotic stunt* luteoviruses in chickpea from Iran

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Abstract

Chickpea (*Cicer arietinum* L.) is an important legume crop and widely cultivated in northwestern provinces of Iran. During a survey in the 2015 growing season a total of 170 selected chickpea plants with general yellowing symptoms including stunting and leaf bronzing were collected. Serological Elisa and tissue blot immunoassay (TIBA) tests revealed the presence of *Bean leaf roll virus* (BLRV) and *Chickpea chlorotic stunt virus* (CpCSV) as the predominant viruses in the region. Some serologically positive samples of BLRV and CpCSV were selected and rechecked by RT-PCR. The results of amplified PCR products using a specific pair of primers towards the *Cp* gene region of the viruses were approximately 413 bp for CpCSV and 391 bp for BLRV. Results obtained from sequence comparison of BLRV (IR-F-Lor-5) isolate form two subgroups with eight other BLRV isolates from GeneBank indicating a high homology of 96% with isolates from Argentina, Germany, Tunisia, USA, Spain, and Colombia. An isolate from Norabad (Iran) (IR-Nor) had 98% homology with HQ840727 Libyan isolate. CpCSV sequence comparison with six other GeneBank isolates indicated 98% homology with isolates from Tunisia and Azerbaijan. The overall results of this research revealed the CpCSV and BLRV (luteoviruses) associated with the yellowing disease syndrome of chickpea crops in the surveyed region.

Key words: BLRV, CpCSV, phylogeny, serology, sequence comparison

Introduction

Chickpea (*Cicer arietinum* L.) is the second most important legume cultivated generally on residual soil moisture in arid and semi-arid regions of the world (Jukanti *et al*. 2012). Chickpea is also an important legume crop in Iran and is widely cultivated in the northwestern provinces of Lorestan, Kermanshah, West Azarbaijan, Zanjan, Arak, Eilam and Hamedan, with an estimated cultivated area of 472,000 ha in the 2013–2014 growing season (Anonymous 2013).

Several diseases caused by plant viruses have recently emerged to cause economic losses (Subbarao *et al*. 2015; Minicka *et al*. 2016). Viruses are known to infect chickpea plants worldwide, some of which cause serious economic losses (Bos *et al*. 1988; Najar *et al*. 2000; Chen *et al*. 2011). These viruses are genetically diverse but collectively share several features clearly distinct from other plant viruses (Smith and Barker 1999; D'Arcy *et al*. 2000).

Luteoviruses cause significant yield losses around the world in economically important crops such as beets, cereals, faba beans, peas, lentils, potatoes and a variety of legumes, especially chickpeas (D'Arcy *et al*. 2000; Abraham *et al*. 2006). Surveys conducted in many countries in the WANA region (North and West Africa) during the last three decades (Kaiser 1972; Horn *et al*. 1993, 1995; El-Muadhidi *et al*. 2001; Makkouk and Kumari 2001; Makkouk *et al*. 2002) established that the most important cool legume viruses are: *Faba bean necrotic yellows virus* (FBNYV: genus Nanovirus; family Nanoviridae), *Bean leaf roll virus* (BLRV: genus Luteovirus; family Luteoviridae), *Beet western yellows virus* (BWYV: genus Polerovirus; family Luteoviridae),

Soybean dwarf virus (SbDV: genus Luteovirus; family Luteoviridae) and *Chickpea chlorotic stunt virus* (CpCSV: genus Polerovirus; family Luteoviridae) which affect legume crops (Smith *et al.* 1999; D'Arcy *et al.* 2000; Leslie *et al.* 2002).

To date, five luteoviruses have been reported to infect faba bean and/or chickpea in different parts of the world (D'Arcy *et al.* 2005), namely BLRV (Makkouk *et al.* 1993), BWYV (Fortass *et al.* 1997), SbDV (Tamada *et al.* 1977), *Pea enation mosaic virus*-1 (PEMV-1) (Demler *et al.* 1995), and *Chickpea stunt disease associated virus* (CpSDaV). The latter has been reported only in India and is closely related to, but distinct from, BWYV (Naidu *et al.* 1997). During a survey of chickpea crops in western provinces of Iran in July 2009 (Bananej *et al.* 2010), leaf samples from yellow and stunted plants were collected from fields in the provinces of Kermanshah and Lorestan. Symptoms suggested infections by luteoviruses, such as BWYV and CpCSV, a virus first described in Ethiopia (Abraham *et al.* 2006) and West Asia and North Africa (Abraham *et al.* 2009).

Chickpea plants showing symptoms suggestive of viral infection such as leaf rolling, yellowing, reduction in overall plant growth, dwarf stem, foliar bronzing and stunting, were observed in Lorestan, Kermanshah, Hamedan and West Azerbaijan regions of Iran. Serological tests showed the presence of four different viruses, BLRV, BWYV, CpCSV and SbDV, in these regions (Shahraeen *et al.* 2016).

There were reports of chickpea infection by several viral diseases in Iran (Kaiser 1972; Kaiser and Danesh 1972; Makkouk *et al.* 2002; Hydari *et al.* 2011; Shahraeen *et al.* 2012, 2016). The aim of this study was to apply a rapid reverse transcription-polymerase chain reaction (RT-PCR) based method for detection and differentiation of BLRV and CpCSV Iranian strains, the most frequent occurring luteoviruses in cool season legume crops (Shahraeen *et al.* 2016).

Materials and Methods

Source of materials

Naturally infected chickpea samples were collected from five locations in Lorestan, 12 locations in Kermanshah and two locations in Hamedan, Iran (Makkouk *et al.* 2003). Therewere three farms per location and 170 plants were collected from each location. The luteovirus-like isolates were obtained from chickpea plants showing identical symptoms suggestive of viral infection such as foliar yellowing, chlorosis or leaf reddening, stunting, growth reduction, phloem browning, and leaf narrowing.

Identification of the isolated viruses

All collected samples were tested for luteovirus infection using tissue-blot immunoassay (TIBA), triple antibody sandwich (TAS) and antigen coated plate (ACP)-indirect Elisa procedure using a battery of rabbit poly and monoclonal antibodies for CpCSV, BLRV(5G4), BLRV(6G4), and polyclonal antisera for BWYV and SbDV (Kumari *et al.* 2008; Shahraeen *et al.* 2016). To identify the BLRV and CpCSV-luteovirus affecting chickpea in farmers' fields, infected samples that gave a positive reaction to the recent antibodies were tested by RT-PCR using specific primers as described (Table 1).

RNA extraction

Fifteen chickpea samples that reacted serologically positive with BLRV and CpCSV antibodies were selected for further testing by RT-PCR. Total RNA was extracted from 50 to 100 mg virus infected young leaves and stems by using RNX-Plus solution kit (Sinaclone, Iran, Cat No. RN7713C). Purified RNA was stored as a solution in water at –80°C.

Table 1. Specific primers used in this study and polymerase chain reaction (PCR) thermocycling conditions

Sequence	Primers	PCR profile	Amplicon size [bp]	Reference
BLRV-3 BLRV-5	5′-TCCAGCAATCTTGGCATCTC-3′ 5′-GAAGATCAAGCCAGGTTCA-3′	95°C, 2 min 95°C, 30 s 56°C, 30 s 72°C, 30 s } × 40 72°C, 10 min	391	Oritz *et al.* 2005
CpCSV-F CpCSV-R	5′-TAGGCGTACTGTTCAGCGGG-3′ 5′-TCCTTTGTCCATTCGAGGTGA-3′	95°C, 2 min 95°C, 30 s 53°C, 30 s 72°C, 30 s } × 30 72°C, 10 min	413	Abraham *et al.* 2006

Reverse transcription – polymerase chain reaction (RT-PCR)

Reverse transcription PCR was performed as described for both viruses (BLRV and CpCSV) (Oritz *et al.* 2005; Abraham 2005; Abraham *et al.* 2006). Seven µl total RNA, 2 µl reverse primer (Sinaclon, Iran) and 3.5 µl water, were heated at 70°C for 5 min. The reaction was cooled on ice for 2 min and the following reagents added: 4 µl 5X first-strand buffer, 2 µl dNTP (Fermentase, Germany), 0.5 µl ribonuclease inhibitor and 1 µl reverse transcriptase enzyme (Sinaclone, Iran). The reaction was incubated at 37°C for 5 min, then 42°C for 60 min before being inactivated at 70°C for 10 min. PCR reactions using generic luteovirus (BLRV and CpCSV) primers (Table 1) consisted of 1 µl $MgCl_2$, 1 µl forward primer, 1 µl reverse primer and 12.5 µl Red Taq (mini kit, Pishgam BC, Iran). Thermocycling conditions were as follows in two different procedures (Table 1). The presence and identity of viruses in cDNA from infected plant samples were checked by PCR with specific primers as described (Table 1). PCR reactions were replicated at least five times. All PCR products were analyzed in 1–3% agarose gels (Kumari *et al.* 2008; Chomic *et al.* 2010).

Results

The collected samples which showed positive reactions to generic luteovirus monoclonal antibodies (BLRV--5G4 and 6G4) (DSMZ-Germany) and subsequently polyclonal antibody specific to BLRV were used in Elisa tests. When 10 selected samples were tested by RT--PCR using two specific primer pairs (Table 1), chickpea samples numbers 1, 5 and 6 (Nour Abad, Aleshtar and Khoram Abad regions) yielded amplicons of 391 bp product with BLRV primers (Fig. 1). Furthermore, only one sample, number 20 (Sar Aroud region), yielded amplicons of 413-bp product with CpCSV primers (Fig. 2). No amplification was observed from healthy plant extracts.

Two BLRV samples (Nos. 1 and 5) and one CpCSV (No. 20) were sent to Faza Pajoh company (Iran) for sequencing. The target sequences were Blast in Gen Bank and compared, respectively, with 7 BLRV and 6 CpCSV of *Cp* gene sequences of the virus strains registered in Gen Bank (Table 2). *Potato leaf roll virus* isolate Cole-25 (Accession No. HQ171923) was selected as an out group member. Sequence alignment was performed using the Clustal X, Ver. 1.83 program and the phylogenetic tree was drawn using the Mega 4 program (Thompson *et al.* 1994).

Coat protein gene amplification, sequencing and sequence analysis

In molecular assays, approximately ~391 bp products were amplified using specific primers for BLRV and 413 bp products for CpCSV (Table 1). Annealing temperatures for a primer pair were 56°C for BLRV and 53°C for CpCSV. Two nucleotide sequences for BLRV (Figs. 1, 2) and one for CpCSV (Figs. 3, 4) species comprising partial *Cp* gene were obtained after RT-PCR amplification reactions with the appropriate primers. Each of the nucleotide sequences of the CpCSV and BLRV Iranian isolates was compared with corresponding *Cp*

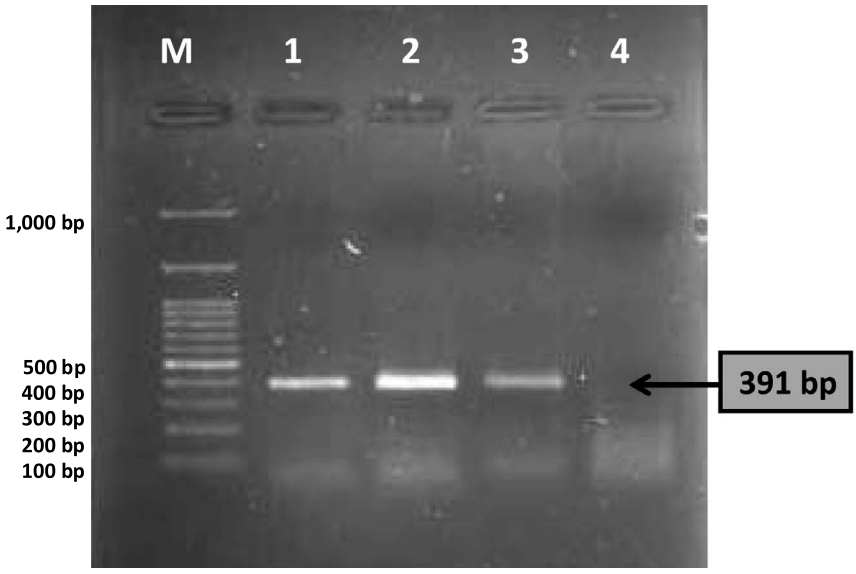

Fig. 1. PCR amplification products using specific BLRV primers: lane M – GeneRuler™ 100 bp DNA ladder (Fermentas), lane 1 – BLRV samples 1 (Nour Abad, Lorestan), lane 2 – BLRV sample 5 (Aleshtar, Lorestan), lane 3 – BLRV sample 6 (Takane, Lorestan), lane 4 – negative (water). PCR products were analysed in 3% agarose gel

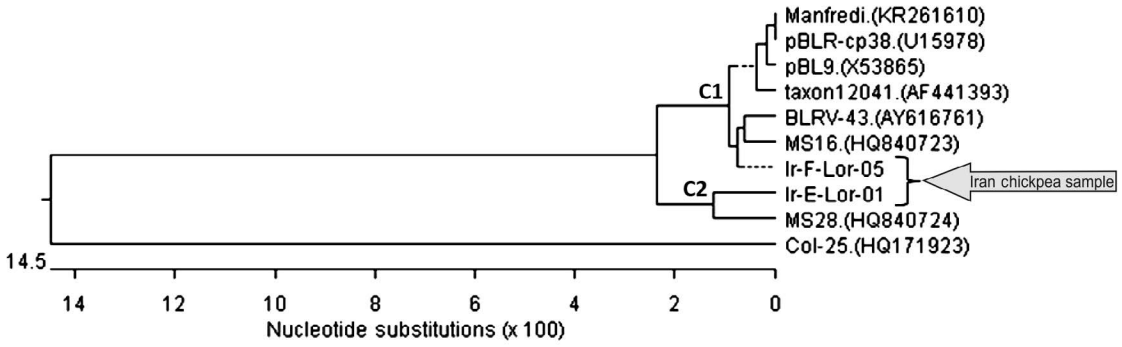

Fig. 2. Phylogenetic tree reconstructed based on comparison sequences isolate of chickpea BLRV and seven different isolates. C1 – cluster 1, C2 – cluster 2

gene sequences of a few representative isolates from other parts of the world (Table 2). Blast algorithm (Altschul *et al.* 1997) and grouping according to a specified isolate were used. Phylogenetic analysis (Megalighn software) based on partial nucleotide sequences (Fig. 2) for BLRV revealed two main clusters. The first cluster contained two subgroups which included isolates from the Alshtar-Lorestan region (IR-F-lor-05-and) besides Tunisian and Spanish isolates. The second BLRV sub-group consisted of an isolate from Noorabad (Lorestan-IR-E-Lor-01) which grouped with the Libyan isolate. CpCSV sequence analysis formed one cluster with two subgroups (Fig. 3). CpCSV Kermanshah isolate from the present study was placed in the first subgroup along with isolates from Tunesia and Azerbaijan. The previously reported CpCSV Iranian isolates (Bananej *et al.* 2010) were placed in the second subgroup along with the Syrian isolate. For both viruses, *Potato leaf roll virus*-isolate (Col 25) was used as an out group sample member (Table 2).

Sequence comparison of the studied isolates of BLRV with similar isolates in NCBI, using Meg Align software, revealed the highest identity (99.4%) between Ir-F-Lor-05 isolate and Manfredi (Acc.No-KR261610), pBLR-cp38(Acc.No-U15978), toxon-12041(Acc.No--AF441393) and BLRV-43(Acc.No-AY616761) from Argentina, Spain, USA and Spain, respectively. The lowest similarity (95.2%) was observed between one

Table 2. *Bean leaf roll virus* (BLRV) and *Chickpea stunt disease associated virus* (CpCSV) isolate comparison

Isolates	Host	Country	NCBI GenBank Accession Number	Percentage similarity with chickpea sample from Iran
BLRV-Isolates				
MS28	*Vicia faba* L.	Libya	HQ840724	98
pBL9	–	Germany	X53865	96
Manfredi	*Medicago sativa* L.	Argentina	KR261610	96
MS16	*Cicer arietinum* L.	Tunisia	HQ840723	96
taxon12041	–	USA	AF441393	96
BLRV-43	*V. faba*	Spain	AY616761	96
pBLR-cp38	–	USA	U15978	96
PLRV-Col-25	*Solanum betaceum* Cav.	Colombia	HQ171923	–
CpCSV-Isolates				
Az296-07	*Legumes*	Azerbaijan	HQ199305	98
Ir-ch-Ker-31	*C. arietinum*	Iran	GU930838	98
Ir-ch-Lor-25	*C. arietinum*	Iran	GU930837	98
Az Cp50-07	*Legumes*	Azerbaijan	HQ180354	98
TuC215-201	*Legumes*	Tunisia	HQ199307	98
16Sy-fb1-03	North Africa and West Asia	Syria	EU541270	98
PLRV-Col-25	*S. betaceum*	Colombia	HQ171923	–

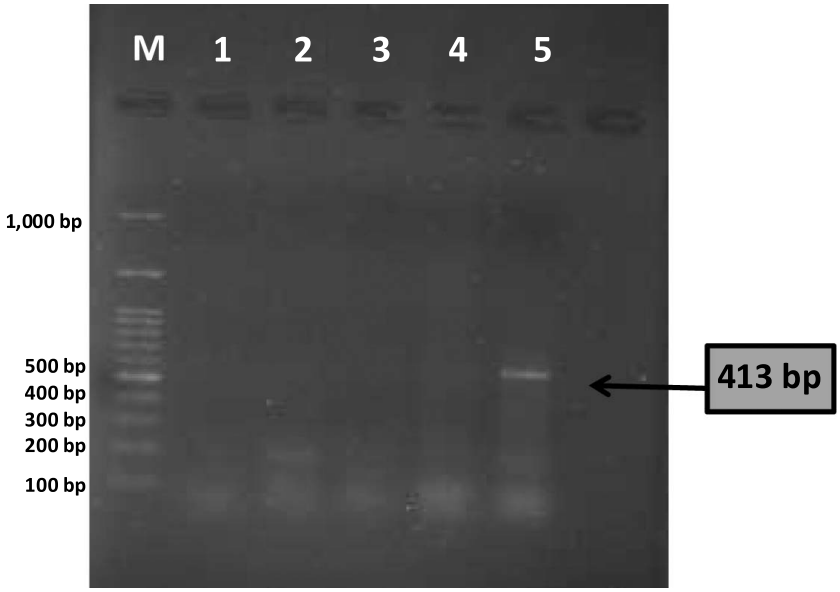

Fig. 3. PCR amplification products using specific CpCSV primers: lane M – GeneRuler™ 100 bp DNA Ladder (Fermentas), lane 1 – negative (water), lane 2 – CpCSV sample 5 (Aleshtar, Lorestan), lane 3 – CpCSV sample 14 (Ravansar, Kermanshah), lane 4 – CpCSV sample 27 (Harsin2, Kermanshah), lane 5 – CpCSV sample 20 (Sar Aroud, Kermanshah). Only sample 20 (lane 5) was positive to CpCSV primer. PCR products were analysed in 3% agarose gel

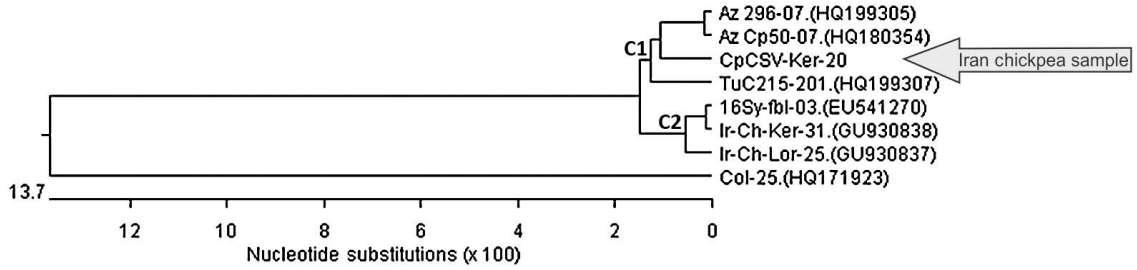

Fig. 4. Phylogenetic tree reconstructed based on comparison sequences isolate of chickpea CpCSV and seven different isolates. C1 – cluster 1, C2 – cluster 2

of the studied isolates (Ir-Lor-05) and MS28(Acc.No--HQ840724) isolate from Libya whereas the similarity between the two studied isolates was 96.4% (Table 3).

In the case of CpCSV the highest identity was 97.8% between the studied isolate and Az296-07(Acc.No--HQ199305) isolate from Azerbaijan and Ir-Ch-Lor--25(Acc.No-GU930837) which was previously reported in Iran. The lowest similarity was 97.3% between the studied isolate and 16Sy-fbl-03(Acc.No-EU541270) (Table 4). There are different reports of similarities in sequence homology of CpCSV Iranian isolates (Bananej *et al.* 2010; Shahraeen *et al.* 2016). Based on the phylogram, it is clear that clustering of the present luteovirus isolates with other strains/isolates was not based on host or geographic region. Evidence for recombination has not been evaluated and there is a need to determine if there is any recombination.

Discussion

Several viruses are known to infect chickpea worldwide, some of which cause serious economic losses (Bos *et al.* 1988; Horn *et al.* 1993, 1995; El-Muadhidi *et al.* 2001; Makkouk *et al.* 2002; Chen *et al.* 2011). There have been reports of chickpea infection by several viral diseases in Iran causing symptoms such as foliar yellowing, chlorosis or leaf reddening, stunting, growth reduction, phloem browning, and leaf narrowing (Kaiser and Danesh 1972; Makkouk *et al.* 2002; Bananej *et al.* 2010; Hydari *et al.* 2011; Shahraeen *et al.* 2012, 2016).

The present study revealed some serological and molecular properties of BLRV and CpCSV (luteoviruses) infecting chickpea in some parts of Iran. Our

Table 3. Percent similarity between analyzed *Bean leaf roll virus* (BLRV) strains

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	
1		72.5	95.8	99.4	98.8	98.8	94.6	98.5	98.8	98.8	1
2	28.1		71.3	72.5	72.5	70.8	70.2	72.5	72.5	72.5	2
3	4.0	28.9		96.4	95.8	95.8	97.6	96.1	95.8	95.8	3
4	0.6	28.1	3.4		99.4	98.8	95.2	99.1	99.4	99.4	4
5	1.2	28.1	4.0	0.6		98.2	94.6	99.7	100.0	99.4	5
6	1.2	29.8	4.0	1.2	1.8		95.8	97.9	98.2	98.2	6
7	5.3	29.8	2.4	4.6	5.3	4.0		94.9	94.6	94.6	7
8	1.5	28.1	3.7	0.9	0.3	2.1	5.0		99.7	99.1	8
9	1.2	28.1	4.0	0.6	0.0	1.8	5.3	0.3		99.4	9
10	1.2	28.1	4.0	0.6	0.6	1.8	5.3	0.9	0.6		10
	1	2	3	4	5	6	7	8	9	10	

1 BLRV-43.(AY616761)
2 Col-25.(HQ171923)
3 Ir-E-Lor-01
4 Ir-F-Lor-05
5 Manfredi.(KR261610)
6 MS16.(HQ840723)
7 MS28.(HQ840724)
8 pBL9.(X53865)
9 pBLR-cp38.(U15978)
10 taxon12041.(AF441393)

Table 4. Percent similarity between analyzed *Chickpea chlorotic stunt virus* (CpCSV) strains

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	
1		96.9	96.6	74.4	97.3	99.8	99.0	96.4	1
2	3.0		99.8	73.7	97.8	96.6	96.9	97.6	2
3	3.2	0.2		73.3	97.6	96.4	96.6	97.3	3
4	26.9	28.0	28.6		73.3	74.0	74.8	74.0	4
5	2.5	2.0	2.2	28.0		97.6	97.8	97.6	5
6	0.2	3.2	3.5	27.5	2.2		98.8	96.1	6
7	1.0	3.0	3.2	26.4	2.0	1.2		96.6	7
8	3.5	2.5	2.7	27.5	2.5	3.7	3.5		8
	1	2	3	4	5	6	7	8	

1 16Sy-fbl-03.(EU541270)
2 Az 296-07.(HQ199305)
3 Az Cp50-07.(HQ180354)
4 Col-25.(HQ171923)
5 CpCSV-Ker-20
6 Ir-Ch-Ker-31.(GU930838)
7 Ir-Ch-Lor-25.(GU930837)
8 TuC215-201.(HQ199307)

findings from sequence comparisons and phylogeny suggest that these two luteoviruses may have been introduced and/or established in the region through diverse agricultural cropping systems, specific vector population activity, susceptible hosts/variety and changes in climate.

Further study is required to investigate the evolution of cool season food legume, (chickpea and lentil) luteovirus like populations in Iran. In this study and in Elisa tests more plant samples showing yellowing symptoms react with BLRV-6G4 batch of monoclonal antibodies than 5G4(DSMZ-Germany) (Shahraeen *et al.* 2016). This suggests that there is a unique epitope to a group of isolates and hence can be used for discrimination of a specific group of BLRV isolates. These limited data suggest that there may be a genetically, biologically and geographically distinct strain group (population) of BLRV and CpCSV infecting cool season food legumes (chickpea) in Iran which require thorough investigation.

Prevalence and variations according to the geographical origin have been reported for other plant viruses from Iran (Shahraeen *et al.* 2002, 2005; Alimoradian *et al.* 2016).

Beet western yellows virus and *Soybean dwarf virus* were also detected in our samplings and serological assays but they are not described in this paper (Shahraeen, unpublished). These two viruses with their detection in samples collected as early as 2002, 2001 (Makkouk *et al.* 2002) in the northern part of the country indicate that the BLRV and CpCSV (luteoviruses), from the cool season food legumes, are not recent (Shahraeen *et al.* 2016). They have probably been infecting the legume plants for decades but remained undetected or were wrongly identified as a different virus of legumes possibly due to the lack of appropriate diagnostic tools or cross-reacting antibodies. Further study on both the incidence and variability of CpCSV and BLRV in the country or in a region is required for determining the economic importance.

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