Iranian Journal of Virology 2021;15 (2): 47-53

Original Article

An Investigation on Natural Occurrence of Soybean Dwarf Virus in Alfalfa Fields

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Abstract

Background and Aims: Alfalfa (Medicago sativa) is the most cultivated forage legume in Iran. Viruses belonging to the Luteoviridae family are among the important yellowing and stunting diseases in food and forage legume crops. Despite the important role of yellowing viruses, limited information is available on the occurrence of viruses from the family Luteoviridae in forage legume fields in Iran. In this study, a survey was conducted to detect Soybean dwarf virus (SbDV, from Luteoviridae family) in alfalfa fields in West Iran.

Materials and Methods: Twenty-one alfalfa fields were surveyed in the Lorestan province (West Iran) in 2011. Plants exhibiting symptoms of dwarfing, yellowing and reddening were collected and tested by tissue-blot immunoassay using monoclonal antibody 5G4. Thirty positive samples with 5G4 were further characterized by RT-PCR using total RNA and a pair of primers to amplify a 370 bp DNA fragment in the coding region of SbDV coat protein (CP) gene. The nucleotide sequence of PCR amplicons was determined and compared with the previously reported data.

Results: Forty-three out of the 127 (33.8 %) leaf samples reacted with antibody 5G4. A DNA fragment of the expected size was amplified in seven out of thirty (23.3%) symptomatic leaf samples, but not in healthy plants. Furthermore, a biological assay using grafting of Vicia faba induced interveinal yellows, one month after grafting and their infection with SbDV was confirmed by RT-PCR. Analysis of the sequences revealed the presence of 370 nucleotides of the SbDV partial coat protein gene. Phylogenetic analysis using the neighbor-joining (NJ) method clustered SbDV isolates into two main groups (D and Y), and SbDV-Irn1 and SbDV-Irn2 isolates fell into group D.

Conclusion: In Iran, the natural infection of SbDV has been reported from chickpea and lentil crops, but there was not any data about its occurrence on other crops and phylogenetic properties. Our results showed for the first time the occurrence of SbDV in alfalfa fields in West Iran using different biological and molecular approaches. Partial nucleotide sequence of CP gene of two Iranian SbDV isolates from alfalfa (Ir.Alf-D1 and Ir.Alf-D2) was determined and revealed that both isolates fell into the type D group of SbDV. The SbDV-D strain causes dwarfing and significant yield losses in a range of economically important food legume crops, so its occurrence in alfalfa fields should serve as an important warning to monitor its occurrence in respective food crops.

Keywords: Serology; Luteoviruses; RT-PCR; Iran

Introduction

Alfalfa (*Medicago sativa* L.) is the most cultivated forage legume in the world. Alfalfa is an important field crop worldwide and is native to a warmer temperate climate such as that of Iran, where it is thought to have originated. It is planted on over 630,000 ha under varied climate conditions in Iran (1). Thirty-two viruses representing thirteen virus groups have been reported to systemically infect alfalfa (2). Several viruses belonging to the various taxa are known to be associated with yellowing and stunting diseases in food and forage legume crops, which includes the viruses belonging to the *Luteoviridae* family. Three genera, namely *Luteovirus*, *Polerovirus*, and *Enamovirus* belong to this family.

Luteoviruses, probably cause greater losses

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than any other group of viruses in plants. Members of this family are phloem-limited with positive-strand RNA (3) and are circulatively-persistently transmitted by several aphid species. Some important luteoviruses such as *Bean leaf roll virus* (BLRV) (4), *Beet western yellows virus* (BWYV) and *Soybean dwarf virus* (SbDV) (5, 6) have been reported to infect alfalfa plants in USA, Australia and European countries. The infection causes degeneration or necrosis of phloem cells, disrupting the movement of assimilates in plants.

Soybean dwarf virus (SbDV) is an aphid-transmitted *Luteovirus* that can cause high soybean yield losses especially in Japan (7) and is capable of infecting more than 50 leguminous species like chickpea, lentil, and bean (6, 8). SbDV, like other luteoviruses, is transmitted by aphids in a circulative, persistent manner, and icosahedral particles of virus are localized in the phloem with low concentration (5). SbDV isolates have been grouped into two main strain types, dwarfing (SbDV-D) and yellowing (SbDV-Y), on the basis of host range and symptoms (9). Furthermore, at least four unique strains of SbDV have been described in Japan based on symptoms and their vector including SbDV-DS (dwarfing, solani), DP (dwarfing, pisum), YS (yellowing, solani), and YP (yellowing, pisum) (10).

In spite of the important role of yellowing viruses, limited information is available on the occurrence of viruses from the family *Luteoviridae* in forage legume fields (e.g. alfalfa) in Iran. Recently, *Bean leafroll virus* (BLRV), the other member of the *Luteovirus* genus, has been reported from alfalfa fields in Southeast Iran (11). Although SbDV has been reported from annual food legumes including chickpea and lentil in Iran (12), little is known about the incidence of this virus in alfalfa fields.

Methods

Sampling: To determine luteoviruses in alfalfa in Iran during a survey in the 2011 growing season, 127 symptomatic leaf samples of alfalfa showing mosaic, mottling, dwarfing, yellowing, and reddening symptoms were collected from 21 fields in Lorestan province (Fig. 1).

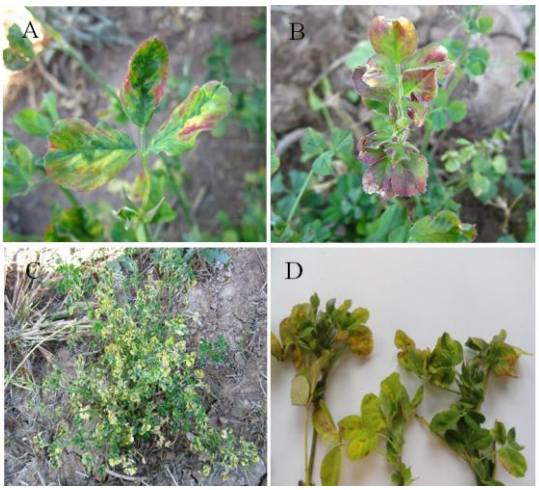

Fig. 1. Yellowing (A), reddening (B), dwarfing (C) and malformation (D) symptoms observed in alfalfa fields.

Samples were immediately transported in labeled plastic bags on ice in cold boxes to the laboratory and kept at 4°C prior to being tested. Virus disease incidence in each randomly chosen field was estimated according to the reactions of leaf sample extracts in diagnostic assays initially by serological assays followed by the RT-PCR as described below.

Tissue Blot Immune Assay (TBIA): Leaf extracts were tested by tissue blot immunoassay (TBIA) according to Makkouk and Kumari (13), using 5G4 monoclonal antibody which has been reported to react with a broad-spectrum of legume infecting luteoviruses (14). Petioles were cut with a new razor blade in a steady motion to obtain a single plane cut surface and pressed on a Nitrocellulose membrane (BA-45, Schleicher & Schuell, Germany). The membrane was washed three times with phosphate-buffered saline (PBS) (3 mM KCl, 3 mM NaN3, 8 mM Na2HPO4, 1 mM NaH2PO4, and 0.13 M NaCl, pH 7.4) containing 0.05% Tween 20 (PBST) at 5-min intervals, blocked in 1-µg/ml polyvinyl alcohol in PBST, and incubated 30 min at room temperature (RT). After washing, the membrane was placed in 1:1,000 dilution of monoclonal antibody 5G4 which has been reported to react with a broad-spectrum of

legume infecting luteoviruses in conjugate buffer (2% polyvinylpyrrolidone-24000, 0.05% Tween 20, 0.2% bovine serum albumin, and 1 mM MgCl2 in PBS, pH 7.4). The membrane was washed and incubated in 1:10,000 dilution of alkaline phosphatase-conjugated goat antimouse IgG (Sigma Chemical Co., St. Louis, MO) for one hour at RT. The membrane was washed, and directly placed in color development solution (0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl, 5 mM MgCl2, 0.33 mg/ml nitro blue tetrazolium, and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) prepared just before use. All buffers used contained 0.02% sodium azide as a preservative. Development of blue-purple color in dotted areas was interpreted as infected samples. SbDV infected chickpea (11) was used as positive control and young healthy alfalfa plants cultivated in insect-free greenhouse was used as the negative control. Positive samples for BLRV, BWYV, and SbDV were used as previously reported (12).

Graft inoculation test: Seventy-five healthy faba bean (*Vicia fabae*) plants were grafted with TBIA positive (SbDV infected) alfalfa samples. One to two nodes of each SbDV infected alfalfa stem are selected and leaves and apical tip were removed to reduce evaporation and early abort of the scion. The vascular system of the scion was exposed by a long cut on two sides to create a wedge. In the healthy faba bean plants, leaves around the scion insertion side were removed and a longitudinal cut was made into the stem in which the wedge cut scion was inserted so that both vascular systems join. The graft insertion side was taped tightly with parafilm to prevent desiccation and the grafted plant was protected from excessive evaporation by a plastic bag cover. This cover was removed after 2-3 days (3). The infection of grafted plants was checked by RT-PCR.

RT-PCR and sequence analysis: Thirty samples showing positive reaction with 5G4 were selected and further tested by the reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was extracted from 100 mg of leaf tissue using 1 ml of RNA extraction solution TRI Reagent® (Sigma Chemical, St. Louis, MO) according to the manufacturer's instructions and dissolved in 20 µl of diethylpyrocarbonate (DEPC)-treated water after a brief air drying.

Table 1. Information of selected Soybean dwarf virus (SbDV) sequences used in this study for phylogenetic analysis.

No	GenBank Acc. No.	Isolate name	Country origin	Strain type
1	EU306587	PA	USA	D
2	EU306582	MD8	USA	D
3	EU306581	MD7	USA	D
4	DQ145545	Wisc3	USA	D
5	EU306585	NY	USA	D
6	EU306588	SC1	USA	D
7	EU306578	MD2-D	USA	D
8	EU306591	MD9-D	USA	D
9	EU306589	VA	USA	D
10	EU306580	MD3-D	USA	D
11	AB038147	YS	Japan	Y
12	NC-003056	YS	Japan	Y
13	EU306576	KY-1	USA	Y
14	EU306590	VA.Y	USA	Y
15	EU306579	MD2-Y	USA	Y
16	EU306586	NC-Y	USA	Y
17	EU306584	MS.Y	USA	Y
18	EU306577	MD-1.Y	USA	Y
19	EU306583	MD8.Y	USA	Y
20	L20835	Y	USA	Y
21	Ir.Alf-D1	Ir.Alf-D1	Iran(this study)	D
22	Ir-Alf-D2	Ir.Alf-D2	Iran(this study)	D

First-strand cDNA synthesis was performed using M-MuLV reverse transcriptase (Fermentas, Lithuania) with the previously described primers (15). Synthesized cDNA was used as template DNA, with *Taq* polymerase (SinaClon, Iran) in PCR. Cycle conditions were one cycle of 95°C for 5 min; 35 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 2 min; and one cycle of 72°C for 10 min. The PCR products were analyzed by electrophoresis in a 1 % agarose gel. PCR products of four SbDV isolates were separated by electrophoresis in agarose gels. The expected fragments were excised from the gels and cleaned by the QIAquick Gel Extraction Kit (QIAGen) according to the manufacturer's recommendations. The DNA amplicons were sequenced in both directions by Sanger's dideoxynucleotide chain termination method.

Nucleotide sequences of two SbDV isolates (obtained in this study) together with 20 available sequences of SbDV in GenBank were aligned using CLUSTAL X (16) with Trans-align (17) for optimal alignment (Table 1). The phylogenetic tree was inferred using the NJ method implemented in MEGA X (18).

Results

The use of the TBIA technique greatly facilitated the detection of such a broad array of samples tested. The reactions of diseased and non-diseased plant samples in TBIA with the antisera used were clearly different. Serological results showed 43 of the 127 (33.8 %) leaf samples reacted with the general *Luteovirus* monoclonal antibody 5G4 (Fig. 2).

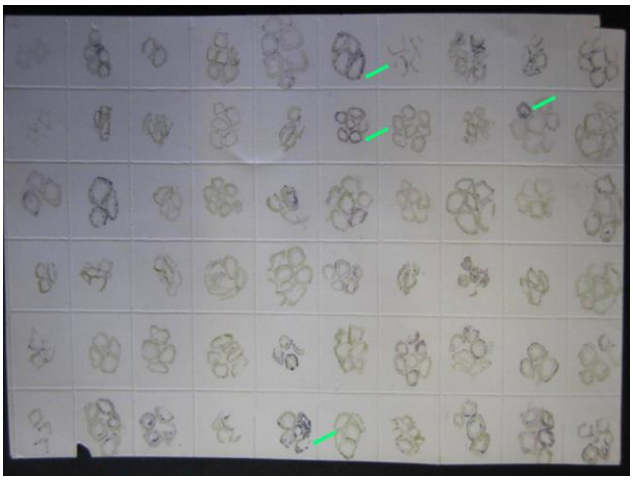

Fig. 2. Results of tissue blot immunoassay (TBIA) of alfalfa tissue using 5G4 monoclonal antibody. Luteovirus infected samples developed a purple-blue color (shown by arrows), whereas non-infected ones did not.

The reactions of plant samples in TBIA with the antibody used were clearly different between diseased and non-diseased ones, i.e., a positive reaction was indicated by the development of a blue-purple color, whereas in negative reaction tissue blots remain green. Surveys were conducted in 21 alfalfa fields distributed over Lorestan province West Iran.
The most prevalent symptoms associated with alfalfa plants affected by SbDV were yellows, dwarfing, and reddening. SbDV infections of alfalfa samples were also successfully transmitted by grafting to *V. faba* and interveinal chlorosis was observed three weeks post-inoculation which their SbDV infection was verified by RT-PCR. This confirms the symptoms previously reported for SbDV (19).
Thirty samples showing positive reaction with 5G4 were selected and further tested by reverse transcription-polymerase chain reaction (RT-PCR) method using specific primers designed to amplify a DNA fragment in SbDV coat protein (CP) gene. Amplification of a DNA fragment with the expected size (372 bp) was observed in 7 out of 30 (23.3%) symptomatic leaf samples, but not in healthy plants (Fig. 3).

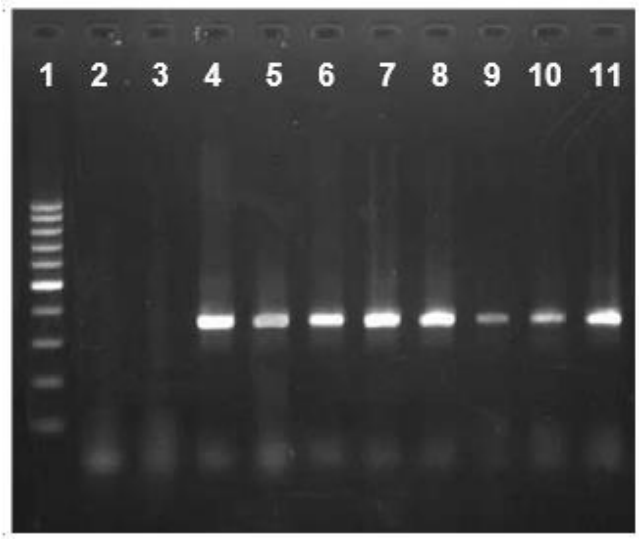

Fig. 3. Agarose gel electrophoresis of PCR products using specific primers designed to amplify a DNA fragment of ca. 370 bp of SbDV CP gene. Lane 1, 100 bp DNA ladder (ThermoFisher scientific, Lithuania), Lanes 2 and 3, healthy plant extracts, lane 4, positive control (SbDV infected), lanes 5-11, alfalfa samples infected with SbDV.

Nucleotide sequence of PCR amplicons was determined for two isolates (Ir.Alf-D1 and Ir.Alf-D2). Phylogenetic analysis based on CP nucleotide sequences showed that two Ir.Alf-D1 and Ir.Alf-D2 isolates fell in type D group of SbDV (Fig. 4). Overall CP partial nucleotide identity between SbDV isolates in this study ranged from 91.8% to 100%. However, Comparative sequence analysis revealed that Ir.Alf-D1 and Ir.Alf-D2 isolates shares the highest nucleotide sequence identity (97.9-98.7%) with EU306580 (MD3 isolate from USA) and the lowest nucleotide sequence identity (91.8-92.4%) with AB038147 and NC-003056 (both from Japan) (Fig. 5)

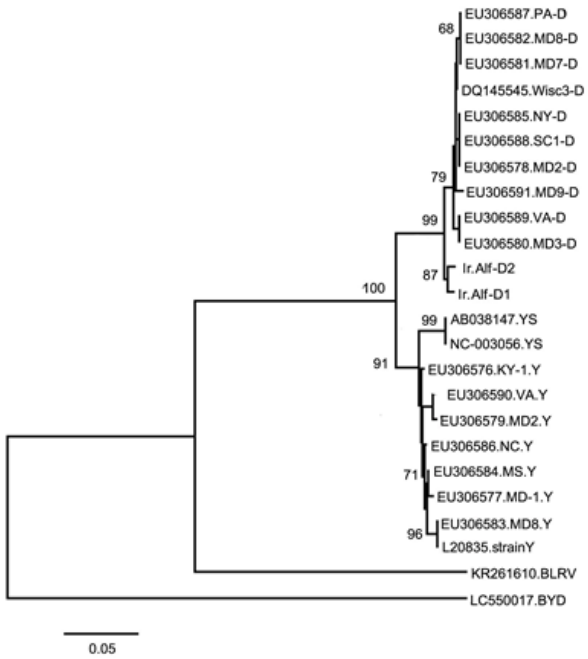

Fig. 4. Phylogenetic tree reconstructed based on the nucleotide sequences of SbDV coat protein gene. Bean leaf roll virus (BLRV) and Barley yellow dwarf virus (BYDV) are the chosen outgroups.

Discussion

Serological results showed 43 of the 127 (33.8 %) leaf samples reacted with the general *Luteovirus* monoclonal antibody 5G4 (a monoclonal antibody against a wide range of legume infecting luteoviruses) in TBIA. This result shows that the luteoviruses are one of the most predominant problems in all alfalfa fields surveyed in Lorestan province. Furthermore, it confirmed the infection of alfalfa fields by luteoviruses as previously reported (5, 6). However, twenty-three symptomatic samples with positive reaction with 5G4 antibody in TBIA did not produce any DNA fragment in RT-PCR using SbDV specific primers. Further studies are required to determine possible other virus(es) infections in these symptomatic samples.

The incidence of infections differed among the regions surveyed. This variation could be caused by many ecological factors such as the presence or abundance of virus reservoirs. In this study, the most important alfalfa aphids including the green peach aphid (*Myzus persicae*), spotted aphid (*Therioaphis maculata*) and pea aphid (*Acyrthosiphon pisum*) were observed. The aphids as vectors of luteoviruses colonize the newly grown spring plants and multiply rapidly and can lead to the rapid spread of the disease (14). In addition, differences in the rate of infections may be due to the uneven distribution of inoculum sources and/or the different climatic conditions in alfalfa areas surveyed.

Perennial legumes like alfalfa can provide an unremitting inoculums reservoir and are hence of great significance to the epidemiology of viruses. In spite of using insecticides in food legume crops (particularly against aphids), less or no insecticide used for alfalfa and in many cases alfalfa fields left without any attention to the control of pests. Aphids persistently transmit SbDV and many plants may become infected near the site where the viruliferous aphids land and move about. Also relatively short rotations commonly employed in annual food legumes production lead to the occurrence of overlapping of these crops with legume pasture species (e.g. alfalfa) or weed species and a greater likelihood of volunteer plants from unharvested seed which can act as virus reservoirs (2). A biological assay using indicator plant *V. faba* confirmed the presence of SbDV in alfalfa fields. However, no biological differentiation was found among Iranian SbDV isolates.

Previous phylogenetic studies using coat protein sequences showed that SbDV population fell into two main groups (Y and D) (20). Phylogenetic tree reconstructed using CP sequence of 22 SbDV isolates including Ir.Alf-D1 and Ir.Alf-D2 (this study), confirm the previous reports and indicating that the phylogenetic clustering of SbDV strains based on CP is not clearly related to the geographic separation of the isolates. SbDV has been reported from chickpea and lentils from Iran (11), but there is not any data regarding their genomic nucleotide sequences. However, more SbDV isolates from various hosts and locations should be sequenced and analyzed for a better understanding of phylogenetic structure of SbDV population.

Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
	100.0	100.0	99.7	99.5	99.5	99.5	99.2	99.2	99.2	97.9	98.2	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	1	EU306587.PA-D
		100.0	99.7	99.5	99.5	99.5	99.2	99.2	99.2	97.9	98.2	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	2	EU306582.MD8-D
			99.7	99.5	99.5	99.5	99.2	99.2	99.2	97.9	98.2	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	3	EU306581.MD7-D
				99.7	99.7	99.7	99.5	99.5	99.5	98.2	98.4	92.9	92.9	94.5	93.2	93.4	93.9	93.9	93.7	93.4	93.4	4	DQ145545.Wisc3
					100.0	100.0	99.2	99.2	99.2	98.4	98.7	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	5	EU306585.NY-D
						100.0	99.2	99.2	99.2	98.4	98.7	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	6	EU306588.SC1-D
							99.2	99.2	99.2	98.4	98.7	92.6	92.6	94.2	92.9	93.2	93.7	93.7	93.4	93.2	93.2	7	EU306578.MD2-D
								98.9	98.9	97.6	98.4	92.4	92.4	93.9	93.2	93.4	93.4	93.4	93.2	92.9	92.9	8	EU306591.MD9-D.
									100.0	98.7	97.9	92.4	92.4	93.9	92.6	92.9	93.4	93.4	93.2	92.9	92.9	9	EU306589.VA-D
										98.7	97.9	92.4	92.4	93.9	92.6	92.9	93.4	93.4	93.2	92.9	92.9	10	EU306580.MD3-D
											99.2	91.8	91.8	93.9	92.6	92.9	93.4	93.9	93.7	93.4	93.4	11	Ir.Alf-D2
												92.1	92.1	94.2	93.4	93.7	93.7	94.2	93.9	93.7	93.7	12	Ir.Alf-D1
													100.0	97.4	96.6	97.4	97.4	97.4	97.1	97.1	97.1	13	AB038147
														97.4	96.6	97.4	97.4	97.4	97.1	97.1	97.1	14	NC_003056.strain
															98.2	98.9	99.5	99.5	99.2	98.7	98.7	15	EU306576.KY-1
																99.2	98.2	98.2	97.9	97.4	97.4	16	EU306590.VA-Y
																	98.9	98.9	98.7	98.2	98.2	17	EU306579.MD2-Y
																		99.5	99.2	98.7	98.7	18	EU306586.NC-Y
																			99.7	99.2	99.2	19	EU306584.MS-Y
																				98.9	98.9	20	EU306577.MD-1
																					100.0	21	EU306583.MD8-Y
																						22	L20835.strain

Fig.5. Percent nucleotide sequence identity of coat protein gene among 22 Soybean dwarf virus isolates including two Iranian SbDV isolates from alfalfa (Ir.Alf-D1 and Ir.Alf-D2). Details of the isolates are presented in table 1.

This survey was restricted to main alfalfa-growing areas in Lorestan province. An extension of this survey to other areas of Iran may reveal the presence of more viruses and viral strains in the crop. The importance of forage legumes especially alfalfa crops to the economy of these regions illustrate the significance of gathering information on the viral spread and subsequent crop losses. Characterization of the virus population structure and determination of its genetic diversity are important factors to the development of efficient and permanent control measures for alfalfa viruses and provides the opportunity for us to implement preventive strategies and may prevent spread to other susceptible crops.

Conclusion

Natural occurrence of SbDV infection on chickpea and lentil crops have been previously reported from Iran (12), however, there was not any data about its occurrence on other crops and phylogenetic properties of Iranian SbDV isolates. Our results showed for the first time the occurrence of SbDV in alfalfa fields in West Iran using different biological and molecular approaches. Partial nucleotide sequence of CP gene of two Iranian SbDV isolates from alfalfa (Ir.Alf-D1 and Ir.Alf-D2) revealed that both isolates fell into the type D group of SbDV. The SbDV-D strain causes dwarfing and significant yield losses in a range of economically important food legume crops worldwide, so its occurrence in alfalfa fields should serve as an important warning to monitor its occurrence in respective food crops.

Acknowledgment

We would like to thank the plant virus research department of Iranian research institute of plant protection for availing the facility to carry out the study.

Conflict of interest

No conflict of interest is declared

Funding

This work was supported in part by The Iranian Research Institute of Plant Protection (IRIPP) [agreement No. 5068-90/12/09].

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