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Detection and Identification of Some Soybean Viral Mosaic Viruses, Using Molecular Techniques in Lorestan Province, South West of Iran

[1]A. Naghavi, [2]M.K. Habibi and [3]F.N. Firouzabadi
[1]Plant Protection Group, Faculty of Agriculture, Lorestan University,
P.O. Box 465, Khorramabad, Iran
[2]Plant Protection Group, Faculty of Agriculture, Tehran University, P.O. Box 4111, Karaj, Iran
[3]Agronomy and Plant Breeding Group, Faculty of Agriculture,
Lorestan University, P.O. Box 465, Khorramabad, Iran

Abstract: In this study to identify and characterize the causal agents of the soybean mosaic viral disease in Lorestan province, South west of Iran. Soybean (*Glycine max* L.) is frequently attacked by many devastating mosaic viral diseases. A total number of 254 samples of infected soybean plants showing mosaic, deformation and leaf roll symptoms were collected from soybean fields. The Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay (DAS-ELISA) and Antigen Coated Plate-ELISA (ACP-ELISA) techniques were used to test the collected samples for the presence of the following viruses, *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Soybean mosaic virus* (SMV), *Bean yellow mosaic* (BYMV), *Bean common mosaic virus* (BCMV), *Potyvirus*, *Tomato spotted wilt virus* (TSV), *Tobacco ring spot virus* (TRSV). Four viruses namely AMV, SMV, BCMV and CMV were detected by molecular techniques out of them AMV was found the most prevalent virus in Lorestan province. The Western blot analysis using infected plant samples confirmed the association of presence of expressed viral proteins and viral disease symptoms. Proteins about 30 and 27 kDa were identified which corresponded well to the expected molecular weight of AMV and SMV Coat Proteins (CP), respectively. The Immunocapture-Revers Transcription-Polymerase Chain Reaction (IC-RT-PCR) was performed using SMV-CPr and SMV-CPf primer pairs. An approximately 500 bp fragment was amplified. In order to differentiate the SMV strains, SMV-G_2 and SMV-G_7 primer pairs were used in IC-RT-PCR. None of the strains showed reaction with G_2 strain primers and no fragment was amplified but all of the strains amplified a 300 bp fragment with G_7 strain primers.

Key words: Soybean, mosaic, virus, ELISA, IC-RT-PCR

INTRODUCTION

Soybean (*Glycine max* L.) is an annual plant belonging to the Leguminosae family, which provides both protein and oil for human nutrition (Dragoljob *et al*., 1999). Soybean viral diseases are among devastating diseases with significant impact on yield loss, alteration of seed composition and seed coat mottling (Giesler *et al*., 2002). More than 111 viruses/strains, belonging to different virus genera and families are able to infect soybean under natural conditions (Hartman *et al*., 1999). The SMV, one of the most economically damaging viruses, transmitted through seed. It is also the alternate natural host of several other viruses including BPMV, BYMV, TSV and TRSV, which are also naturally widespread and occasionally represent a threat to soybean production (Dragoljob *et al*., 1999). Viral symptoms range from latent infections to plant death. Viruses may induce stunting, rugosity, mosaic patterns, yellowing of foliage and necrosis. The way in which two or more viruses interact in a single plant can be additive, synergistic or cross-protective (Hartman *et al*., 1999). Some symptoms may lead to deterministic field diagnosis of the disease. However, most symptoms overlap and often require laboratory techniques, such as serology or Polymerase Chain Reaction (PCR) for diagnosis. Investigations of incidence and distribution of soybean viruses are very important in developing diagnostic systems and appropriate control measures. There have been a few previous studies on soybean viral diseases and their distribution in Iran. In Golestan and Mazandaran provinces, North of Iran, TSV and

Corresponding Author: F.N. Firouzabadi, Agronomy and Plant Breeding Group, Faculty of Agriculture, Lorestan University, P.O. Box 465, Khorramabad, Iran

TRSV were isolated from soybean plant showing Pod Set Failure syndrome (PSF) and appeared to be of great concerns to farmers in some seasons (Rahimian *et al*., 1995). SMV has also been reported previously from Iran (Golnaraghi *et al*., 2004). Since Lorestan province is one of the most important regions of soybean cultivation in Iran, this study was conducted to recognize and determine the incidence and distribution of the causal agents of soybean mosaic viral diseases using serological and molecular methods. The results of this study can be used for precise identification and control of these agents in soybean fields.

MATERIALS AND METHODS

Sample collection: During two consecutive summers (2004 and 2005), a total of 254 symptomatic leaf samples showing mosaic, deformation and leaf roll were collected from 25 different soybean fields across the whole region (Table 1). Each sample was collected from a different plant and immediately bagged and transported in cold boxes. Collected samples were stored at 4°C for further analysis. Following molecular and serological experiments were done at laboratory of plant pathology of Tehran University, Kraj, Iran.

Virus identification: DAS-ELISA was performed, using a polyclonal antiserum against SMV (DSMZ, AS-0543), AMV (DSMZ, AS-0779), CMV (DSMZ, AS-0475), BCMV (DSMZ, AS-0241), BYMV (DSMZ, AS-0471), PVY (DSMZ, AS-0137) and TSWV (DSMZ, AS-0105) according to the Clark and Adams method (1977). Each step of ELISA was followed by 4 h incubation at 37°C. Samples were washed with a PBST washing buffer (8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g NaN_3, 0.2 g KCl g L^{-1} containing 0.05% Tween-20, pH 7.4). Ten milliliters of sample buffer (PBST containing 2% polyvinylpyrolidine (PVP-2400)) was added to 1 g grounded tissue samples, subsequently, 200 μL of supernatant was loaded onto each well. The reaction was read using a colorimeter at 405 nm after adding conjugate incubation with substrate for 1 h. Samples with absorbance values greater than or equal to three times of negative samples were considered infected (positive).

Table 1: Numbers of field and sample collected from different regions of Lorestan province

Location	Field	Leaf samples
Khorammabad	6	60
Borojerd	3	14
Aleshtar	12	160
Chaghalvandi	4	20

Host range infection: The Soybean leaf sample with positive reaction in ELISA were selected and grounded in 0.1 M sodium-phosphate buffer, containing 0.2% sodium sulfate at pH 7.0. In the host range trial, at least three plant species or cultivars from Chenopodiaceae, Fabaceae and Solanaceae families were mechanically inoculated. The plants were kept in a greenhouse at 25±5°C, 50 to 70% relative humidity and observed after inoculation for one to three weeks. The presence of related viruses in inoculated plants was checked by ELISA.

Viral proteins separation: Purified virion suspensions and plant infected samples with SMV and AMV isolates were incubated in sample buffer and boiled at 100°C for 5 min. Twenty five microliters of supernatants were loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with coomassie brilliant blue. Consequently, proteins were separated electrophoretically and transferred to a nitrocellulose membrane using the buffer styles of Sambrook *et al*. (1989). A dilution of 1:1000 (v/v) of anti-SMV and AMV sera were used to detect viral proteins.

IC-RT-PCR analysis: IC-RT-PCR was performed to detect CMV (Han-Xin *et al*., 2004), AMV (Martinez-Priego *et al*., 2004), BCMV (Paiambar *et al*., 2004 unpublished data) and SMV (Wang and Ghabrial, 2002) and its strains (Omunyin *et al*., 1996). One hundred milligrams of infected leaf materials was grounded in 1 mL extraction buffer. Then 100 μL of the supernatant was added to a tube coated with antisera and kept for 2 to 4 h at 37°C. Tubes were washed with PBST. Subsequently, cDNA was made in the same tube containing the RNA as template for Reverse Transcriptase. A final volume of 20 μL, including 12 μL deionized H_2O, 4 μL 5xRT buffer, 1 μL DTT, 1 μL dNTPs, 0.5 μL RNase inhibitor, 1 μL reveres primer (Table 2). The reaction was incubated at 42°C with 0.5 μL

Table 2: Genomic region of the different viruses and the feature of primers used for amplification by IC-RT-PCR

Pathogen	Size	Genomic region	Length	Reference
SMV	469 bp	CP gene	23(F), 21(R)	Wang and Ghabrial (2002)
SMV-G7	277 bp	CP gene	20(F), 20(R)	Omunyin *et al*. (1996)
AMV	700 bp	CP gene	20(F), 21(R)	Martinez-Priego *et al*. (2004)
CMV	678 bp	CP gene	23(F), 21(R)	Han xin *et al*. (2004)
BCMV	700 bp	CP and Nib gene	20(F), 20(R)	Paiambary *et al*. (2004)

Cp: Coat protein; R: Reverse primer; F: Forward primer; NIb: Nuclear inclusion protein b

Table 3: The thermal programs of PCR for CMV, AMV, BCMV and SMV and its strains

PCR steps	SMV	SMV strains (G2 and G7)	AMV	CMV and BCMC
Denaturing	94°C/1 min	94°C/1 min	94°C/45 sec	94°C/1 min
Annealing	55°C/1 min	56°C/45 sec	55°C/30 sec	52°C/1 min
extension	72°C/1 min	72°C/1 min	72°C/30 sec	72°C/3 min
No. of cycle	30	30	35	35

Cll: chlorotic local lesion; nll: necrotic local lesion; ld: Leaf distortion; m: mosaic; sm: seed mottling

MMLV reverse transcriptase for 1 h. For all viruses except SMV and its strains, PCR-reaction was as follow: 5 μL of the of cDNA was added to PCR reaction including a 2.5 μL 10x PCR buffer, 1 μL $MgCl_2$, 0.5 μL reverse primer, 5 μL cDNA, 0.6 μL forward primer, 0.5 μL dNTPs and 0.3 μL Taq polymerase.

For SMV and its strains PCR performed by using 10.2 μL H_2O, 2 μL PCR buffer 10x, 0.8 μL $MgCl_2$, 5 μL cDNA, 1 μL dNTPs, 0.3 μL reveres primer, 0.4 μL forward primer (10 pmole μL^{-1}) and 0.3 μL Taq polymerase. The thermal PCR programs are showed in Table 3.

RESULTS

AMV frequency detection: ELISA analysis showed that the viral disease incidence in a decreasing order was AMV (23.62), SMV (6.26), BCMV (1.57) and CMV (0.78%). Field symptoms associated with virus infection included mosaic, mottling, vein clearing and vein necrosis with SMV, mosaic and mottling with AMV (Fig. 1a, b), CMV and BCMV. Although plants were frequently infected with more than one virus, it was not possible to pinpoint every specific symptom with a particular virus.

Host range infection: Although the biological assay is suitable for virus detection, it is not generally sufficient for identification of the viruses. In order to identify the host range of SMV, AMV and CMV, a biological assay was carried out according to description of the viruses (Hartman *et al*., 1999; Anonymous, 2002; Palukaitis and Garcia-Arenal, 2003). Soybean seedlings were systematically infected (Fig. 2a, b). Although inoculation of *Chenopodium quinoa* and *C. album* with SMV isolates resulted in chlorotic local lesion symptoms (Fig. 2c), the same isolates could not infect *C. amaranticolor*, *Nicotiana rustica* and *N. glutinosa*. Soybean seeds were found with frequent mottling symptoms (Fig. 2i). Interestingly and in contrast to SMV, AMV isolated from soybean systematically infected *C. amaranticolor*, *C. quinoa*, *C. album* and *Vigna unguiclata* (Table 4, Fig. 2d-f). The CMV readily gave mosaic symptoms on soybean as well as on *N. rustica* (Table 4, Fig. 2 g, h).

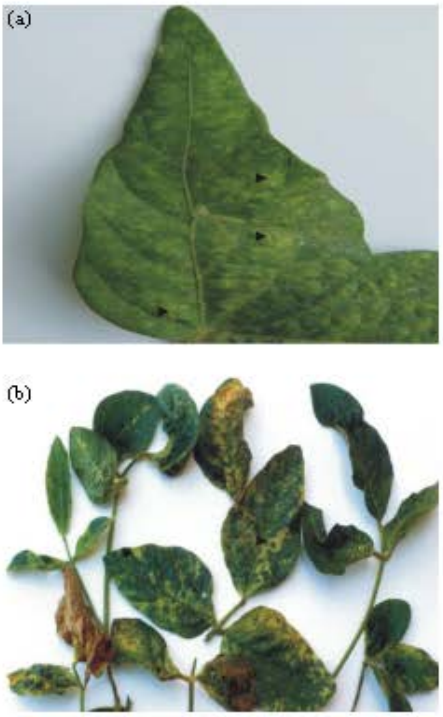

Fig. 1: Symptoms of mosaic disease caused by SMV (a) and (b)AMV

Table 4: Reaction of selected indicator plant species to different viruses infecting soybean

Viruses	Indicator plant	Symptoms
SMV	*Chenopodium quinoa*	Cll
	Chenopodium album	Cll
	Chenopodium amaranticolor	-
	Nicotiana rustica	-
	Nicotiana glutinosa	-
	Glycine max	m, ld, sm
AMV	*Chenopodium quinoa*	m
	Chenopodium album	m
	Chenopodium amaranticolor	m
	Vigna unguiculata	nll
	Nicotiana glutinosa	-
	Nicotiana rustica	-
	Glycine max	m
	Medicago sativa	m
CMV	*Nicotiana rustica*	m
	Vigna unguiculata	nll
	Lycopersicon esculentum	m
	Glycine max	m
	Cucumis sativus	m

Capsid proteins detection: The Electrophoretic analysis of viral capsid proteins revealed the presence of two proteins. A 30 and 27 kDa proteins were recognized, using polyclonal antibodies. These proteins corresponded well with an expected molecular weight of SMV and AMV coat proteins, respectively. Interestingly, the same proteins were identified with coomassie blue staining, indicating the high amount of such proteins. No band(s) was detected with control plants (Fig. 3, 4).

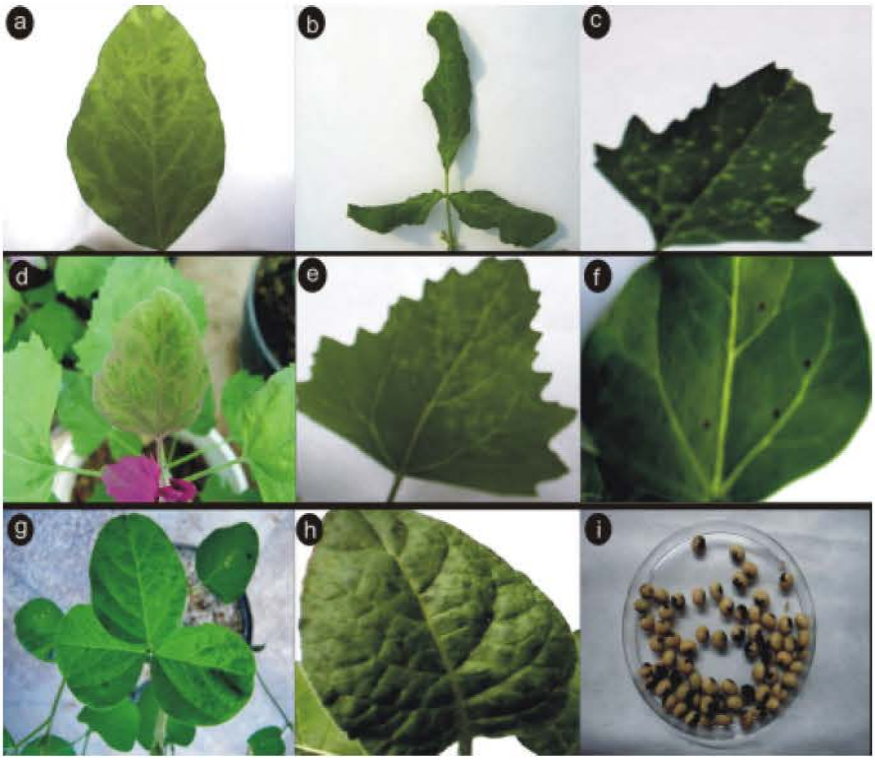

Fig. 2: Photograph of the viral symptoms on different plant hosts leaves SMV on *G. max* (a, b) and *C. quinoa* (c, d, e and f) AMV on *C. amaranticolor*, *C. quinoa* and *V. unguiculata* (g and h) CMV on *G. max* and *N. rustica* (i) SMV on *G. max*

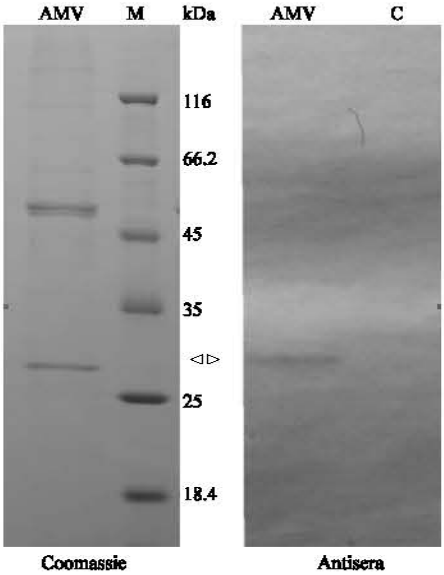

Fig. 3: Denaturing polyacrylamide gel electrophoresis and electro blot immunoassay of capsid protein with specific antiserums of every virus for infected samples to SMV. M and C show size marker and negative control, respectively

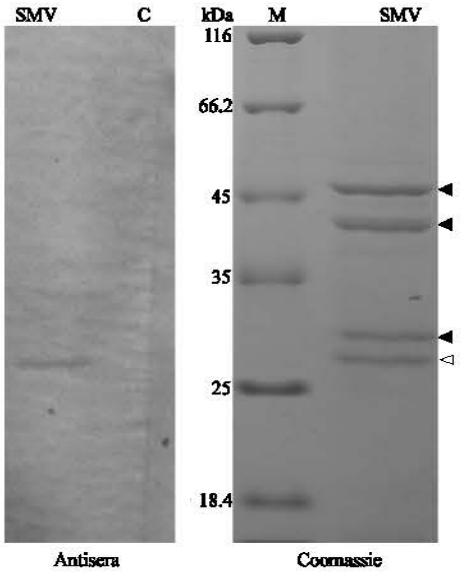

Fig. 4: Denaturing polyacrylamide gel electrophoresis and electro blot immunoassay of capsid protein with specific antisera of virus infected samples by AMV. Marker proteins (M), negative control (C) and purified virus (AMV)

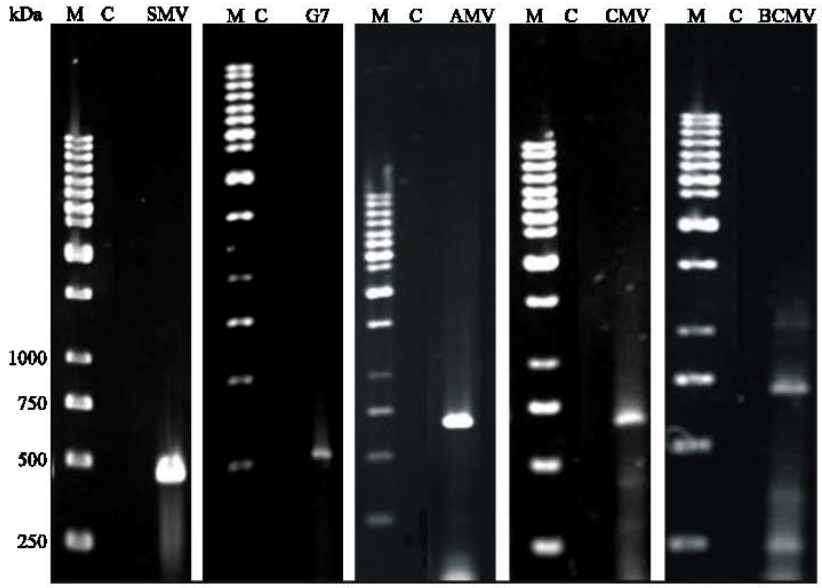

Fig. 5: A 2% agarose gel electrophoresis of IC-RT-PCR products amplified with specific primer pairs for SMV and AMV, BCMV and CMV strains. M and C show size marker and negative control, respectively. For negative control, materials were isolated from a healthy leaf

Viral coat protein analysis: IC-RT-PCR with specific SMV coat protein primers (Table 2) resulted in an approximately 500 bp fragment, which was in accordance with the SMV coat protein gene. To differentiate the SMV strains, specific SMV-G2 and SMV-G7 primer pairs were used for amplification. SMV-G2 primer pairs could not fish out any fragment. Interestingly, SMV-G7 primers amplified an approximately 300 bp fragment, using all SMV isolates. The same procedure was employed with specific AMV and CMV coat protein gene primers. For both viruses, an approximately 7 00 bp fragment was amplified. BCMV specific coat protein gene primers gave rise to an approximately 700 bp fragment (Fig. 5).

DISCUSSION

Golnaraghi *et al.* (2004) reported that SMV had the highest incidence in all Iranian provinces tested, including Lorestan. In contrast to their research, AMV is the most prevalent virus in Lorestan province among all the viruses tested. Since these authors have not checked the infection rate of cultivated seeds, they might have been working with pre-infected soybean fields. Another reason could be associated with plant cultivars. Lorestan province Southern regions have the highest forage more specifically an alfalfa cultivation area in comparison to Northern provinces. This could lead to distribution of AMV from alfalfa to soybean.

Soybean AMV derived isolates had a wider host range in comparison to the other viruses tested in this study. However, it is difficult to draw a concrete conclusion on this. More data are needed to elucidate the host range differences among these viruses.

Although Jasper and Bos (1980) reported a chlorotic local lesion, systemic chlorotic and necrotic flecks symptoms, our AMV isolates could systemically infect *C. quinoa* and *C. album.* A reason for observation of mosaic symptoms caused by such isolates was not expected and may be explained by different AMV isolates used in both experiments which may have different potential to induce different symptoms. The SDS-PAGE pattern showed the presence of proteins of molecular mass of 30 and 27 kDa, respectively the expected sizes for the CP from SMV and AMV, which are in accordance to previous findings (Eggenbrger *et al.*,1989; Anonymous, 2002). The IC-RT-PCR of SMV performed and an approximately 469 bp fragment amplified, which is reported by Wang and Ghabrial (2002). In order to differentiate the SMV strains, IC-RT-PCR was used, the results revealed that none of the G2 strains amplified any fragment but all of the G7 strains amplified a 277 bp fragment with specific primers in line with finding of Omunyin *et al.* (1996). Coat proteins of AMV and CMV isolates were amplified as expected fragments in accordance with the results of Martinez-Priego *et al.* (2004) and Han-Xin *et al.* (2004), respectively.

Despite the fact SMV is found as the main soybean virus, our findings imply that the distribution and incidence of a virus may change. Therefore, to investigate the incidence of plant viruses, one should be caution about the distribution of other host plants in the region which can potentially be a host for a virus.

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