Isolation of Alfalfa Mosaic Virus from four Pepper Cultivar in Riyadh K.S.A Using RAPD-PCR Technique

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Abstract: Random Amplification of Polymorphic DNA (RAPD-PCR) technique was used for isolation and identification of alfalfa mosaic virus (AMV) in homogenates of four common Saudi varieties of pepper *Capsicum annum.* Two sweet pepper (Sirtaki and S.P.KING) and two hot pepper (Cruise and E48.192). Ten virus specific primers were used to amplify DNA fragments. Specific bands of PCR products were observed at the position were corresponding to the expected size of DNA amplification product. The results indicated that primers could permit the specific detection of AMV and RAPD-PCR technique is efficient to distinguish between pepper varieties in their infection by alfalfa mosaic virus. Identifying the primary sources of the inoculum may help to reduce AMV spread and the severity of effects on pepper production. The observed symptoms showed that, two sweet varieties were very sensitive to virus infection. Meanwhile the hot varieties were tolerant or resistant to virus infection. In E48.192 and S.P.king varieties, virus was transferred by seeds**.**

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1.Introduction

Field grown pepper (*Capsicum annum* L.) is attacked by 35 viruses which reduce pepper production in many parts of the world *(Green and Kim, 1994; Ken et al.,* 2003). There are several viruses transmitted to peppers by aphids. Cucumber mosaic virus (CMV) is the most important in the northeast, while tobacco etch (TEV), alfalfa mosaic virus (AMV) and potato virus Y (PVY) are less common or destructive. *Alfalfa mosaic virus* (AMV) is the type member of the genus *Alfamovirus* in the *Bromoviridae* family of plant viruses. AMV has a very wide host range. This virus can naturally infect many herbaceous and some woody plant hosts (150 species in 22 families) and is transmissible to over 430 species of 51 dicotyledonous families (Jaspars and Bos,1980). Alfalfa mosaic virus is transmitted to peppers by several species of aphids. The virus has a wide host range and several different strains. It occurs commonly where pepper planted near alfalfa or after alfalfa in rotation. Symptoms appear in leaves as white blotches in a mosaic pattern. Early heavy infections in pepper may cause stunting and distorted fruit. Cucumber mosaic virus (CMV; genus Cucumovirus, family Bromoviridae) is one of the most devastating viruses of pepper and has created difficulties for pepper growers' worldwide (Greenleaf, 1986; Palukaitis *et al.,* 1992). CMV infects more than 1200 plant species in 100 families (Edwardson and Christie, 1991), and has the largest host range of any RNA virus, making it one of the most economically important plant virus in pepper (Tomlinson, 1987). Seed transmission plays a pivotal role in the survival of viruses from season to season (Johansen *et al.,*1994; Meinke, 1994). Transmission of viruses through seed, even at a very low rate, can be important for virus perpetuation, overwintering and long range dissemination. Seed transmission provides an initial source of inoculum for vector transmission of the virus that may have a considerable impact on crop yield.

The recent outbreaks of AMV in Saudi Arabia renewed the interest for this virus as a threat to vegetables production. Little is known on AMV strains from Saudi Arabia and how they correlate with those already described elsewhere and characterized in more detail (Al-Shahwan, 2002; Al-Shahwan *et al*., 2007). Information on the variability of this virus can supply basic knowledge for studying the evolution of resident population and can assist in determining useful criteria for selecting cultivars for AMV resistance. Many researchers studied the susceptibility of pepper cultivars for infection with AMV (Horvath, 1986, Chod *et al*., 1994 and Al-Shahwan *et al*., 2007)
The goal of this research was to evaluate whether alfalfa mosaic virus is seed borne and can be transmitted through pepper seed, which could serve as a primary source of inoculum in the field for AMV transmission by various aphid vectors. Identifying the primary sources of inoculum may help to reduce AMV spread and the severity of effects on pepper production. Random Amplification of Polymorphic DNA (RAPD-PCR) was used to distinguish between seeds of four pepper cultivars in their infection by alfalfa mosaic virus.

2.Materials and methods

2.1. Pepper varieties

Seeds of four common Saudi varieties of Pepper *(Capaicum annum* L), two sweet pepper (Sirtaki and S.P.KING) and two hot pepper (Cruise and E48.192). were used in this study.

2.2. Isolation of Genomic DNA from plant tissue

Seeds of pepper were ground into fine powder using morter and pestle. 40 mg of the powder was transferred to 1.5 ml micro centrifuge tube. 600 µl of Nuclei Lysis solution was added to each tube and vortex for 1-3 seconds to wet the sample then incubated at 65 ^{o} C for 15 min. 3 µl of RNase solution was added to the cell lysate and the sample mixed by inverting the tube 2-5 times. The resultant mixtures were incubated at 37^{o} C for 15 min. then allowed to cool for 5 min. at room temperature. 200 µl of protein precipitation solution was added and vortex vigorously at high speed for 20 seconds then centrifuged for 3 min. at 13,000-16,000 xg. The resultant supernatant containing DNA was carefully transferred to a clean 1.5 ml micro centrifuge tube containing 600 µl of isopropanol, the solution was then mixed gently by inversion until thread-like strands of DNA form a visible mass. Resultant solution was centrifuged at 13,000-16,000 xg for 1 minute at room temperature. Supernatant was decanted and the resulting pellets were resuspended in 600 µl of 70% ethanol and the tube inverted gently several times to wash the DNA, then aspirate the ethanol carefully using sequencing pipette tip. The tubes were inverted into clean absorbent papers and air-dried the pellet for 15 minutes. DNA solution then rehydrated at 65^{o} C for 1 hour and DNA stored at 2-8 ^{o} C until further use

2.3.Random Amplification of Polymorphic DNA(RAPD-PCR) amplification

Virus detection was carried out at Agricultural Genetic Engineering Research Institute (**AGERI**) by RAPD-PCR according to the procedure given (Jaspars and Bos,1980) with minor modifications. The amplification reaction was carried out in 25 µl reaction volume containing 1 X PCR buffer, 1.5 mM Mg $Cl_{2,}$ 0.2 mM d NTPs, 1 uM primer (Table 1), 1U tag DNA polymerase and 25 ng template DNA.

2.4.Theromocyling Profile and detection of the PCR products

PCR amplification was performed in a Perkin-Elmer/ Gene Amp^{R} PCR system 9700 (PE applied biosystems) programmed to fulfill 40 cycles after an initial denaturantion cycle for 5 min. at 94^{o} C. Each cycle consisted of a denaturation step at 94^{o} C for 1 min., an annealing step at 36^{o} C for 1 min, and an elongation step at 72^{o} C for 1.5 min. The primer extension segment was extended to 7 min. at 72^{o} C in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1 X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

Table (1): Sequence of primers assayed in RAPD-PCR.

Primer*	Sequence (5'-3')	Primer	Sequence (5'-3')
OPA-08	GTGACGTAGG	OPB-06	TGCTCTGCCC
OPA-14	TCTGTGCTGG	OPB-11	GTAGACCCGT
OPA-19	CAAACGTCGG	OPB-10	CTGCTGGAC
OPA-11	CAATCGCCGT	O-02	ACGTAGCGTC
OPA-02	TGCCGAGCTG		
OPA-06	GGTCCCTGAC		

Primer designed polymerase chain reaction based on coat protein gene sequence of alfalfa mosaic virus.

3.Results .

The Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) method was used to investigate the dynamic of alfalfa seed-borne mosaic virus (ASBMV) in seed homogenate of four common Saudi varieties of pepper. Ten virus specific primers were used to amplify DNA fragments. Specific bands of PCR products were observed at the position corresponding to the expected size of DNA amplification product (Fig.1)

Amplification of DNA using different primers.

1) Primer OPB-06.

The four varieties shared six bands were observed at 430, 700, 880, 1100, 1700 and 2000 bp. However, three bands were found at 490, 600 and 1350 for Cruise variety and are absent in the other varieties. Moreover one band was observed in Varity (S.P.KING) at 750 bp and another one at 770 for (E48.192) and they were not present in the other varieties. Those bands existed in the samples of pepper varieties were corresponding to the expected size of DNA amplification products for mosaic virus.

2) Primer OPB-11.

Comparing, the four varieties three bands were observed at 460, 500 and 600 bp. Two bands at 780 bp and 900 bp were absent in variety Cruise and E48.192, respectively compared with the other varieties. Moreover a band at 450 bp was existed in Cruise and

was absent in the other three varieties. In addition, a band at 230 bp was observed in E48.192 while it was absent in the other varieties.

3) Primer OPB-10.

Seven bands were observed in the samples of the four varieties. Meanwhile a band at 750 bp was observed in three varieties and absent in Cruise. Furthermore, three bands at 190, 420 and 560 bp were absent in samples of S.P.KING compared with other varieties. While a band at 430 bp was present in S.P.KING and absent in the other varieties.

4) Primer OPA-06.

Three bands were observed at 560, 870 and 1070 bp in all tested varieties. While a band at 670 bp was present in S.P.KING and a band at 310 bp in Sirtaki and absent in the other varieties. Moreover, a band at 600 bp was present in Cruise, Sirtaki and S.P.KING except E48.192 variety. Comparing susceptible varieties, two bands were observed at 900 and 600 bp.

5) Primer OPA-19

Comparing the four varieties, eight bands are existed in all varieties, six of them between 380-650 bp and two at 1100 and 1250 bp. In the mean time, two bands were observed in variety Cruise and Sirtaki at 700 and 290 bp and were disappeared in the other varieties.

6) Primer OPA-08

Three bands were observed at 870, 700 and 550 bp in the four varieties. While a band at 1350 was observed in Cruise variety and absent in the other varieties. In the mean time three bands were diminished in Cruise, Sirtaki and E48.192 samples at 500 and 1070 and 330 bp, respectively compared with the other varieties.

7) Primer OPA-14

Seven bands were observed in all tested varieties. However, a band at 750bp was observed in the samples of varieties Cruise, S.P.KING and E48.192 except S3. Meanwhile a band at 430bp was observed in S.P.KING and was absent in the other three varieties.

8) Primer OPA-02

Comparing the four varieties, two bands at 890 and 650 bp were existed in the samples of Cruise variety and absent in the other three varieties. Meanwhile a band at 360 bp was present in Sirtaki and absent in the other three varieties. The four varieties shared six bands at 230, 350, 375, 500, 500 and 700 bp. Furthermore, a band was observed at 890 bp in Sirtaki, S.P.KING and E48.192 varieties and absent in Cruise.

9) Primer OPO-02

Three bands were observed in the four varieties at 250, 570 and 1500 bp. In the meantime three bands were existed in Sirtaki at 1300, 900 and 820 bp and were absent in the other three varieties. A band was observed at 280 in S.P.KING and was absent in the other three varieties. In addition, a band at 450 was diminished in S.P.KING while it was found in the other varieties.

10) Primer OPA-11

All varieties were analogous in the presence of three bands at 760, 650 and 360 bp . Two bands were detected in 310 and 2000 bp in Cruise and Sirtaki, respectively and absent in the other varieties. Moreover two bands at 560 and 420 bp was absent in Sirtaki compared with other varieties. In the meantime, two bands were diminished in S.P.KING at 390 and 230 bp compared with the other varieties.

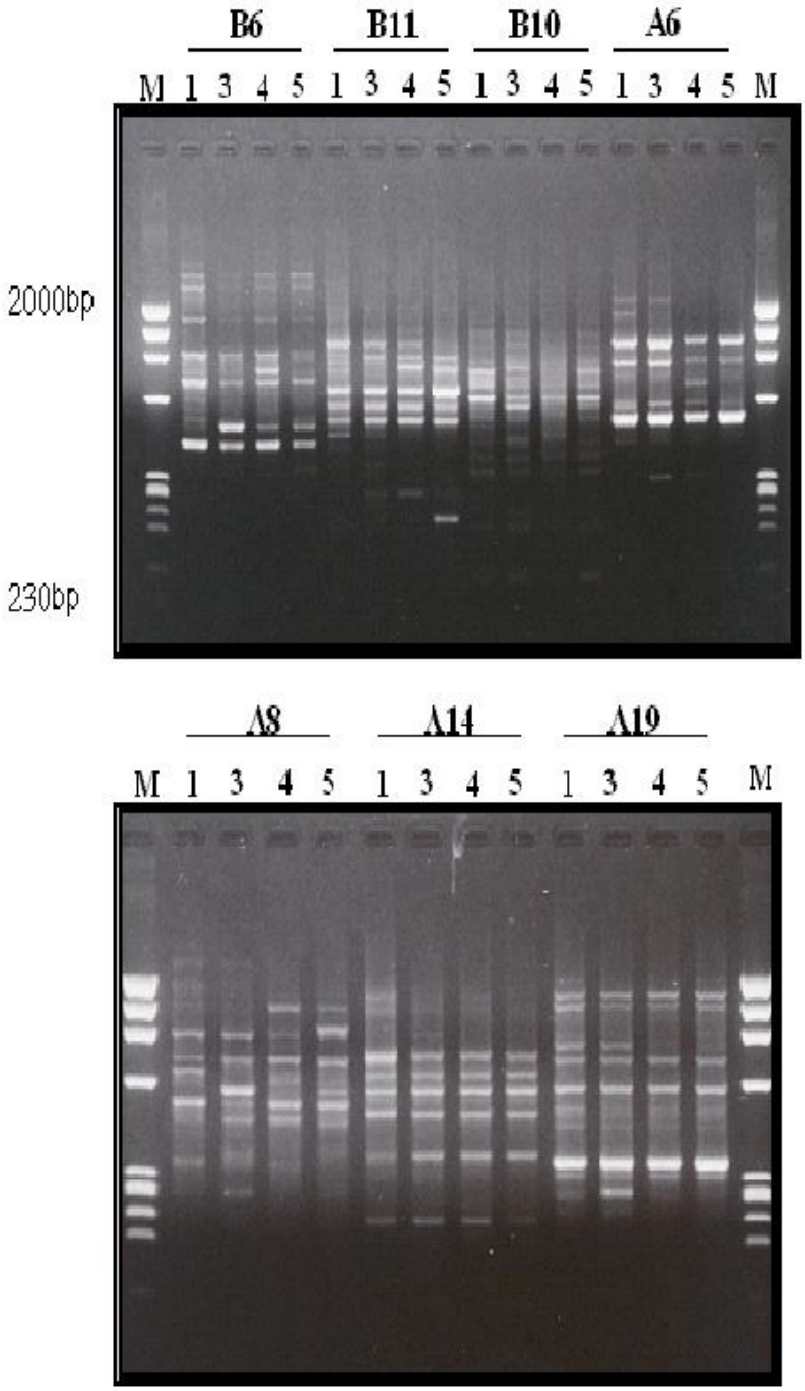
The percent of similarity between the four varieties of pepper reached 78% between. While it was 81.4% between S.P.KING (S4) and E48.192 (S5) varieties. Similarity was reduced to 81.2% between Cruise (S1) and Sirtaki (S3) (Table 2).

The observed symptoms revealed that the two sweet varieties were very sensitive to virus infection. Meanwhile the hot varieties were tolerant or resistant to virus infection. In E48.192 and S.P.king varieties, virus was transferred by seeds**.**

4. Discussion

RAPD-PCR has a potential application for detecting AMV in bulk samples in which the incidence of virus infection might be low. There always has been a need for rapid procedures to detect virus infection in seeds. This technique appears to be useful. Analysis of amplified sequences offers a simple and accurate means of grouping AMV isolates and could prove useful for the analysis of AMV dynamics in natural populations. From the previous results, it is obvious that primers are extremely important for RAPD-PCR detection of AMW virus. The results indicated that primers could permit the specific detection of AMW of infected pepper. This agreed with results that the RT-PCR with degenerate primers is an efficient method for virus detection (Routh *et al.,* 1998). In addition, Niimi *et al.,* (2003), showed that RT-PCR is more sensitive method than ELISA to determine the presence of viruses in Lilium plants of various species. Xu and Nie (2006), reported that, RT-PCR followed by restriction fragment length polymorphism analysis may be a useful approach for screening potato samples on a large scale for the presence of AMV. The results showed that this molecular technique is more reliable for the detection of the above mentioned alfalfa viruses in seeds of pepper. Burgmans *et al.,* (1986) reported the susceptibility of pepper cultivars to AMV infection that caused about 3.8% loss of pepper yield. The reduction of pepper yield was due to the reduction of number, weight and quality of pepper fruits. Al-Shahwan (2002) and Al-Shahwan *et al.* (2007) pointed to avoidance of cultivating very susceptible pepper Varity such as Sirtaki beside other pepper varieties.

They added that Chica pepper Varity is more tolerant to AMV. Moreover, the production of hot pepper was not significantly affected by AMV. RAPD-PCR might be widely used to detect this virus in other plant species, since the degenerated primers used designed according to various strains of virus in different plants. OPA19 and OPA-14 are good for detecting and distinguishing the presence of AMV in pepper varieties.



Figure(1):DNA products amplified in PCR from different varieties of pepper
*****Primers (B6, B11, B10, A6, A8, A14, A19, A2, O2 and A11), M (markers)

* Pepper cultivar, 1, 3,4, 5,

Table (2) Similarity matrix

Variety	S1	S3	S4	S5
S1	1.00	-		
S3	0.81	1.00		
S4	0.765	0.779	1.00	
S5	0.768	0.808	0.818	1.00

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