

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 used to amplify DNA fragments. Specific bands of PCR products 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 inoculum may help to reduce AMV spread and the severity of effects on pepper production. The observed symptoms showed two sweet varieties are very sensitive to virus infection. Meanwhile the hot varieties are tolerant (cruise) or resistant (E48.192) 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 *Alfavirus* 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 is 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 researches 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 seedborne 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.

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 grinding into fine powder using mortar and pestle. 40 mg of the powder was transferred to 1.5 ml microcentrifuge tube. 600 ul of Nuclei Lysis solution was added to each tube and vortex for 1-3 seconds to wet the sample then incubated at 65 °C for 15 min. 3 ul 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°C for 15 minutes then allow cooling for 5 min. at room temperature. 200 ul of protein precipitation solution was added and vortex vigorously at high speed for 20 seconds then centrifuged for 3 minutes at 13,000-16,000 xg. The resultant supernatant containing DNA was carefully transferred to a clean 1.5 ml microcentrifuge tube containing 600 ul 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 ul 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 onto clean absorbent papers and

air-dried the pellet for 15 minutes. DNA solution then rehydrated at 65°C for 1 hour and DNA stored at 2-8 °C until further use

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

Virus detection was carried out at Agricultural Genetic Engineering Research Institut (AGERI) by RAPD-PCR according to the procedure given (Alibrahem,1990) with minor modifications. The amplification reaction was carried out in 25 ul reaction volume containing 1 X PCR buffer, 1.5 mM Mg Cl₂, 0.2 mM d NTPs, 1 uM primer (Table 1), 1U tag DNA polymerase and 25 ng template DNA.

2.4. Thermocycling 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 denaturation cycle for 5 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 36 °C for 1 min, and an elongation step at 72 °C for 1.5 min. The primer extension segment was extended to 7 min at 72 °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 are observed at 430, 700, 880, 1100, 1700 and 2000 bp. However, three bands are present at 490, 600 and 1350 for Cruise variety and are absent in the other varieties. Moreover one band is observed in variety (S.P.KING) at 750 bp and another one at 770 for (E48.192) and they are not present in the other varieties. Those bands existed in the samples of pepper varieties are corresponding to the expected size of DNA amplification products for mosaic virus.

2) Primer OPB-11.

Comparing, the four varieties three bands observed at 460, 500 and 600 bp. Two bands at 780bp

and 900bp were absent in variety Cruise and E48.192, respectively compared with the other varieties. Moreover a band at 450 bp are existed in Cruise and was absent in the other three varieties. Also a band at 230 bp observed in E48.192 while it was absent in the other varieties.

3) Primer OPB-10.

Seven bands 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 560bp were absent in samples of S.P.KING comparing 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 observed at 560, 870 and 1070bp in all tested varieties. While a band at 670bp was present in S.P.KING and a band at 310 bp in Sirtaki and absent in the other varieties. Moreover, a band at 600bp 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 380-650bp and two at 1100 and 1250bp. In the mean time two bands are observed in variety Cruise and Sirtaki at 700 and 290bp and were disappeared in the other varieties.

6) Primer OPA-08

Three bands are observed at 870, 700 and 550bp 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 are diminished in Cruise, Sirtaki and E48.192 samples at 500 and 1070 and 330 bp, respectively, comparing 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 360bp was present in Sirtaki and absent in the other three varieties. The four varieties are shared six bands at 230, 350, 375, 500, 500 and 700 bp. Furthermore a band observed at 890bp 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 1500bp. In the meantime three bands was existed in Sirtaki at 1300, 900 and 820bp and

were absent in the other three varieties. A band observed at 280 in S.P.KING and was absent in the other three varieties. Also a band at 450 was diminished in S.P.KING while it was present in the other varieties.

10) Primer OPA-11

All varieties are analogous in the presence of three bands at 760, 650 and 360bp. Two bands are present in 310 and 2000bp in Cruise and Sirtaki, respectively and absent in the other varieties. Moreover two bands at 560 and 420 was absent in Sirtaki compared with other varieties. In the meantime two bands were diminished in S.P.KING at 390 and 230bp 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 reduced to 81.2% between Cruise (S1) and Sirtaki (S3) (Table 2).

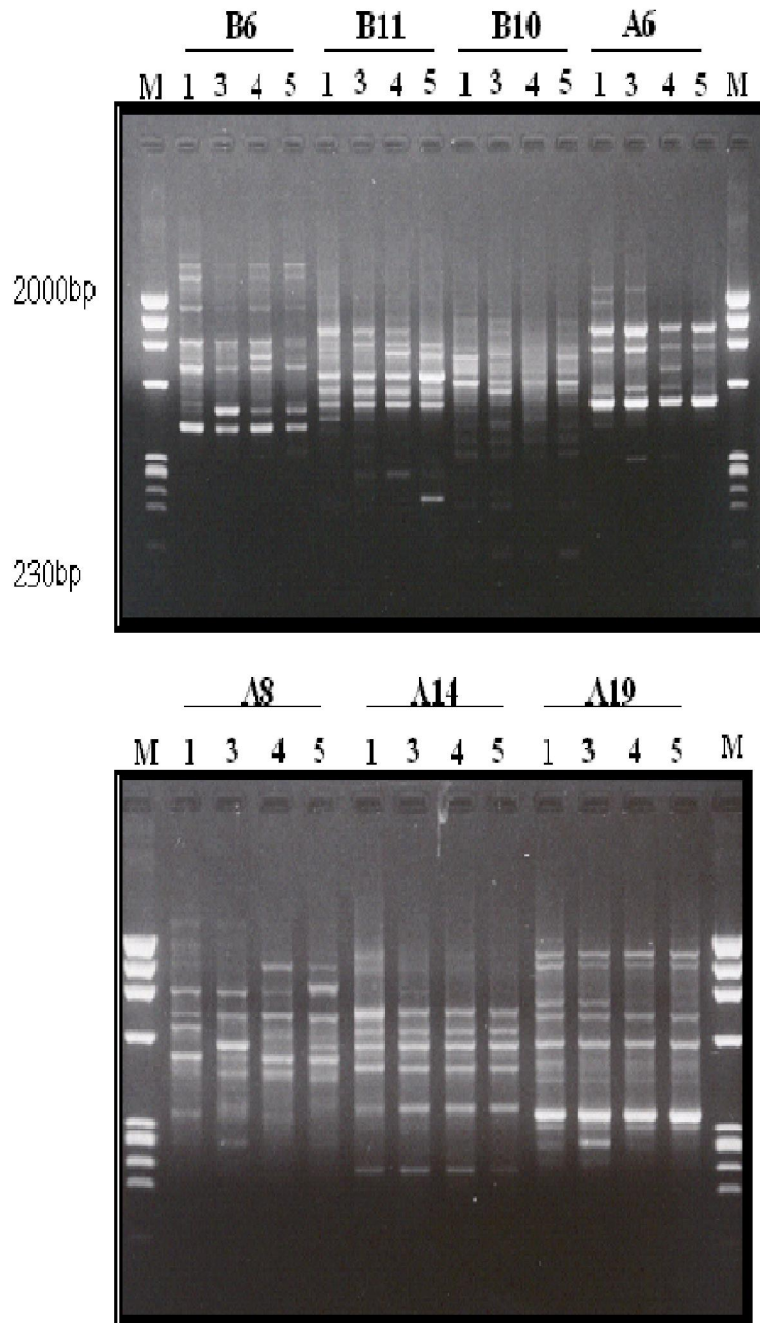
The observed symptoms revealed that the two sweet varieties are very sensitive to virus infection. Meanwhile the hot varieties are tolerant (Cruise) or resistant (E48.192) to virus infection. In E48.192 and S.P.KING varieties, virus was transferred by seeds.

4. Discussion

RAPD-PCR have 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 AMV virus. The results indicated that primers could permit the specific detection of AMV 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). Also, Niimi *et al.*, 2003, showed that RT-PCR is more sensitive method than ELISA to determine the presence of viruses in Liliun 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 show that this molecular technique is more reliable for the detection of the above mentioned alfalfa virus in seeds of pepper. Burgmans *et al.*, 1986, reported the susceptibility of pepper cultivars to AMV infection and 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 point to

avoidance of cultivating very susceptible pepper variety such as Sirtaki beside other pepper varieties. They added that Chica pepper variety is more tolerant to AMV. Moreover, the production of hot pepper does not significantly affected by AMV. RAPD-PCR might

be widely used to detect this virus in other plant species, since the degenrated primers used designed according to various strains of virus in different palnts. OPA19 and OPA-14 are good for detecting and distinguish the presence of AMV in pepper varities.



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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