Serological and molecular detection of *Prune dwarf* virus infecting stone fruits of Charmahal-va-Bakhtiari province, a central region of Iran

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Abstract

Prune dwarf virus (PDV) is one of the major positive RNA viruses which cause economical damages in stone fruit trees. The symptoms of PDV vary between different stone fruits namely sour and sweet cherry, almond, peach, apricot and plum including leaf narrowing, leaf chlorosis, vein clearing, mosaic, leaf whitening, leathery leaf, bushy branches and stunt trees. During the years 2011 and 2012, 251 leaf samples were collected for detection of PDV in stone fruit orchards of Charmahal-va-Bakhtiari province. DAS-ELISA test proved PDV presence serologically. Then, total RNA were extracted and tested by two-step RT-PCR which replicated partial and full coat protein sequence of PDV. One hundred and eighty one out of total samples (251 samples) showed PDV infection using serological and two-step RT-PCR assays, hence, incidence of PDV in Charmahal-va-Bakhtiari province was confirmed. This is the first report of PDV in stone fruit orchards of Charmahal-va-Bakhtiari province and in Iran.

Introduction

Viral diseases are one of the reducing factors of stone fruits yield, which RNA viruses play an important role in this loss. Charmahal-va-Bakhtiari province has great areas under stone fruits cultivation in which viral diseases decrease production and fruits quality.

RNA viruses cause most of stone fruits economical damages. PDV whether solely or in mix infection causes loss of stone fruit products.[1] The virus belongs to the Bromoviridae and 4th subgroup of *Ilarvirus*, that has tripartite genome, single strand positive sense RNA and 5 ORFs. ORFs encode structural proteins, viral silencer suppressor, movement and capsid proteins.[2]

PDV has no biological vector and cannot be transmitted by seed and pollen. Grafting infected scions and grafts is the main mood of its worldwide distribution.[3,4] Cool nights and warm days increase symptoms development.[5] PDV shows different symptoms in various stone fruits, stunting is observed in peach; yellows in sour cherry; stunting and leaf malformations in plums; and chlorotic spots, rings, and fruit rain cracking in sweet cherry.[4,5] During various time periods of sample collection observed that at the end of growing season, viral concentration will be lowered, though, the symptoms are still visible.[6] In mixed infections with *Prunus necrotic ringspot virus* (PNRSV) more damage will appear called stunting, but some of mixed infections with *Apple mosaic virus* (ApMV), *Apple chlorotic leaf spot virus* (ACLSV), *American plum line pattern virus* (APLPV) and *Plum pox virus* (PPV) has decreasing or no effect on PDV infection.[7,8] Iran is the first producer of stone fruits and its derivatives in the world with 202,000 tones production.[9]

Charmahal-va-Bakhtiari province is one of the major producers of stone fruits namely almond and peach in the country with 19,300 and 12,600 tones productions, respectively.[10]

The objective of this research is investigating PDV on leaf samples of stone fruit orchards of Charmahal-va-Bakhtiari province by ELISA and PCR techniques.

Materials and Methods

Sample collection

During 2011 and 2012, 286 leaf samples of different stone fruit orchards of Charmahal-va-Bakhtiari province were collected. Suspicious viral infected leaves were collected from four compass points of the trees with the symptoms as described in Table 1, and were carried on ice to lab.

Mechanical transmission

Curcubita maxima, Chenopodium album, Nicotiana tabacum var Turkish and *N. rustica* were inoculated by crude leaf homogenate of stone fruit leaves which were extracted in 0.1 M phosphate buffer, pH 7 containing 0.1% 2-mercaptoethanol. The homogenate was rubbed on the leaves coated with carborundum powder. The inoculated plants were kept under observation for two weeks to record potential symptoms.

DAS-ELISA

Serological test carried out by DAS-ELISA using commercial polyclonal antibody of Prune dwarf virus (Bioreba, Switzerland) and with Clark and Adams method (1977). ELISA Plate was pre-coated with coating antibody that was diluted in coating buffer (0.01 M Na_2CO_3, 0.03 M $NaHCO_3$, 3 mM NaN_3 per L, pH 9.6) at 1:1000, and incubated overnight at 4°C. The plate was washed with PBST buffer (0.1 M NaCl, 0.001 M KH_2PO_4, 0.02 M $Na_2HPO_4.12H_2O$, 0.002 M KCl, 0.003 M NaN_3, 0.5 mL Tween 20 per L, pH 7.4) three times, each time for 3 min. 150 mg of leaf samples were grounded by liquid nitrogen and mixed with 1 mL of extraction buffer [PBST+2% Polyvniylpyrrolidone (PVP), pH 7.4] and 200 mL of the homogenates were placed in wells. Plate was incubated at 4°C overnight and washed five times with PBST buffer. Then Plate was coated with alkaline phosphatase conjugated antibody diluted in conjugate buffer (PBST, 2% PVP, 0.2% egg albumin, pH 7.4) at 1:1000, and incubated overnight at 37°C. After washing, mixture of 4-nitrophenyl phosphate diluted 1:1000 in substrate buffer (97 mL diethanolamine, 0.003M NaN_3, pH 9.8) was added to each well and incubated in a dark place at room temperature for 30-60 min. The reaction was detected at OD_{405nm} using ELISA reader (STAT FAX 2100, USA).

Total RNA extraction

Total RNA of the both stone fruit leaves used as viral source of inoculation and inoculated indicator plants showed PDV infection, were isolated according to the LiCl method described by Channuntapipat *et al.* (2001) with some modifications. Approximately 100 mg of infected leaves were grounded in liquid nitrogen and mixed with 1ml of extraction buffer

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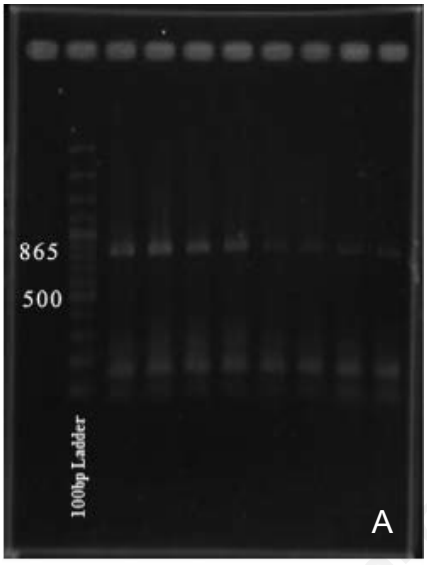
(0.1 M LiCl, 0.1 M Tris-HCl, pH 8.0, 0.01 M EDTA, pH 8.0, and 1% SDS, PVP-40) 5% w/v and sodium metabisulaphite 2% w/v). 800 μL of a mixture of Tris-saturated phenol, pH 8.0: chloroform: isoamyl alcohol (25:24:1) were added to the tube and vortexed for 1 min, followed by centrifugation at 14,000 rpm for 15 min at room temperature (RT). The upper aqueous layer was transferred to a new tube and mixed with an equal volume of 4.0 M LiCl. RNA was allowed to precipitate at –20°C for 1-24h to check the effect of freezing on RNA percipitation and its quality, which followed by centrifugation at 4°C for 15 min at 14,000 rpm. The pellet was dissolved in 400 μL of sterile water and 2 volumes of cold 70% ethanol (–20°C) in the presence of 40 μL of 3.0 M NaOAc (pH 5.4). RNA was recovered by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was washed with 500 μL cold 70% ethanol by centrifugation at 4000 rpm for 5 min at 4°C, dissolved in 40 μL of sterile water, and stored at –20°C (or –80°C for long-term storage). The quantity of RNA ($A_{260/280}$) was measured with Genova Spectrophotometer (Jenway, UK).

Primers

The conserved regions of PDV coat protein published on the GenBank database were aligned by using Mega5.1 (The Biodesign Institute, AZ, USA) software. Oligonucleotide primers were designed by Primer3 (Whitehead Institute for Biomedical Research, MA, USA) software with the results of aligned conserved regions. Choosing two pairs of primers for both partial and full sequence of PDV coat protein replication provided more accuracy and valid confirmation of molecular detection. The expected amplification products for partial and full sequence of PDV coat protein were 381 bps and 865 bps, respectively (Table 2, Figure 1).

cDNA synthesis and two-step RT-PCR

Complementary DNA synthesis was carried out in 20 μL total volume containing 5 μL of extracted RNA, 1 μL of random hexamer primer (100 pmole)(Genet Bio, Korea), 6 μL of RNase-free water. This mixture was incubated for 10 min at 70ºC. Then 4 μL of 5X reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 μL of dNTP mix (10 mM), and 2 μL of M-MuLV-Reverse transcriptase (40 units)(Genet Bio, Korea) were added. Reverse transcription was continued at 20-25°C for 10 min and followed by 42°C for 1 h and finally at 70°C for 10 min. PCR mixture



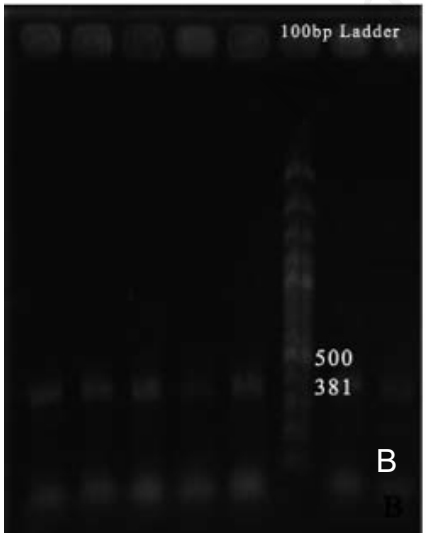


Figure 1. Amplified products with expected sizes. A) 865 bps amplified products, B) 381 bps amplified products.

Table 1. Natural host range of PDV and its symptoms.

Host	Viral symptoms in leaves
Prunus domestica	Yellowing, malformation, narrowing
P. avium	Marginally whitening, wrinkling
P. cerasus	Yellowing
P. armeniaca	Mosaic and yellowing
P. persica	Mosaic, yellowing, malformation
P. amygdalus	Mosaic, narrowing, malformation, leathery, bushy branches, stunt tree

Table 2. Primers designed to detect PDV coat protein.

Primers	Primer sequence	Expected size	Primer length
PDV1F	5- GGAAAGCCTACTGCCCGATCAC		22
PDV1R	5- CCTACGTTGTAGGGGATTAGG	381 bps	21
PDV5F	5-GAAGCTTTTGGTGTAACGATTGG		23
PDV5R	5-CAGTGACAAAATCTGAATGGTGG	865 bps	23

Table 3. Mechanical transmission of PDV and its respective results.

Indicator plants	Inoculated leaves as PDV source	Inoculated indicator plants	Latent period (days)	Leaf growth period	Symptoms in indicators
Cucumis sativus	Almond, sweet and sour cherry, plum, peach	3	7	Two leaves	Mosaic, local chlorosis in margins and veins
Cucurbita maxima	Almond, sweet and sour cherry, plum, peach	4	7	Two leaves	Mosaic, chlorosis in margins and veins
Nicotiana tabacum var Turkish	Almond, sweet cherry, sour cherry	15	10	Two to four leaves	Local chlorosis
N. rustica	Almond, sweet cherry, sour cherry	15	10	Two to four leaves	Local necrosis and malformation
Chenopodium album	Almond, sweet cherry, sour cherry	6	7-10	Four to six leaves	Local chlorosis

in 20 μL total volume containing 1 μL of the cDNA, 0.8 μL of $MgCl_2$ (50 mM), 0.6 μL of dNTPs (10 mM), 1 μL of each forward and reverse primer (10 pmole each), 2 μL of 10X PCR reaction buffer (Genet Bio, Korea), 0.25 μL of Taq-polymerase (5 U per μL), and 13.4 μL of water were added. The cycling parameters for PDV5 primer were as follows: initial denaturation of 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 40 sec at 45°C, 1 min at 72°C and final extension to 5 min at 72°C. All the parameters for PDV1 primer is the same as PDV5 except annealing temperature which changed to 50°C. The amplification products were analyzed using electrophoresis through 1.5% agarose gels in 1× TBE buffer (pH 8.0) at a current of 30-40 mA. After electrophoresis, gels were stained with ethidium bromide (0.5 g/mL), and visualised under UV light.

Results

Mechanical transmission

Inoculation of indicator plants with homogenate of infected stone fruit leaves showed different symptoms on different hosts as illustrated in Figure 2. *Cucurbita maxima* showed mosaic, vein yellowing and chlorosis; symptoms of *Chenopodium album* were in range of local chlorosis and the predominant symptoms of both *Nicotiana* species were local chlorosis and local necrosis. The stone fruits used as PDV source were successfully transmitted to indicator plants during their 2 to 6 leaves. Latent period of virus varied about 7-10 days among different indicator plants. Complementary information about PDV transmission is described in Table 3.

DAS-ELISA

The results of 251 tested samples revealed 72.1% positive infection to PDV. Measuring OD405 nm provided information of PDV titre in tested stone fruit hosts. One of the almond leaf samples had OD about 1.5, which in comparison with PDV positive control with OD 1.6 was remarkable. After almond samples sweet cherry and peach had the most PDV titre in their leaf samples (Table 4).

RNA extraction and two-step RT-PCR

The range of extracted total RNA of both stone fruit leaves used as viral source of inoculation and inoculated indicator plants showed PDV infection in $A_{260/280}$ was 1.8. The fragments with partial and full sequence of coat protein, respectively, 381 and 865 bps were amplified. Therefore, presence of PDV was confirmed with designed primers in both host and indicator plants. Instead of using negative controls, utilizing two pairs of primers was only for confident of PDV presence in leaf samples which may be mistaken with unwanted amplified plant genes.

Discussion

As symptoms of viral infection retained in leaves, at the end of the growth season and by increasing temperature, viral concentration decreases. As reported, In this situation pericarp (fruit skin) is preferable compared to leaf and other plant tissues.[6] Reaching to a definite result needs to know accurate time of sampling which help to collect infected leaves with high viral concentrations. Symptomology studies on Charmahal-va-Bakhtiari stone fruits samples showed diverse and wide range of symptoms including mosaic, yellowing, malformation, narrowing, whitening, bushy branches and stunt tree.

Both the *C. maxima* and *C. sativus* as indicator plants were more sensitive to PDV than other three indicators because of viral latent

Table 4. Infection percentage of PDV on various collected samples of stone fruits.

Stone fruits	Total n. of collected samples	Total n. infected samples	Infection percentage	Most OD_{405nm}
Almond	182	126	69.2	1.5
Apricot	6	3	50	0.145
Sweet cherry	19	19	100	1.01
Sour cherry	6	6	100	0.35
Peach	16	13	81.2	0.67
Plum	22	14	63.6	0.455
Total	251	181	72.1	

Positive control=1.6

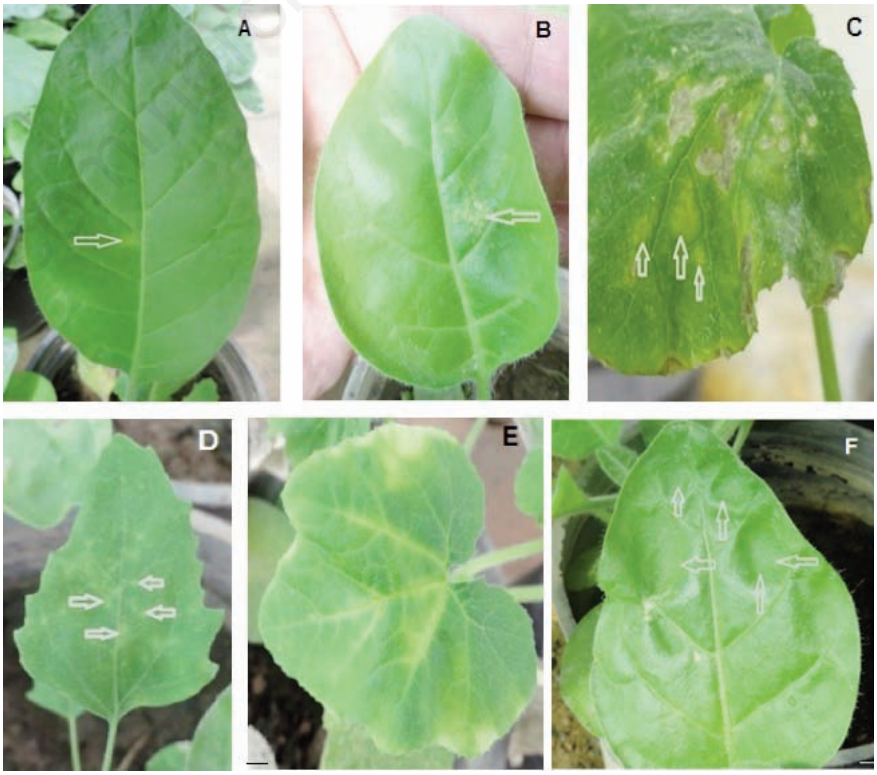


Figure 2. Different symtoms of PDV inoculation to indicator plants. A) Local chlorosis (Arrow) on *N. tabacum* var.Turkish, B) Local necrosis (Arrow) on *N. rustica*, C) Mosaic, local chlorosis (Arrows) on *C. sativus*, D) Local chlorosis (Arrows) on *C. album*. E) Mosaic, chlorosis and vein yellowing (Arrows) on *C. maxima*. F) Leaf malformation (Arrows) on *N. rustica*.

period and symptoms development on leaf surface. Although, some factors like uneven distribution, leaf age differences and seasonal fluctuation make ELISA as an inaccurate and inefficient method but this is the most routine approach for viral detection.[7] To confirm the results of DAS-ELISA, two-step RT-PCR with specific coat protein primers was carried out.

Serological and molecular results indicate that high incidence of PDV (72.1%) has been taken place among stone fruits of the province. According to viral concentration in leaf tissue, among mentioned stone fruits in Charmahal-va-Bakhtiari province almond, sweet cherry and peach had the most PDV infection and plum, sour cherry and apricot have little infection to PDV. Although, sour cherry has 100% infection in our results of experiments but in serological and molecular methods revealed little virus titre (Table 4).

Since no natural vectors for spreading this virus have been described, and mechanical transmission is the main factor of transmission, the production of virus-free reproductive and planting material and its usage for the establishment of new plantings are important for effective virus control. However, the eradication of infected mother plants is a significant part of the certification system of nursery material in Charmahal-va-Bakhtiari province.

Studying about genetic diversity of PDV coat protein in stone fruits of Charmahal-va-Bakhtiari province or a broader area, in Iran, not only extends probably iranian isolate of PDV genome library but also would help in future controlling methods of this virus.

Both of these items could be the next field of study.

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