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# Molecular Detection of Potato Spindle Tuber Viroid in Razavi and Northern Khorasan Provinces

[1]Yazarlou Arezou, [1]Behrouz Jafarpour, [1]Mahrokh Falahati Rastegar and [2]Ali Javadmanesh
[1]Department of Plant Pathology, Agriculture Faculty, Ferdowsi University of Mashhad,
P.O. Box 91775-1163, Mashhad, Iran
[2]Departmet of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

**Abstract:** The aim of this study was to identify the Potato Spindle Tuber Viroid (PSTVd) by Reverse Transcriptase-PCR. Among all plant diseases which are caused by viroids, PSTVd was the first viroid which is recognized by plant pathologist. Suspended potato tubers from Razavi and Northern Khorasan provinces collected and cultured. Leaves of cultured tubers were used for RNA extraction by $PEG_{6000}$ Precipitation method. Subsequent RT-PCR reaction has been carried out using specific primers. A 359 bp fragment has been appeared after electrophoresis which has not appeared in healthy tuber samples. Digestion of obtained fragment with *Bam*HI confirmed the sequence by producing two fragments of 119 and 240 bp. Sequencing has been done to certify the PSTVd. The sequencing result showed that infection in tested areas is caused by mild strain of PSTVd. In this project from 250 samples, 14 tubers were infected. This was the first report of occurrence of mild strain of PSTVd in Iran.

**Key words:** Potato, PSTVd, reverse transcriptase-PCR

## INTRODUCTION

Viroids are small, circular, single-stranded RNA molecules that cause serious diseases in economically important crops (potato, tomato, cucumber and hop), fruit trees (citrus, apple, peach, grape, coconut and avocado) and ornamental plants (*Chrysanthemum* and *Coleus*) (Nakahara *et al*., 1999). Because of their small size (246-401 nucleotides) Viroids lack sufficient information to code for even one protein. These pathogens replicate autonomously and spread in the plant by recruiting host proteins via functional motifs encoded in their RNA genome (Tabler and Tsagris, 2004). Viroids have no protein coat and they depend on plant host enzymes for their replication and other functions. They have been implicated in several economically important diseases of higher plants (Gora-Sochacka, 2004). Twenty and seven distinct viroids have been recognized and they have been placed in seven genera (Hull, 2002). To date, any human or animal diseases that caused by viroids has not been distinguished, in spite of this, there is probability that viroids can be the factor of some unknown human and animal diseases (Agrios, 1996).

Of all plant diseases now known to be caused by viroids, the potato spindle tuber viroid disease was the first to be recognized and studied by plant pathologists. Thus, the potato spindle tuber disease truly represents the prototype of all viroid diseases (Diener, 1987). PSTVd is small, circular, single-stranded RNA, with considerable secondary structures and is capable of autonomous replication when inoculated into a host. This viroid is commonly 359 nucleotides in length. Infected plants are upright and smaller than healthy plants. Leaves are slight dark green and angels between stems are more acute than normal and leaflets with fluted margins, tend to curve inward (Hooker, 1990).

The symptoms are most distinct and severe at high temperatures, while almost masked at lower temperatures. The severe strain causes enhanced symptoms, twisting of leaflets and rugosity of leaf surface (Hadidi *et al*., 2003). In potato, mild strains with indistinct symptoms outnumber severe strain by a ratio of 10:1 and cause yield losses of 15-25%, whereas severe strains with distinct symptoms cause about 65% yield losses (Hooker, 1990). Transmission is largely mechanical. PSTVd seems to be transmitted also by pollen and true seed (Hooker, 1990). Various diagnostic techniques have been examined (e.g., bioassay and gel electrophoresis) (Nakahara *et al*., 1999). Because of the relative simplicity and high sensitivity, RT-PCR became a useful and popular method for the practical diagnosis of viroids. This technique has been used for the identification of all groups of viroids except

**Corresponding Author:** Yazarlou, Arezou, Department of Plant Pathology, Agriculture Faculty, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, Iran Fax: +98 511 8787430

*Coleviroid* (Bostan *et al*., 2004). The aim of this study was to identify the Potato Spindle Tuber Viroid (PSTVd) by Reverse Transcriptase-PCR.

## MATERIALS AND METHODS

**Sampling:** In summer and fall of 2004, Suspended potato tubers from Razavi and Northern Khorasan provinces collected. In total, 250 tubers were obtained and cultured. Leaf of the cultured potato plants were used as a source viroid for RNA extraction.

**RNA extraction:** RNA extraction was done by $PEG_{6000}$ Precipitation method (Schmitz, 2003). To extract the RNA, 200 mg of fresh potato leaf tissues ground with mortar and pestle in liquid nitrogen. Then immediately 200 μL phenol and 200 μL TNE-buffer (Tris/HCl 100 mM, NaCl 100 mM, EDTA 10 mM, 2% SDS, 2% 2-Mercaptoethanal) were added to 100 mg of grounded leaf. After shaking vigorously and centrifuge for 1 min at 13000 rpm two phases were separated. Upper phase transferred to a new tube then 100 μL phenol and 100 μL TNE-buffer were added. Repeat vigorous shaking, centrifuging and transferring aqueous phase into a new tube then 200 μL phenol and 200 μL TNE-buffer were added, again shaking, centrifuge and transferring the aqueous phase. The aqueous phase treated with $PEG_{6000}$ precipitation to separate the nucleic acids from the other molecules. 15.09 μL 50% PEG6000 and 10.69 μL 5 M NaCl were added to 100 μL of aqueous phase. Samples incubated for 10 min at room-temperature then for 10 min on ice. After centrifuge at 13000 rpm for 10 min supernatant-containing small RNA and PSTVd- transferred into a new tube. By adding 39.15 μL 50% PEG6000 and 4.56 μL 5 M NaCl concentration of PEG6000 increased to 16% and NaCl to 500 mM. After incubation for 1 h on ice and centrifuge for 10 min at 13000 rpm, supernatant discarded promptly. Twenty hundred microliter of 70% Ethanol was added and centrifuged for 10 min at 13000 rpm. Supernatant was discarded, 100 μL of 70% Ethanol was added and centrifuged for 10 min at 13000 rpm. Supernatant discarded and the pellet was dried at room temperature over night or for 30 min at 37°C to get rid of phenol. Finally the pellet dissolved in 15 μL of 5 mM Tris/HCl, pH 8.0 for further cDNA synthesis.

**cDNA synthesis:** To make the cDNA following components were used. Template RNA 1.5 μL, reverse strand of primer 20 pMol (2 μL), deionized water to 11 μL. The mixture incubated at 70°C for 5 min and chilled on ice. Then 4 μL of 5X reaction buffer, 2 μL of 10 mM dNTP's mix, 20 U Ribonuclease inhibitor (0.5 μL), deionized water to 19 μL were added. Samples incubated at 37°C for 5 min and 200 U (1 μL) of Revert Aid$^{Tm}$M-MuLv Reverse Transcriptase (Fermentas, Lithuania) added. Reaction mixture incubated at 42°C for 60 min. Finally reaction stopped by heating at 70°C for 10 min and chilling on ice.

**PCR:** PCR was done in total volume of 20 μL in T-personal Thermal cycler (T Personal, Biometra Co., Germany). Reaction components were: primers (Forward and Reverse) 20 pmol $\mu L^{-1}$, $MgCl_2$ 0.7 mM, 0.2 mM dNTPs mix, 10× PCR reaction buffer (2 μL), 1 U of *Taq* polymerase and 2 μL of cDNA. Amplification program started with 3 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 69°C, 30 sec at 72°C and finished with a final extension for 5 min at 72°C.

Sequences of primers were:

| | |
|---|---|
| QFV6 | 3'-TCGCGCCCGCAGGACCAC-5' |
| RGV5 | 3'-GGAAGGACACCCGAAGAAAGGAAGGGTGGAAA-5' |
| Asltr | 3'-GGCCGACAGGAGTAATTCCC-5' |
| AS2 | 3'-GCTGGGCACTCCCCAC-5' |

PCR with QFV6 and RGV5, ASltr and AS2 should yield full length PSTVd (359 bp).

**Sequencing:** For primary confirmation of amplified product, the PCR product was digested with *Bam*HI restriction enzyme. Digestion performed in a total volume of 20 μL. Four units of enzyme, 5 μL of PCR product and 2 μL of buffer were mixed and incubated in 37°C for 12 h. Digestion should produce 2 fragments of 240 and 119 bp. For final approval of the sequence of PCR product, it has been sent for sequencing to GENterprise Gmblt Company (Germany).

## RESULTS AND DISCUSSION

The specific viroid band of 359 bp was showed in Fig. 1.

The digesting of PCR products with *Bam*H I produced two fragments with size of 119 and 240 bp (Fig. 2). Sequencing result of the amplified fragment determined is shown in Fig. 3.

It has been aligned with the BLAST tool in NCBI gene bank. Result of alignment demonstrated 100% homology with the sequence of mild strain of potato spindle tuber viroid with accession number of M14814.

Reverse Transcriptase-PCR, digestion with restriction enzyme and sequencing tests accompany with infected plant symptomology including malformation, elongation and small tubers, with numerous eyes and characteristic

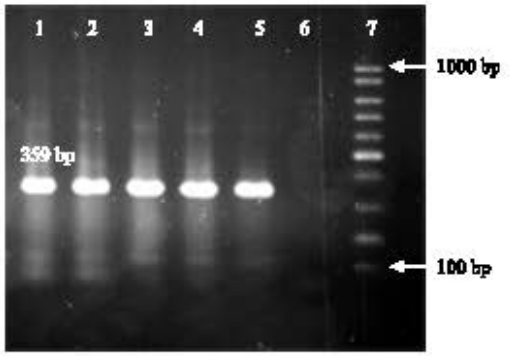


Fig. 1: Agarose electrophoresis of PCR products (359 bp) from suspended Potato tubers (Lanes 1-5), Lane 6 is negative control and Lane 7 is M100 bp molecular marker (1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp)

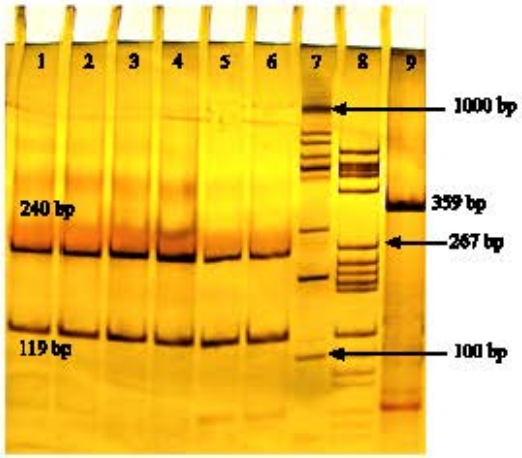


Fig. 2: Digestion with *Bam*HI enzyme on 5% polyacrylamide gel. Lanes 1-6 are digested products, lane 9 is undigested PCR product, lane 7 is M100 bp and 8 is pBR322/*Hae*III size markers

heavy eyebrows as well as foliage symptoms such as small, erect and twisting leaves indicate the presence of mild strain of this viroid in Northern and Razavi provinces in this study. Also the other studies employing RT-PCR have demonstrated the presence of this viroid in Tehran (Esmaeeli Far *et al*., 2002) and Mazandaran provinces (Rahimian, 1989), although the strain of viroid was unknown because sequencing method has not done. Fortunately losses of this viroid are low in potato growing area in these provinces, but in consideration the probability of natural mutation in viroid genome and producing severe strain, this viroid can be a real danger in

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1 cggaactaaa ctcgtggttc ctgtggttca cacctgacct cctgagcaga aaagaaaaaa
61 gaaggcggct cggaggagcg cttcagggat ccccgggggaa acctggagcg aactggcaat
121 aaggacggtg gggagtgccc agcggccgac aggagtaatt cccgccgaaa cagggtttt
181 accettcctt tcttcgggtg tccttcctcg cgccgcgagg accacccctc gccccctttg
241 cgctgtcgct tcggctacta cccggtggaa acaactgaag ctcccgagaa ccgcttttc
301 tctatcttte tttgcttcgg ggcgaggggtg tttagccctt ggaaccgcag ttggttcct
```

Fig. 3: Sequence of detected PSTVd in suspended potato tuber from Northern and Razavi Provinces

the future for potato industry in these provinces. Therefore research must widely perform on detection of this viroid in those places that presence of viroid has not been yet confirmed. In addition because of readily mechanical transmission and also transmission via pollen and true seed that cause widely distribution of the viroid in the field, the precautionary measures must be taken for control of PSTVd, such as planting only viroid free potato tubers and preventing spread of viroid during handling and planting of crop.

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