Reverse transcription-polymerase chain reaction (RT-PCR) assay for detection and characterization of Pea seed-borne mosaic virus in legumes applied by ICARDA's GHU

Safaa G. Kumari and Abdelrahman Moukahel
Seed Health Laboratory, International Center for Agricultural Research in the Dry Areas (ICARDA), Terbol Station, Zahle, Lebanon

DESCRIPTION:

Pea seed-borne mosaic virus (PSbMV, genus *Potyvirus*, family *Potyviridae*) is the most important virus infecting lentil both in terms of economic importance and geographical distribution. It was reported that PSbMV can even lead up to 61% yield loss. Therefore, developing and implementing effective management strategies for PSbMV is very important to reduce yield and quality loss. However, accurate detection of virus disease is the first essential step for effective management strategies for control of this disease. Molecular is efficient tool to accurately detect and monitor the occurrence and development of crop diseases. This report describes a validated reverse transcription-polymerase chain reaction (RT-PCR) assay for PSbMV detection and characterization applied by ICARDA's GHU.

Keywords: *Legume, virus disease, seed-borne, RT-PCR, plant health.*

MATERIALS:

- PSbMV infected plants/seedlings.
- 70% ethanol for disinfection of surfaces and equipment.
- Balance: capable of weighing to the nearest 0.001 g.
- pH meter: capable of being read to the nearest 0.01 pH unit.
- Pipettes: capable of pipetting to the nearest 0.001 ml.
- Eppendorf tubes: 2 and 1.5 ml.
- Specific PCR Primers.
- Thermal cycler.
- Agarose electrophoresis equipment.
- Sterile pipette tips.

METHODS:

1. *Tested samples*

- Infected legume plants at different plant stages can be used in molecular characterization.
- Samples can be tested serologically against PSbMV specific antibodies using ELISA or TBIA tests (Kumari *et al*., 2022) before using molecular tools.

2. *Molecular characterization*

2.1 RNA Isolation

- As the detected virus is RNA virus, thus all working surfaces must be disinfected with 70% Ethanol and all working tools must be autoclaved before using.
- Disrupt a maximum of 100 mg plant tissue from each sample in 2 ml Eppendorf tube.
- Extract the RNA following the MacKenzie RNA extraction protocol (MacKenzie *et al.,* 1997) with RNeasy Plant Mini-Kit (Cat No. 74904, Qiagen) or any available RNA extraction kit.
- In addition to the RNA of tested samples, a positive control (RNA for identified virus isolate) and a negative control (RNase free water) should be used as references.

2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

- Synthesize the cDNA using any RT-PCR kit as per the manufacturer's instruction using reverse primer PSbMV-1R: 5'-TACATCTAGATTACATGGCTCTCATTCCGAGAAG-3' (Roberts *et al.,* 2003) (Table 1)
- Prepare RT-PCR reaction mixture using any PCR kit available (Table 2) using the following primer pairs (Roberts *et al.,* 2003)

PSbMV-2F (5'-CAAACGCGTGACGAAACCAAGGATGATGAAAG-3')
PSbMV-1R (5'-TACATCTAGATTACATGGCTCTCATTCCGAGAAG-3')

- Carry out the RT-PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 10 µl (9 µl reaction mixture + 1 µl synthesized cDNA) (Table 2).
- PCR profile: an initial denaturation of 95 °C for 1 min, followed by 35 cycles of (95 °C for 30 sec, 56 °C for 10 sec, and 72 °C for 30 sec) and a final extension of 72°C for 5 min.
- Fractionate 10 µl of the PCR products by gel electrophoresis for 1 h at 120 V on a 1.5% agarose gel in 0.5x Tris borate EDTA (TBE buffer) stained with RedSafe™ Nucleic Acid Staining Solution 20,000x) (Cat. No. 21141, iNtRON, South Korea), at final concentration of 5%. Include a 100 bp ladder (Solis BioDyne, Cat No. 07-11-0000S).
- Analyze the amplification products for a PSbMV specific product of 888 bp (Figure 1).

Note: It is possible to detect PSbMV by RT-PCR using one-step RT-PCR kit instead of cDNA synthesis then RT-PCR as illustrated in table 3 and analyze the PCR products on agarose gel as previous, but it is a bite costly for routine detection.

SOLUTIONS & BUFFERS:

McKenzie lysis buffer

- 4 M guanidine isothiocyanate
- 0.2 M sodium acetate, pH 5.0
- 25 mM EDTA
- 2.5% (w/v) PVP -40 (polyvinylpyrollidone, average molecular weight, 40,000)
- 1% (v/v) β-mercaptoethanol (β-ME); add immediately before use.

10x TBE (1 liter)

- Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water.
- Add 40 ml 0.5 M Na_2EDTA (pH 8.0) (alternatively use 9.3 g Na_2EDTA)
- Adjust volume to 1 Liter.
- Store at room temperature.

Prepare with DNase-free H_2O. Dilute 100 ml to 1 L to make gel running buffer.

Table 1. Reverse Transcription (cDNA synthesis)

cDNA kit: M-MLV Reverse Transcriptase from Invitrogen Cat No. 28025013

1. Add to the tubes:	
Mix 1	**Vol per tube (1x) µL**
Total RNA template	3
dNTPs (10 µM)	1.0
dH_2O	1.0
Reverse primer (PSbMV-1R) (10 pmol)	1.0
Total	**6.0 µl**
2. Heat mixture to 65°C for 5 minutes and quick chill on ice. Collect the contents of the tube by brief centrifugation. 3. Prepare a master mix, add to tubes in step 2:	
Mix 2	**Vol per tube (1x) µL**
5X First-Strand Buffer	2.0
0.1M DTT	1.0
dH_2O	0.5
Total	**3.5 µl**
4. Mix contents of the tube gently and incubate at 37°C for 2 minutes. 5. Add 0.5 µL (100 units) of M-MLV RT, and mix by pipetting gently up and down. 6. Incubate 50 minutes at 37°C. 7. Inactivate the reaction by heating at 70°C for 15 minutes. 8. Store at -20 °C or -80°C.	

Table 2. Reaction mixture for RT-PCR
Kit used: MyTaq™ Red DNA Polymerase from-Bioline-UK, Cat No# BIO-21108

PCR Reaction Mix	Volume per tube (1x) µl
5X PCR buffer	2.0
Reverse primer (PSbMV-1R) (10 pmol)	0.5
Forward primer (PSbMV-2F) (10 pmol)	0.5
Taq DNA Polymerase	0.1
dH_2O	5.9
cDNA template	1.0
Total	**10 µl**

Table 3. Reaction mixture for one step RT-PCR
Kit used: SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, Cat. No. 12574-026)

PCR Reaction Mix	Volume per tube (1x) µl
2X Reaction Mix	2.5
Reverse primer (PSbMV-1R) (10 pmol)	0.5
Forward primer (PSbMV-2F) (10 pmol)	0.5
SuperScript™ III RT/Platinum™ Taq Mix	0.1
dH_2O	3.4
RNA template	3.0
Total	**10 µl**

PCR Profile: 48˚C for 45 min then 95˚C for 2 min, then 35 cycles of (95°C for 30 sec, 56°C for 10 sec, and 72°C for 30 sec) then 1 cycle of 72˚C for 5 min, then 1 cycle of 25˚C for 3 min and 15˚C (pause)

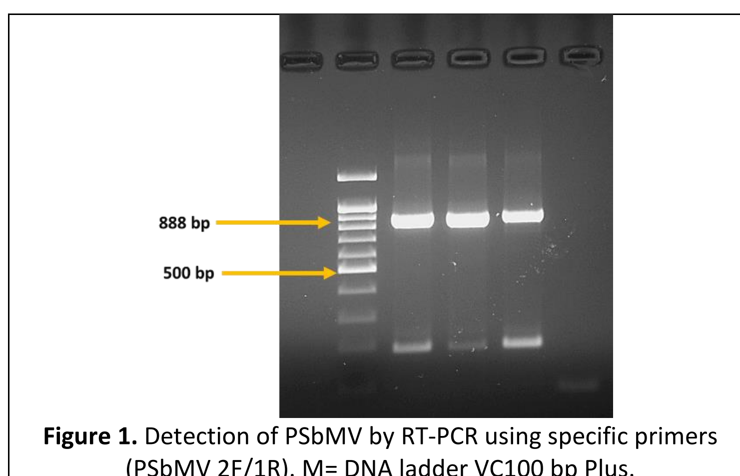

Figure 1. Detection of PSbMV by RT-PCR using specific primers (PSbMV 2F/1R). M= DNA ladder VC100 bp Plus.

References

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