Detection of Four Legume Viruses (AMV, CMV, BYMV and PSbMV) in One Multiplex RT-PCR Test Applied at ICARDA's Seed Health and Virology Laboratory

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BACKGROUND

Many viruses have been reported affecting legume crops in West Asia and North Africa (WANA region) (Kumari and Makkouk, 2007; Kumari *et al*., 2009; Makkouk *et al*., 2012), including Alfalfa mosaic virus (AMV, genus Alfamovirus, family *Bromoviridae*), Cucumber mosaic virus (CMV, genus *Cucumovirus*, family *Bromoviridae*), Bean yellow mosaic virus (BYMV) and Pea seed-borne mosaic virus (PSbMV) (genus *Potyvirus*, family *Potyviridae*). The nucleic acid of these four viruses is RNA and all are transmitted by aphids in a non-persistent manner and via seeds (including faba bean, lentil and chickpea), and pose a significant threat to the productivity of food and feed legume crops. Currently, virus detection methods rely primarily on serological tests such as ELISA and TBIA to detect viral infections. However, screening each sample for all viruses via individual virus-specific tests is both cost-prohibitive and labor-intensive. Additionally, antisera for some of these viruses are difficult to obtain. Thus, there is a clear need for a more efficient assay capable of detecting and differentiating the maximum number of these viruses in a single test, with high sensitivity to process large number of samples. The Reverse transcription polymerase chain reaction (RT-PCR) is a powerful technique for amplifying specific RNA sequences, enabling the detection of minute quantities of target molecules. Multiplex RT-PCR has been successfully applied to detect simultaneously different RNA viruses with high sensitivity (Bariana *et al*., 1994; Moukahel *et al*., 2021).

This report describes a validated molecular tool for a sensitive RT-PCR assay capable of detecting and identifying four legume viruses (AMV, CMV, BYMV, and PSbMV) in a single, straightforward test applied by ICARDA's seed health and Virology Laboratory.

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

MATERIALS

- Infected plants/seedlings with one or more of AMV, CMV, BYMV and PSbMV.
- 70% ethanol for disinfection of surfaces and equipment.
- Balance: capable of weighing to the nearest 0.001 g.
- pH meter: capable of reading to the nearest 0.01 pH unit.
- Pipettes: capable of pipetting to the nearest 0.001 ml.
- Sterile pipette tips.
- Eppendorf tubes: 2 and 1.5 ml.
- Specific polyclonal antibodies.
- AMV, BYMV, CMV and PSbMV specific PCR Primers sets.
- Eppendorf refrigerated centrifuge.
- PCR tubes: 200 µl.
- PCR Thermocycler.
- Agarose electrophoresis equipment.

METHODS

1. Preparation of tested samples

- Infected legume plants at different stages (fresh or lyophilized) can be used for molecular characterization.
- Samples can be tested serologically against AMV, CMV, BYMV, and PSbMV specific antibodies using ELISA or TBIA tests (Kumari *et al*., 2022) before using molecular tools.

A. Grow-out in greenhouse

- Clean and disinfect thoroughly all planting trays that will be used.

- Fill the trays with well-watered potting perlite and make holes of around 2 cm depth in each tray using a planting tag or equivalent.
- Sow approximately 50-100 seeds (according to seed size) from the tested seed samples in one separate tray.
- Cover the seeds in all trays with a thin layer of perlite and place the trays in an insect-proof greenhouse, with adequate space between each other.
- Maintain the greenhouse temperature at 24–30°C during the day and 16–22°C during the night until seedlings emergence.

B. Samples harvesting and preparation

- After 10-15 days of planting, harvest the samples by disinfected scissors or blade.
- It is optional to test the harvested samples serologically to minimize the number of samples subjected to molecular test. For more details about serological detection, check Kumari *et al.*(2022).
- The tested samples may be grouped (10-20 samples in one representative sample to be subjected to molecular testing), or all harvested samples may be subjected individually to molecular testing.
- Lyophilize the samples in the freeze dryer for 48 hrs, and then store at -20°C. Fresh plant samples can be tested immediately.

2. Molecular detection procedure

2.1. Conventional RT-PCR

2.1.1. RNA Isolation

- As the detected viruses are RNA viruses, thus all working surfaces must be disinfected with 70% ethanol and all working tools must be autoclaved before use.
- Cut a maximum of 100 mg plant tissue from each sample and place in a 2 ml Eppendorf tube.
- Extract the RNA 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 PCR control (RNase free water) should be used as references in addition to RNA of healthy plant tissue as a negative control.

2.1.2. Multiplex reverse transcription-polymerase chain reaction (Multiplex RT-PCR)

- Synthesize the cDNA using any RT-PCR kit as per the manufacturer's instructions using random primer (GeneOn Cat No.# S300) (Table 1).
- Prepare multiplex RT-PCR reaction mixture using any appropriate PCR kit available by applying a set of specific primer pairs (Table 2).
- Carry out the RT-PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 25 µl (23 µl reaction mixture + 2 µl synthesized cDNA) (Table 3).
- PCR profile: an initial denaturation of 94 °C for 1 min, followed by 35 cycles (45 sec. at 94°C, 45 sec. at 54°C and 1 min at 72°C), and a final extension for 7 min at 72°C and 15˚C (pause).
- Fractionate 25 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 buffer (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 AMV, BYMV, CMV and PSbMV specific products that should have bands of different size (Figure 1, Table 2).

*Note: It is possible to detect all four viruses 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 mentioned above; it is faster but a bit costly for routine detection.*

Table 1. Reverse Transcription (cDNA synthesis)
cDNA kit: M-MLV Reverse Transcriptase from Invitrogen Cat. No. 28025013.

1. Add to the 0.2 ml tubes:	
Mix 1	**Vol per tube (1x) µL**
Total RNA template	6.0
dNTPs (10 µM)	2.0
dH_2O	2.0
Random primer (10 µM)	2.0
Total	**12.0 µl**
2. Heat mixture to 65°C for 5 minutes and quickly 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	4.0
0.1M DTT	2.0
dH_2O	1.0
Total	**7.0 µl**
4. Mix contents of the tube gently and incubate at 37°C for 2 minutes.	
5. Add 1.0 µL (200 units) of M-MLV RT, and mix by pipetting gently up and down.	
6. Incubate for 60 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: Sequence of specific primer pairs and expected size of PCR product for each primer pair when used in multiplex RT-PCR to amplify the appropriate virus template.

Target species	Primers	Sequence (5' to 3')	Product Size (bp)	Reference
AMV	AMV-F	CGTCAGCTTTCGTCGAACA	288	Bariana *et al.,* 1994
	AMV-R	GCCGTCGCGCATGGTAAT		
BYMV	BYMV-F	GGTTTGGCYAGRTATGCTTTTG	240	Bariana *et al.,* 1994
	BYMY-R	GAGAATTTAAAGACGGATA		
CMV	CMV-F	TATGATAAGAAGCTTGTITCGCGCA	500	Bariana *et al.,* 1994
	CMV-R	TTTTAGCCGTAAGCTGGATGGACAACCC		
PSbMV	PSbMV-2F	TACATCTAGATTACATGGCTCTCATTCCGAGAAG	888	Roberts *et al.,* 2003
	PSbMV-1R	CAAACGCGTGACGAAACCAAGGATGATGAAAG		

Table 3. Reaction mixture for multiplex RT-PCR using two PCR kits
(A) PCR kit of COSMO PCR RED Master Mix from-Willowfort-UK, Cat. No. WF10203001

PCR Reaction Mix	Volume per tube (1x) µl
COSMO PCR RED Master Mix	10.0
Reverse primer (AMV-F) (10 µM)	1.0
Forward primer (AMV-R) (10 µM)	1.0
Reverse primer (BYMV-F) (10 µM)	1.0
Forward primer (BYMV-R) (10 µM)	1.0
Reverse primer (CMV-F) (10 µM)	1.0
Forward primer (CMV-R) (10 µM)	1.0
Reverse primer (PSbMV-2F) (10 µM)	1.0
Forward primer (PSbMV-1R) (10 µM)	1.0
dH_2O	5.0
cDNA template	2.0
Total	**25 µl**

(B) PCR kit of 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	15.0
Reverse primer (AMV-F) (10 µM)	1.0
Forward primer (AMV-R) (10 µM)	1.0
Reverse primer (BYMV-F) (10 µM)	1.0
Forward primer (BYMV-R) (10 µM)	1.0
Reverse primer (CMV-F) (10 µM)	1.0
Forward primer (CMV-R) (10 µM)	1.0
Reverse primer (PSbMV-2F) (10 µM)	1.0
Forward primer (PSbMV-1R) (10 µM)	1.0
SuperScript™ III RT/Platinum™ Taq Mix	1.0
dH_2O	1.0
RNA template	5.0
Total	**30 µl**

PCR Profile

48˚C for 45 min for cDNA synthesis then 94˚C for 5 min, then 35 cycles (45 sec. at 94°C, 45 sec. at 54°C and 1 min at 72°C), and a final extension for 5 min at 72°C and 15˚C (pause).

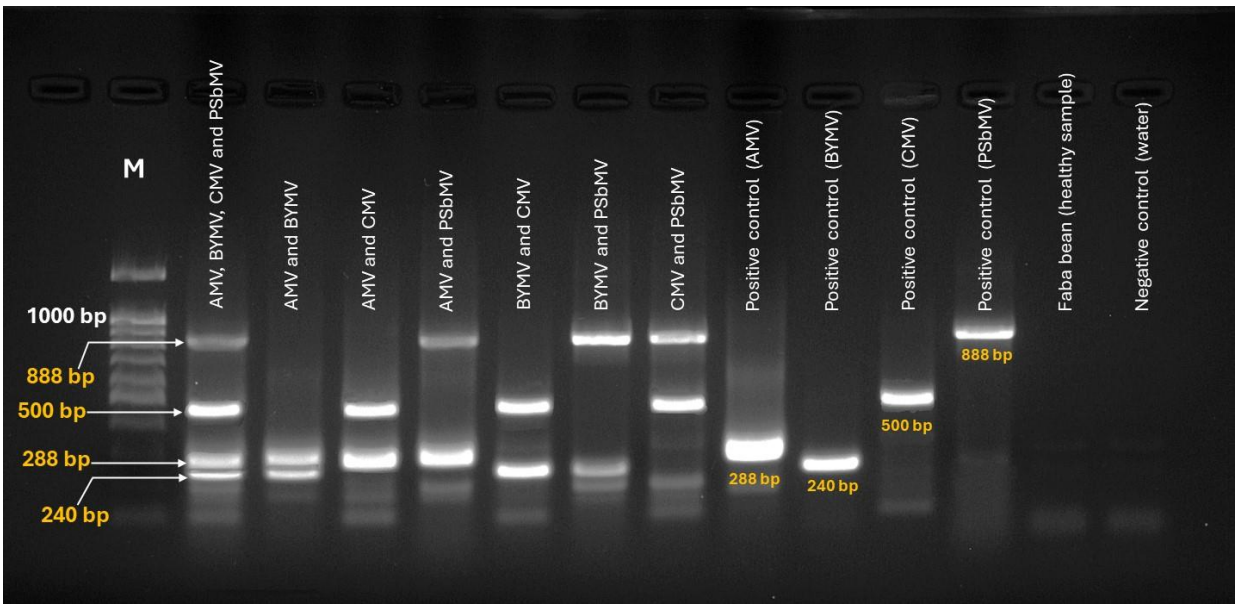

Figure 1. Detection of AMV, BYMV, CMV and PSbMV by multiplex RT-PCR using specific primers (Table 2). M= DNA ladder VC100 bp Plus.

BUFFERS

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.

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