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

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BACKGROUND

Bean yellow mosaic virus (BYMV, genus *Potyvirus*, family *Potyviridae*) is distributed worldwide including CWANA and East African countries, and it is reported to occur on several legumes including faba bean, lentil, chickpea, field pea, grasspea, and a number of forage legumes, as well as a few non-legume hosts. A high incidence, up to 100%, has been noted in some regions of Egypt, Iraq and Sudan (Makkouk *et al*., 2003, 2012; Kumari *et al.,* 2009; Kumari and Makkouk, 2007). BYMV is transmitted through seeds of most pulses, including faba beans, field peas, lentils, and lupins and through seed of several forage legumes and clovers. The virus is also transmitted by several species of aphids in a non-persistent manner.

Early detection and accurate diagnosis of viral diseases is critical for the application of appropriate control measures. The last three decades have witnessed significant developments in improving the sensitivity of the methods to detect seed-borne viruses. The development of polymerase chain reaction (PCR) has greatly improved the sensitivity and utility of hybridization and other nucleic acid-based assays. Immunocapture-RT-PCR (IC-RT-PCR) is another alternative where virus particles are captured with the help of specific antibodies followed by their detection using PCR without isolating RNA. This hybrid diagnostic technique is more sensitive than ELISA and RT-PCR alone (Mulholland 2009; Wetzel *et al.* 1992). In addition, Immunocapture of virus particles step provides a simple method to isolate virus particles from plant tissue, particularly when inhibitory substances are present (Selvaraj *et al*., 2009), and thus enables subsequent use of RT-PCR amplification. Many primers for detecting BYMV viruses by RT-PCR have been reported (Bariana *et al.*, 1994; Uga, 2005).

The current report describes a validated molecular tool (RT-PCR and IC-RT-PCR) for detection and characterization of BYMV in legumes applied by ICARDA's GHU.

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

MATERIALS

- BYMV 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.
- Sterile pipette tips.
- Eppendorf tubes: 2 and 1.5 ml.
- BYMV specific PCR Primers.
- Eppendorf refrigerated centrifuge.
- PCR tubes: 200 µl.
- Thermal cycler.
- Agarose electrophoresis equipment.

For Immunocapture-RT-PCR (IC-RT-PCR), specific BYMV Polyclonal antibody is needed, in addition to the above materials.

METHODS

1. Tested samples

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

2. Molecular detection procedure

2.1. Conventional RT-PCR

2.1.1. 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 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 as negative control.

2.1.2. Reverse transcription-polymerase chain reaction (RT-PCR)

- Synthesize the cDNA using any RT-PCR kit as per the manufacturer's instruction using reverse primer BYMV-CPD (Al-Khalaf *et al.,* 2008) (Table 1).
- Prepare RT-PCR reaction mixture using any PCR kit available (Table 2) by the following primer pairs (Al-Khalaf *et al.,* 2008)

  BYMV-CPU: (5'- GTCGATTTCAATCCGAACAAG -3')

  BYMV-CPD: (5'- GGAGGTGAAACCTCACTAATAC -3')
- Carry out the RT-PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 20 µl (18 µl reaction mixture + 2 µl synthesized cDNA) (Table 2).
- PCR profile: an initial denaturation of 94 °C for 2 min, followed by 35 cycles of (30 s at 94°C, 1 min at 50°C and 2 min at 72°C), and a final extension for 5 min at 72°C and 15˚C (pause).
- Fractionate 20 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 BYMV specific product of 907 bp (Figure 1).

Note: It is possible to detect BYMV 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, it is faster but a little bit costly for routine detection.*

2.2. Immunocapture-RT-PCR (IC-RT-PCR)

2.2.1. Tube coating

- Coat 200 µl- PCR tube with 50 µl of virus-captured antibody (polyclonal antibody) diluted 1:1000 (v:v) in Coating buffer (pH 9.6), close the caps and incubate overnight at 4°C. Antibody-coated tubes may be prepared in advance and stored at −20°C for at least 4 weeks.
- After antibody coating, wash the PCR tubes wells twice with 200 µl of PBST and once with PBS.

2.2.2. Samples extraction

- Extract the tissue (fresh of lyophilized tissues) in sample extraction buffer (SEB, pH 7.4) (dilution 1:10; w:v) using any type of extraction procedure.
- Transfer 1.0 ml of the extraction to 2.0 ml Eppendorf tubes and centrifuge at 15,000 rpm for 5 min and discard the sediment.

2.2.3. BYMV capture

- Add 50 µl of tissue extract to individual precoated tubes from previous step (paragraph # 2.2.1) and incubate overnight at 4°C.
- Remove the extracts from the tubes by knocking the tubes on an absorbent pad.
- Wash the PCR coated tubes wells twice with 200 µl of PBST and once with PBS and final wash with RNase free water.
- Allow the tube to dry and proceed to cDNA synthesis (paragraph # 2.1.2).

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

A) Conventional RT-PCR

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
Reverse primer (BYMV-CPD) (10 pmol)	2.0
Total	**12.0 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	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 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.	

B) Immunocapture-RT-PCR (IC-RT-PCR) – it similar to the above Conventional RT-PCR, except Mix 1

1. Add to the 0.2 ml tubes:	
Mix 1	**Vol per tube (1x) µL**
dNTPs (10 µM)	2.0
dH_2O	8.0
Reverse primer (BYMV-CPD) (10 pmol)	2.0
Total	**12.0 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	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.	
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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
Using the 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 (BYMV-CPD) (10 pmol)	1.0
Forward primer (BYMV-CPU) (10 pmol)	1.0
dH_2O	6.0
cDNA template	2.0
Total	**20 µl**

Table 3. Reaction mixture for one step RT-PCR

Using the 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	5.0
Reverse primer (BYMV-CPD) (10 pmol)	1.0
Forward primer (BYMV-CPU) (10 pmol)	1.0
SuperScript™ III RT/Platinum™ Taq Mix	0.2
dH_2O	6.8
RNA template	6.0
Total	**20 µl**

PCR Profile

48˚C for 45 min for cDNA synthesis then 94˚C for 5 min, then 35 cycles of (30 s at 94°C, 1 min at 50°C and 2 min at 72°C), a final extension for 5 min at 72˚C and 15˚C (pause).

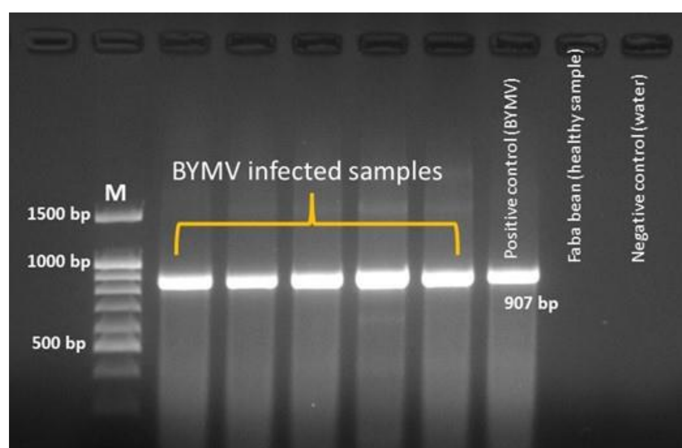

Figure 1. Detection of BYMV by RT-PCR using specific primers BYMV-CPU and BYMV-CPD.
M= DNA ladder VC100 bp Plus.

SOLUTIONS & BUFFERS

Coating buffer (pH 9.6)

- 0.16% Sodium carbonate (Na_2CO_3)
- 0.29% Sodium bicarbonate ($NaHCO_3$)

Phosphate-buffered saline (1x PBS), pH 7.4 (1 liter)

- 8.0 g Sodium chloride (NaCl)
- 0.24 g Potassium phosphate (Monobasic) (KH_2PO4)
- 1.44 g Sodium Phosphate (Dibasic) (Na_2HPO4)
- 0.2 g Potassium chloride (KCl)

Phosphate-buffered saline tween (PBST) (1 liter)

- 1000 ml of 1x PBS (pH 7.4) + 0.5 ml Tween 20

Sample extraction buffer (SEB), pH 7.4

- PBST (pH 7.4)
- 2% (w/v) polyvinylpyrrolidone 25000
- 0.5% (w/v) bovine serum albumin.
- 1% Sodium sulfite (Na_2SO_3)

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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