Reverse transcription-polymerase chain reaction (RT-PCR) assay for detection and characterization of Alfalfa mosaic virus (AMV) in legume crops applied by ICARDA's Seed Health and Virology Laboratory

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BACKGROUND:

Alfalfa mosaic virus (AMV, genus Alfamovirus, family *Bromoviridae*) is a significant plant pathogen affecting a wide range of legume crops across various regions of the world including West Asia and North Africa (WANA) countries (Kumari and Makkouk, 2007; Kumari *et al*., 2009; Makkouk *et al*., 2012). AMV has one of the broadest host range among plant viruses, affecting over 600 species in more than 70 plant families, and it is transmitted by over 14 aphid species, including *Myzus persicae* (green peach aphid), in a non-persistent manner. The virus can also spread through infected seeds of several hosts (including faba bean, chickpea, and lentil), mechanical means, and grafting. Seed transmission makes managing AMV more challenging, as infected seeds can introduce the virus into new areas and lead to infections in the next growing season, even before aphid vectors are active (Makkouk and Attar, 2003). Accordingly, seed testing and the use of certified seeds are key strategies to mitigate the spread of AMV as a seed-borne virus.

For many years, the most commonly used methods to identify viruses in vegetative plant parts and in seeds were the serological tests using antibodies, such as ELISA and TBIA (Ahoonmanesh, 1990; Kumari *et al*., 2009; 2022). 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 plant viruses. The development of polymerase chain reaction (PCR) in addition to other molecular-based techniques such as molecular hybridization has greatly improved the test sensitivity. However, the accuracy and reliability of molecular methods is a fact beyond dispute.

Immunocapture-RT-PCR (IC-RT-PCR) is an advanced diagnostic technique where virus particles are first captured using specific antibodies, and then detected via PCR without the need for prior RNA extraction. This hybrid approach offers greater sensitivity compared to using either ELISA or RT-PCR alone (Mulholland, 2009; Parrella *et al.,* 2011). Additionally, the immunocapture step provides an efficient method to isolate virus particles from plant tissues, particularly when inhibitory substances are present (Selvaraj *et al.,* 2009), facilitating subsequent RT-PCR amplification.

Many primers for detecting AMV viruses by RT-PCR have been reported (Bariana *et al.,* 1994; Xu and Nie, 2006). This report describes a validated molecular tool for AMV detection and characterization applied by ICARDA's Seed Health and Virology Laboratory.

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

MATERIALS:

- AMV-infected field plants/seedlings.
- 70% ethanol for disinfection of surfaces and equipment.
- Balance: accuracy 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.
- Specific polyclonal antibodies.
- AMV specific RT-PCR Primers.
- Eppendorf refrigerated centrifuge.
- PCR tubes: 200 µl.
- PCR Thermocycler.
- Agarose electrophoresis equipment.
- For Immunocapture-RT-PCR (IC-RT-PCR), specific AMV polyclonal antibody is needed, in addition to the above materials.

METHODS

1. Preparation of tested samples

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

A. Grow-out test 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 deep 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 with adequate space between each other, in an insect-proof greenhouse.
- Maintain the greenhouse temperature at 24–30°C during the day and 16–22°C during the night until seedlings emerge.

B. Samples harvesting and preparation

- 10-15 days after planting, harvest the samples by using 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, please see Kumari *et al.* (2022).
- The tested samples may be grouped (10-20 samples in one compound 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, then store them at -20°C, or one can test fresh plant tissue.

2. Molecular detection procedure

2.1. Conventional RT-PCR

2.1.1. RNA Isolation

- Since AMV is RNA virus, thus all working surfaces must be disinfected with 70% Ethanol and all working tools must be autoclaved before using.
- Place 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 of an 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 AMV-R (Bariana *et al.,* 1994) (Table 1).
- Prepare RT-PCR reaction mixture using any PCR kit available (Table 2) and use the following primer pairs (Bariana *et al.,* 1994):

  AMV-F: (5'- CGTCAGCTTTCGTCGAACA -3')
  AMV-R: (5'- GCCGTCGCGCATGGTAAT -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 1 min, followed by 35 cycles of (45 sec. at 94°C, 45 sec. at 50°C and 1 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 AMV specific product of 288 bp (Figure 1).

Note: *It is possible to detect AMV 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 previously described, which is faster but a bit more 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 or lyophilized) 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 extract to 2.0 ml Eppendorf tube and centrifuge at 15,000 rpm for 5 min and discard the pellet.

2.2.3. AMV capture

- Add 50 µl of tissue extract to individual pre-coated tubes from the 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 (AMV-R) (10 µM)	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 is 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 (AMV-R) (10 µM)	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.	

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 (AMV-F) (10 µM)	1.0
Forward primer (AMV-R) (10 µM)	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 (AMV-F) (10 µM)	1.0
Forward primer (AMV-R) (10 µM)	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 (45 sec. at 94°C, 45 sec. at 50°C and 1 min at 72°C), and a final extension for 5 min at 72°C and 15˚C (pause).

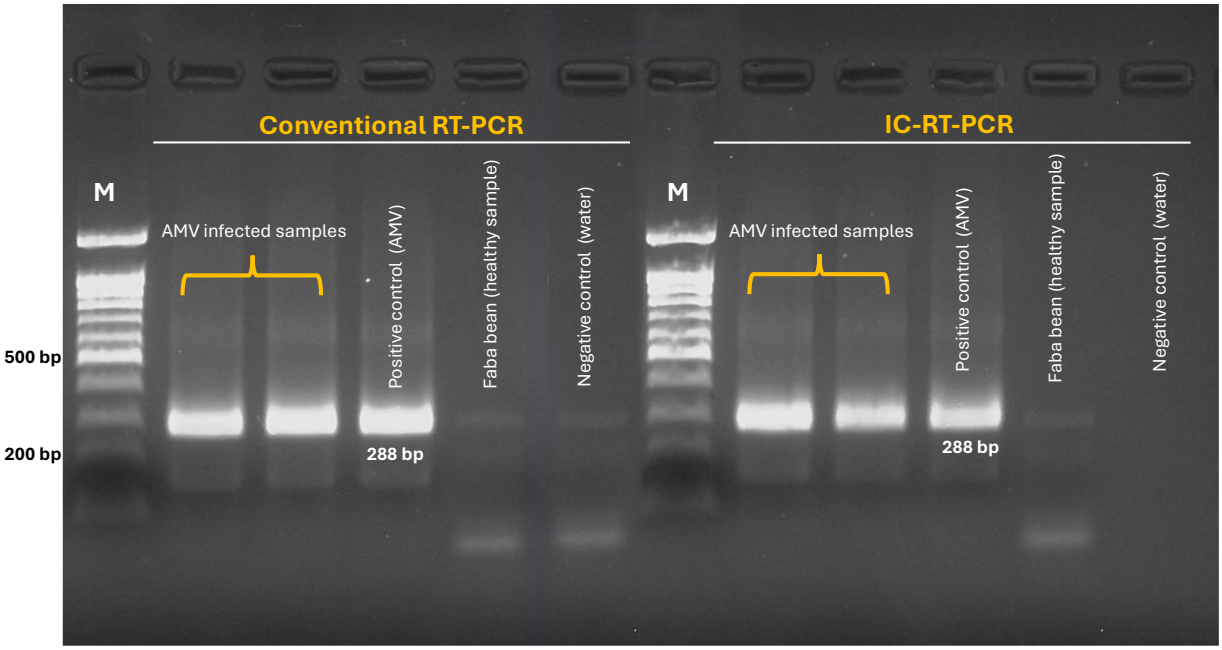

Figure 1. Detection of AMV by RT-PCR using specific primers AMV-F and AMV-R.
M= DNA ladder VC100 bp Plus.

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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