International Seed Federation
Seed is Life

Detection of *Pepino mosaic virus* in Tomato Seed

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Developed by ISHI-Veg

Detection of *Pepino mosaic virus* in Tomato seed

Crop: Tomato (*Solanum lycopersicum*)

Pathogen(s): *Pepino mosaic virus*

Version: 5 (March 2021)

PRINCIPLE

Detection of infectious *Pepino mosaic virus* (PepMV) in seed of tomatoes is done by inoculation of *Nicotiana benthamiana* plants followed by ELISA of the new non-inoculated leaves of the plants to confirm the presence of infectious PepMV.

An ELISA on seed extracts may be used as a pre-screen. The test is complete if no PepMV is detected and the seed lot is considered healthy. However, as ELISA detects both infectious and non-infectious virions, a positive pre-screen ELISA is followed by a bioassay to determine the presence of infectious PepMV. The full method process workflow is presented in Figure 1.

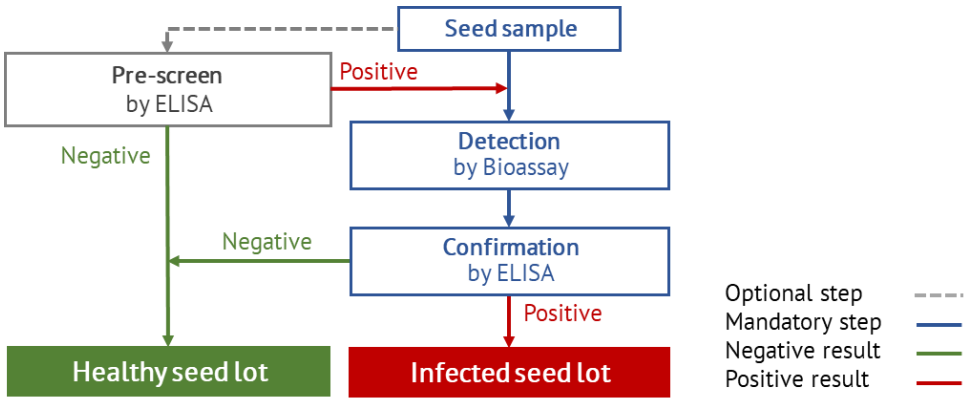

Figure 1. Method process workflow

METHOD VALIDATION

The test method was evaluated in a comparative test organised by ISHI-Veg (Krinkels 2001).

The test for detecting PepMV in tomato seed has been approved by the US National Seed Health System (NSHS) as a Standard B (https://seedhealth.org/seed-health-testing-methods/).

The ELISA has been shown to reproducibly detect a single PepMV infested seed in test samples in a comparative test with six participating laboratories (Krinkels 2001). Using a dilution series of systemically infected leaves in healthy seed extracts, 10,000-fold dilutions were reproducibly detected in both ELISA and bioassay. This sensitivity could vary depending upon laboratory conditions. It is the responsibility of the user to determine sensitivity at the time of running the assay.

RESTRICTIONS ON USE

Technical details on the reagents / material used in the comparative test study (e.g. supplier information) are provided in the protocol. If material and consumables from different suppliers are used, it is necessary to verify their performance.

This test method is suitable for untreated seed.

This test method is suitable for seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for inhibition by analysis, sample spiking, or experimental comparisons.

It is recommended that the seed be checked for traces of any seed treatment that may interfere with the infectivity of the virus. This is done by comparing the infectivity of PepMV in two dilution series: one of the treated-seed extract spiked with infectious PepMV and the other with infectious PepMV in extraction buffer. The latter acts as a control.

Although ELISA is compatible with some seed treatment chemicals (Pataky et al. 2004), seed treatments may affect the performance of this test. It is the responsibility of the user to check for such antagonism by analysis, sample spiking, or experimental comparisons.

METHOD EXECUTION

To ensure process standardisation and valid results, it is strongly recommended to follow the best practices developed by ISHI-Veg for ELISA Assays in Seed Health Tests.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 250 seeds.

It should be noted that infested seeds (a single one being detectable in a sample) rarely lead to infected seedlings. Given this low transmission rate (less than 1 in 1000 (Krinkels 2001)), a sample size of 3,000 seeds is considered to be appropriate.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	October 2003	
2	January 2007	Protocol updated to a new ISF template. 'Method Description' section updated, including a simplification of the ELISA method.
3	January 2009	'Restrictions on Use' section updated to include that the test is suitable for seeds treated with most chemicals with the aim of fungal disinfection with reference to Pataky et al. 2004.

4	August 2011	Crop name *Solanum lycopersicum* added. 'Sensitivity and Restrictions on Use' section updated to include that it is the responsibility of the end-user to check for antagonism and to determine sensitivity of the assays at the time of running the assay.
4.1	July 2017	'Validation' section updated to include the approval from the NSHS as Standard B. 'Method Execution' section added.
5	March 2021	Protocol presented in accordance with ISHI-Veg's protocol guidelines. Crop name *Lycopersicon esculentum* removed. A table with the controls added to the ELISA section.

Protocol for detecting *Pepino mosaic virus* in Tomato Seed

I. PRE-SCREEN BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (OPTIONAL STEP)

Materials

- Seed extraction buffer (Table I.1)
- Controls (Table I.2)
- Grinder
- ELISA necessities
- Lab disposables

Table I.1 Seed extraction buffer (use within 24 h after preparation)

Compound	Amount/L
NaCl	8.0 g
$Na_2HPO_4.12H_2O$	2.9 g
KH_2PO_4	0.2 g
KCl	0.2 g
Na_2SO_3[a]	1.0 g

[a]Add after autoclaving.

Table I.2 Types of controls used

Control type	Description
Positive Process Control (PPC)	Tomato seed with infectious PepMV or an appropriate dilution of reference material including infectious PepMV in seed extraction buffer.
Negative Process Control (NPC)	Tomato seed free of PepMV
Buffer Control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen

1. Seed extraction

1.1. Grind seeds of each subsample, the positive process control (PPC) and the negative process control (NPC), in 10 mL seed extraction buffer.

Note: Process extracts immediately after grinding or store at 4 °C for a maximum of 20 hours. Do not freeze. If the extracts are to be used for the bioassay after ELISA, the ELISA must be completed within 20 hours after extraction. Store the PPC and NPC (Table I.2) under similar conditions as the samples to validate the results.

2. ELISA

2.1. Run a double-antibody-sandwich (DAS-)ELISA on the extracts (Albrechtsen 2006, Clark & Adams 1977).

2.2. A subsample is regarded as positive (infectious PepMV present) when the signal in ELISA is above the decision threshold.

Note: The source of antiserum is critical. In the comparative test study the antiserum supplied by Wageningen Plant Research was used (https://www.wur.nl/en/show/Prime-Diagnostics-2.htm). If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

3. Validity of test results

Test results are only valid when all included controls presented in Table I.2 give the expected result. A positive test result must be followed by a bioassay (described in section II) to determine the presence of infectious PepMV.

II. DETECTION BY BIOASSAY AND CONFIRMATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

- Seed extraction buffer (Table I.1)
- Controls (Table I.2)
- Grinder
- *Nicotiana benthamiana* plants
- Carborundum
- Protective mask
- Gloves
- Tap water
- ELISA necessities
- Lab disposables

General notes

- *Nicotiana benthamiana* is a systemic host for all PepMV strains tested. *N. benthamiana* is preferred over tomato as an assay plant because the systemic movement of the virus in tomato can be erratic. Furthermore, leaves of *N. benthamiana* are more easily inoculated.
- Although PepMV infection of *N. benthamiana* usually results in conspicuous symptoms this is not the case at all times, and symptoms can be caused by other factors than PepMV. Therefore, ELISA of assay plants is required.

1. Seed extraction

1.1. Perform the seed extraction as described in section I.1, or use the seed extracts as obtained in section I.1 when performing the bioassay after the pre-screen ELISA assay (Section I).

2. Inoculation of *Nicotiana benthamiana* assay plants

Assay plants should have 4–7 (nearly) fully expanded leaves, and should have been raised under sufficient light intensity at an average temperature of 20–25 °C. Avoid older assay plants that start to flower. Assay plants should have good turgor at the time of inoculation (Figure II.1).

Figure II.1 An assay plant at the latest stage suitable for inoculation; Leaves to be inoculated are marked by 'x'.

2.1. Inoculate each seed extract, including the positive process control (PPC), and negative process control (NPC), on the two youngest (nearly) fully expanded leaves of two plants, going across the whole surface. Do not use the primary leaf (oldest true leaf).

Inoculate by first dusting the leaves moderately with carborundum (320 mesh grit powder, Fisher Scientific or equivalent), while wearing a protective mask, followed by placing a drop of inoculum (100-200 µL) onto the leaf. Smear the drop with fingers without applying pressure.

Note: Work with gloves and change them between samples or clean hands thoroughly between samples by using alkaline soap.

2.2. Rinse the plants with tap water a few minutes after inoculation.

2.3. In order to allow the assay plants to become systemically infected, incubate them for at least 14 days under controlled conditions at 25±5 °C and with at least 12 hours of light per day.

3. Sampling of *Nicotiana benthamiana* assay plants and leaf extraction

3.1. For each subsample, the PPC, and the NPC, sample and pool leaf material from both assay plants, making sure that the pooled leaves weigh 0.2–0.5 g. Select younger leaves that have expanded during the preceding weeks and not the inoculated leaves.

3.2. Process samples immediately, store at 4° C for at most 48 hours or freeze until use. If the samples were frozen, process them as soon as they have thawed.

3.3. Grind each pooled leaf sample in 10-12 mL ELISA extraction buffer. Process extracts immediately after grinding, store at 4 °C for a maximum of 24 hours or freeze until use.

4. ELISA

4.1. Run a double-antibody-sandwich (DAS-)ELISA (Albrechtsen 2006, Clark & Adams 1977) on the extracts obtained at step 3.3.

4.2. A subsample is regarded as positive (infectious PepMV present) when the signal in ELISA is above the decision threshold.

Note: The source of antiserum is critical. In the comparative test study the antiserum supplied by Wageningen Plant Research was used (https://www.wur.nl/en/show/Prime-Diagnostics-2.htm). If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

5. Validity of test results

Test results are only valid when all included controls presented in Table I.2 give the expected result.

REFERENCES

Albrechtsen, S.E. (2006). Testing methods for seed-transmitted viruses: principles and protocols. Wallingford, UK: CABI Publishing.

Clark, M.F. & Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, **34**, 475–483.

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Pataky, J. K., Block, C. C., Michener, P. M., Shepherd, L. M., McGee, D. C., & White, D. G. (2004). Ability of an ELISA-based seed health test to detect *Erwinia stewartii* in maize seed treated with fungicides and insecticides. *Plant Disease,* **88**, 633–640.