



Method for the Detection of *Pepino mosaic virus* on Tomato seed

Crop:	Tomato (<i>Lycopersicon esculentum</i> L. now <i>Solanum lycopersicum</i>)
Pathogen:	<i>Pepino mosaic virus</i> (PepMV)
Revision history:	Version 4, July 2017

Sample and sub-sample size

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

Principle

- Detection of infectious PepMV by inoculation of *Nicotiana benthamiana* plants followed by ELISA of the plants to confirm infection by PepMV.
- Optionally, seed extracts can be pre-screened with ELISA. If ELISA detects no virus in the seed extracts, the test is considered negative and complete. However, if the ELISA on seed extracts is positive then a bioassay is necessary to determine whether infectious PepMV is present. (An ELISA test detects both infectious and non-infectious virions.)

Sensitivity and Restrictions on Use

- 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 such antagonism and/or 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 (1), 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.
- The ELISA has been shown to reproducibly detect a single PepMV infested seed in test samples in a comparative test with 6 participating laboratories (2).

Note: The method was reviewed recently and found to be fit for purpose. The sections **Validation** and **Method Description** have been updated. A section on **Method Execution** has been added.

- 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 (2)), a sample size of 3000 seeds is considered to be appropriate.
- 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.

Validation

This test method was evaluated in a comparative test organized by ISHI-Veg (2).

This method has also been approved by the US National Seed Health System (NSHS) as a Standard B (see <http://seedhealth.org/seed-health-testing-methods/>).

Method Execution

To ensure process standardization and valid results, it is strongly recommended to follow the best practices developed by ISHI-Veg for *ELISA Assays in Seed Health Tests* (see <http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/>).

Method description

1. Extraction of virus from the seed

- 1.1 Grind seeds of each sub-sample in a 10 ml seed extraction buffer.
 - Process extracts immediately after grinding or store at 4°C for a maximum of 20 hours. Do not freeze. Note that if the extracts are to be used for the bioassay after ELISA, the ELISA must be completed within 20 h after extraction.

2. (Optional) ELISA

- 2.1 Run a double-antibody-sandwich (DAS-)ELISA (3, 4) on the extracts.
 - The source of antiserum is critical. It is recommended that the performance of other antisera be verified against the antiserum supplied by Wageningen Plant Research (<https://www.wur.nl/en/show/Prime-Diagnostics-2.htm>).

3. Inoculation of *Nicotiana benthamiana* assay plants

- Assay plants should have 4-7 (nearly) fully expanded leaves, should not yet have started flowering, and should have been raised under sufficient light intensity at an average temperature of 20 – 25°C. Assay plants should have good turgor at the time of inoculation (Fig. 1).
- 3.1 Inoculate each extract 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).
 - 3.1.1 Dust the leaves moderately with carborundum (320 mesh grit powder, Fisher Scientific or equivalent). Wear a protective mask.
 - 3.1.2 Place a drop of inoculum (100-200 µl) onto the leaf. Smear the drop with fingers without applying pressure.
 - Work with gloves and change them between samples or clean hands thoroughly between samples by using alkaline soap.
 - 3.1.3 Rinse the plants with tap water a few minutes after inoculation.
 - 3.2 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.

3.3 Determine infection of the assay plants by PepMV using ELISA.

- 3.3.1 For each sub-sample, sample and pool leaf material from both assay plants making sure that the pooled leaves weigh 0.2–0.5 g. Select leaves that have expanded during the preceding week (not the inoculated leaves).
 - ❑ 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.
- 3.3.2 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.
- 3.3.3 Run a DAS-ELISA (3, 4) on the extracts.
 - ❑ The source of antiserum is critical. It is recommended that the performance of other antisera be verified against the antiserum supplied by Wageningen Plant Research (<https://www.wur.nl/en/show/Prime-Diagnostics-2.htm>).

Notes

- *N. 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.

Buffers

- Use de-ionized water.
- Autoclave buffers at 121 °C, 115 psi for 15 minutes.

Seed extraction buffer per liter (use within 24 hours after preparation)

Sodium chloride NaCl	8.0 g
Disodium hydrogen phosphate dodecahydrate Na ₂ HPO ₄ .12H ₂ O	2.9 g
Potassium dihydrogen phosphate KH ₂ PO ₄	0.2 g
Potassium chloride KCl	0.2 g
Sodium sulphite Na ₂ SO ₃ ¹	1 g

¹ add after autoclaving

References

1. Pataky, J. K., Block, C. C., Michener, P. M., Shepherd, L. M., McGee, D. C., and 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.
2. Krinkels, M. (2001) Pepino mosaic virus causes sticky problem. Prophyta, The Annual May 2001: 30-33.
3. Albrechtsen, S.E. (2006) Testing methods for seed-transmitted viruses: principles and protocols. Wallingford, UK: CABI Publishing.

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

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

