Method for the Detection of infectious Tobamoviruses on Pepper Seed

Crop:	Pepper (*Capsicum annuum* L.)
Pathogen:	*Tobacco mosaic virus* (TMV), *Pepper mosaic virus* (ToMV) and *Pepper mild mottle virus* (PMMoV)
Revision history:	Version 5, 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 tobamoviruses on pepper seed by using the local lesion assay; inoculation of indicator tobacco plant leaves with pepper seed ground in buffer. Infectious virions will cause a local lesion response (1). *Nicotiana tabacum* cv. Xanthi NN and/or *Nicotiana glutinosa* can be used as assay plants.

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 (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.
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a user chooses to test treated seed using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.
- The sensitivity of the test is reduced significantly under suboptimal bioassay incubation conditions, e.g. insufficient light (2), too high temperature (3), (4), (5).

Validation

The method was evaluated in a comparative test organized by ISHI-Veg (6).

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

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

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 the PBS seed extraction buffer at a rate of 4 ml per 100 seeds. Process seed extracts within 4 hours after grinding.

1.2 If the assay is performed after ELISA pre-screening, grind seed in an alternative ELISA buffer and store at 4°C.

- ❑ It must be demonstrated that local lesions can be obtained with the alternative ELISA buffer and that it is equivalent to PBS.
- ❑ Seed extracts and positive and negative controls must be stored at 4°C until the assay begins. It is strongly recommended the local lesion assay after ELISA testing be done as soon as possible.
- ❑ The final results of the local lesion assay must be validated through comparison of the results given by both controls. The seed extracts and the controls must be prepared at the same time, under the same laboratory conditions and have been stored under the same conditions.
- ❑ If a laboratory routinely uses ELISA as a pre-screen, the correlation between ELISA responses and the number of lesions in the local lesion assay should be well established for the routinely used reference material. This will further establish whether the storage of samples has influenced the assay results.

2. Inoculation of *Nicotiana* assay plants

- ❑ Raise assay plants known to be resistant to all races of TMV or ToMV (*e.g.* *Nicotiana tabacum* cv. Xanthi NN) under sufficient light intensity at a temperature of 20 – 25°C. Choose plants with 4-5 true leaves (6-7 weeks after sowing) with high turgor. Do not use flowering or old plants (8).

2.1 Inoculate the entire surface of two (nearly) fully expanded consecutive leaves of two plants with each seed extract.

- ❑ Do not use the primary leaf (oldest true leaf) since it is different in shape/texture/thickness and thus less susceptible.

2.1.1 Dust the leaves with the appropriate quantity of carborundum (320 mesh grit powder, Fisher Scientific or equivalent) such that there is a very fine layer on the leaf surface. Wear a protective mask.

2.1.2 Place a drop of inoculum (100-200 µl) onto the leaf. Smear the drop with gloved fingers by applying constant, slight pressure but avoid damaging the leaf.

- ❑ Work with gloves and change them between samples or clean hands thoroughly between samples by using alkaline soap or equivalent and then rinse with water to remove residues.

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

2.2 Inoculate positive and negative control plants in a similar manner to the ones used for the seed extracts.

2.2.1 Choose one of the following listed positive controls

- A well-characterized positive seed lot

- Pea seed flour mixed with powdered TMV/ToMV/PMMoV infected *Nicotiana* leaves ground together in a seed extraction buffer (5 mL per 5 gr) or in an alternative ELISA buffer if the assay is performed after ELISA pre-screening
- Liquid extract of TMV/ToMV/PMMoV infected leaves of Solanaceous hosts sufficiently diluted in PBS seed extraction buffer or in an alternative ELISA buffer if the assay is performed after ELISA pre-screening

❑ Depending on the kind of reference material (positive control), adjust the quantity to induce development of countable local lesions. Grinding the reference material into a flour of fine particles must be done to ensure the efficacy of virus extraction.

2.2.2 Use seed extraction buffer or extract from virus-free seed lot as a negative control.

2.3 Incubate the plants for 5-7 days under controlled conditions at 20 – 25 °C and provide at least 12 hours of light.

❑ A hypersensitive response of tobacco plants is not expressed at temperatures higher than 28 °C (7), (8), (9), (10), (11), (12), (13). Thereafter, the test could be less sensitive in greenhouses during the summer period because of high temperature. The sensitivity of the leaves is significantly reduced by downy mildew infection of the tobacco plants. Plants must be free of pathogens and no visible symptoms must be present (Koenraadt H. personal communication).

2.4 Evaluate the inoculated leaves for local lesions (Fig. 1).

❑ When a relatively high number of local lesions is observed, a comparison with the positive and negative controls will readily reveal that most, if not all, of the virus lesions are authentic.

❑ In cases where the number of lesions for a sample is low, it is necessary to confirm a lesion was caused by a virus infection and not due to an artifact (from the mechanical inoculation, use of pesticides, etc.). Cut out the suspect lesion, crush it in a small amount of the seed extraction buffer and inoculate two leaves of two assay plants again. Lesions caused by virus infection contain sufficient amounts of infectious virus to produce multiple lesions in this confirmation test (6).

Buffers

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

Seed extraction buffer (Phosphate Buffered Saline, PBS) pH 7.2 – 7.4 per liter

Sodium chloride NaCl	8.0 g
Disodium hydrogen phosphate Na_2HPO_4	1.15 g
Potassium dihydrogen phosphate KH_2PO_4	0.2 g

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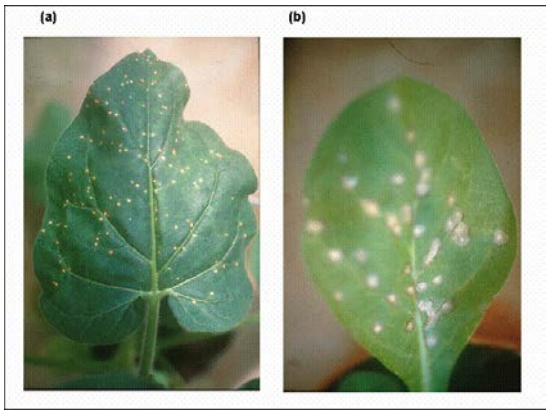
Fig. 1. Local lesions on tobacco indicator plants (a) *Nicotiana glutinosa*, (b) *Nicotiana tabacum* cv. Xanthi NN