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# Detection of Infectious Tobamoviruses in Tomato Seed

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Developed by ISHI-Veg
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# Detection of Infectious Tobamoviruses in Tomato Seed

**Crop:** Tomato (*Solanum lycopersicum*)

**Pathogen(s):** *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Tomato mottle mosaic virus* (ToMMV) and *Tomato brown rugose fruit virus* (ToBRFV)

Note: For many years *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) have been the most prominently perceived tobamoviruses infecting tomato seed. Recently two other tobamoviruses related to but distinct from TMV and ToMV were characterized: *Tomato mottle mosaic virus* (ToMMV) (Li et al., 2013) and *Tomato brown rugose fruit virus* (ToBRFV) (Salem et al., 2016).

These viruses are shown to cause disease in tomato. As in the case of infection with TMV and ToMV, ToMMV (Sui et al., 2017) and ToBRFV (Luria et al., 2017) generate local lesions on leaves of indicator plants containing the N-gene (*Nicotiana tabacum* cv. Xanthi NN and *Nicotiana glutinosa*). Therefore, the method covers these two viruses as well.

**Revision history:** Version 6.2, July 2019

## PRINCIPLE

Detection of infectious tobamoviruses in tomato seed by inoculating leaves of indicator plants *Nicotiana tabacum* cv. Xanthi NN or *Nicotiana glutinosa* with tomato seed extract. Infectious virions cause typical local lesions that demonstrate viability of the virus.

A DAS-ELISA assay may be used to pre-screen a seed extract. The test is complete if no virus is detected and the seed lot is considered healthy. As ELISA detects both infectious virions and non-infectious virus particles, a positive ELISA is followed by a bioassay to determine the presence of infectious tobamovirus.

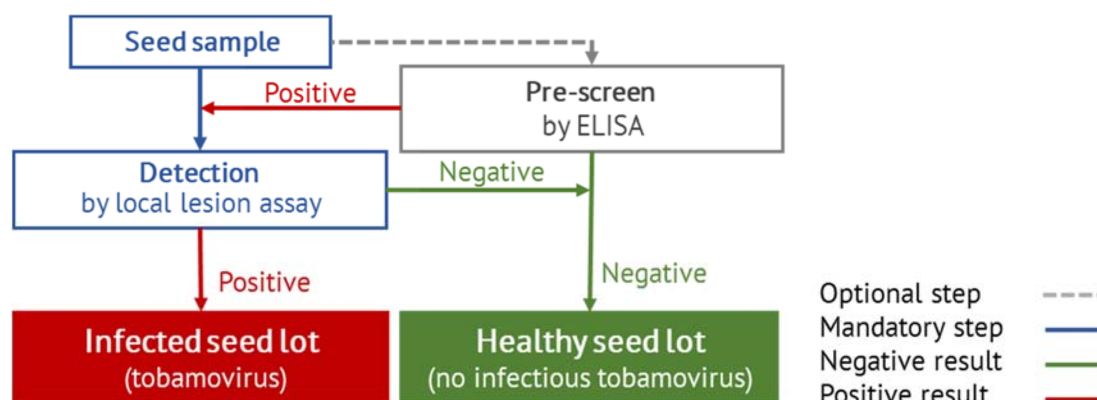


**Figure 1.** Method process flow



NOTE: In this version the buffer volume used to extract the virus from seed in section 2 of the ELISA assay has been corrected

## METHOD VALIDATION

The local lesion assay has been in use by the industry for many years.

The method for detecting infectious *tobacco mosaic virus* and *tomato mosaic virus* in *Solanum lycopersicum* (tomato) seed by the local lesion assay (indexing) on *Nicotiana tabacum* plants is an ISTA Rule since 2011 (www.seedtest.org). The US National Seed Health System (NSHS) (http://seedhealth.org) has approved the method as a Standard B.

## RESTRICTIONS ON USE

This method is suitable for testing untreated seed.

It is also suitable for testing 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 not validated for seed treated with protective chemicals or biological substances. If treated seed is tested, the user is responsible for determining empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

## METHOD EXECUTION

When pre-screening a seed lot using the ELISA test, it is strongly recommended that 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-development/) be followed to ensure process standardization and that the results are valid.

All four tobamoviruses in scope of this method are closely related and it has been shown that polyclonal antisera raised against one tobamovirus may show cross-reactivity with one or more of the other tobamoviruses. Commercial antisera specifically raised against ToMMV and ToBRFV may not yet be available; the antisera for TMV and ToMV may be used provided their reactivity with ToMMV and ToBRFV is verified.

Note: The ELISA seed extract can also be used to perform the SE-qPCR in the ISHI-Veg test: *Detection of Infectious ToBRFV in Tomato and Pepper Seed*.

## SAMPLE AND SUB-SAMPLE SIZE

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

# Protocol for detection of infectious tobamoviruses in tomato seed

This protocol is also available on ISTA's website:
ISTA 7-028: Detection of infectious *tobacco mosaic virus* and *tomato mosaic virus* in *Solanum lycopersicum* (tomato) seed by the local lesion assay (indexing) on *Nicotiana tabacum* plants
https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html

## ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

### 1. General Requirements

If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material that it does not lead to a reduction in the number of lesions obtained.

### 2. Extraction of the virus from the seed

2.1. Grind 250 seeds in each sub-sample in 10 ml of Phosphate Buffered Saline (PBS), the seed extraction buffer in Table 2. Process seed extracts within 4 hours after grinding.

### 3. ELISA

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

## LOCAL LESION ASSAY

### Material

| | |
|---|---|
| Assay plants (*Nicotiana glutinosa* or *Nicotiana tabacum* cv. Xanthi NN) | Carborundum (320 mesh grit powder, Fisher Scientific or equivalent) |
| Controls | Seed extraction buffer |
| Gloves or alkaline soap and water | Protective mask |

<u>Controls</u>

**Table 1: Description of controls**

| | |
|---|---|
| Positive Process Control (PPC) | A well-characterized positive seed lot |
| | Pea seed flour and powdered TMV, ToMV, ToMMV or ToBRFV infected leaves from a systemic host ground together in a seed extraction buffer (5 mL per 0.5 gr). The mix should be ground into a fine flour for efficient virus extraction |
| | Liquid extract of TMV, ToMV, ToMMV or ToBRFV infected leaves of Solanaceous hosts sufficiently diluted in PBS seed extraction buffer |
| Negative Process Control (NPC) | Seed extraction buffer |
| | Tomato seed free of tobamovirus |

Seed extraction buffer

Table 2: 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 |
| Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes | |

## 1. General Requirements

- Seed extracts and controls must be prepared at the same time, under the same laboratory conditions and stored under the same conditions.
- Seed extracts and controls must be stored at 4°C until the assay begins. When performed after pre-screening ELISA it is strongly recommended to perform the local lesion assay within 24 hours after seed-extraction.
- The final results of the local lesion assay must be validated through comparison of the results given by both controls.

## 2. Extraction of the virus from the seed

2.1. Grind seeds of each sub-sample, 250 seeds, in 10 ml of the seed extraction buffer (PBS).

2.2. Process seed extracts within 4 hours after grinding.

## 3. Controls

3.1. Choose one PPCs and NPCs listed.

3.2. Depending on the amount of infectious virus in the PPC, adjust the quantity used to induce development of countable local lesions.

## 4. Inoculation and incubation of *Nicotiana* assay plants

4.1. Raise assay plants (*Nicotiana glutinosa* or *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 (Takahashi, 1975); see Figure 2.
- The primary leaf (oldest true leaf) should not be used as it is different in shape/texture/thickness and therefore less susceptible.
- Leaves of tobacco plants affected by downy mildew are significantly less sensitive. Inoculated plant must have no visible symptoms and be free of disease (Koenraadt H. personal communication).

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

**Figure 2.** Overview of *Nicotiana tabacum* cv. Xanthi NN plants in different growth stages (two pictures taken per plant; pot diameter 14 cm)

4.3. The entire surface of two (nearly) fully expanded consecutive leaves of two plants should be inoculated with each seed extract, a total of 4 leaves for each sub-sample.

4.4. 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.

Note: 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.

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

4.6. Inoculate plants with the PPC and NPC in a similar manner to the ones used for the seed extracts.

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

Note: The hypersensitive reaction of tobacco plants, the development of local lesions, may not be expressed at temperatures higher than 28°C (Samuel, 1931; Takahashi, 1975; Weststeijn, 1981 and Kiraly et al., 2008) and may vary for different tobamoviruses.

## 5. Evaluation of inoculated plants

5.1. Evaluate the inoculated leaves for local lesions (see Figure 3).

**Figure 3.** Local lesions on tobacco leaves.

5.2. If there are many local lesions, a comparison with the PPC and NPC will readily reveal if most, or all, the lesions observed are authentic virus lesions or might be caused by, for instance, rubbing (rubbing damage).

5.3. Optional: If the number of lesions is low, confirm they are a true virus infection and not an artefact (leaf damage during mechanical inoculation, use of pesticides, etc.) by cutting out the suspect lesion, crushing it in a small amount of the seed extraction buffer and inoculating 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 (Hadas, 2000).

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