7-028: Detection of infectious tobamoviruses on *Solanum lycopersicum* (tomato) by the local lesion assay (indexing) on *Nicotiana tabacum* plants

**Disclaimer:** whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description ISTA shall not be liable for any loss damage etc., resulting from the use of this method.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

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7-028: Tobamoviruses on \textit{Solanum lycopersicum}

**Crop:** Tomato (\textit{Solanum lycopersicum})

**Pathogen:** Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV)

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**Revision history:**
Version 1.0, 2011-12-02
Version 1.1, 2014-01-01: scientific name changed to \textit{Solanum lycopersicum}; improved phrasing of "Background"; "indexing" replaced by "local lesion assay"; amendment of "Sample preparation"; different buffer permitted in "Methods"

**Background**
Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV) are seed-borne tobamoviruses commonly found on tomato seed and are transmitted both mechanically and with seed (Lewandowski and Dawson 1998; Hadas et al., 2004, Huttinga and Rast 1995; Demski 1981). ELISA can be used as a detection method but it can not distinguish between infectious and non-infectious virus particles, and this can yield false positive results (Maury et al., 1987; Nolan and Campbell 1984). The local lesion assay is derived from a multi-laboratory comparative test organised by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg). It is based on the detection of infectious virus by mechanical inoculation of resistant \textit{Nicotiana} assay plants with tomato seed extract (Holmes 1929; Hadas 1999; Hadas et al., 2004). In tobacco plants carrying the N gene such as \textit{Nicotiana tabacum} ‘Xanthi NN’ (Stange et al., 2004; Diaz-Griffero et al., 2006) resistance to tobamoviruses (Holmes 1938; Hammond-Kosack and Jones 1996; Erickson et al., 1999a; Whitham et al., 1994; Boovaraghan et al., 2007) is based on a hypersensitive reaction to virus infection (Ehrenfeld et al., 2008; Takahashi 1956; Erickson et al., 1999b; Whitham et al., 1994; Taliantsky et al., 1994), which results in a local necrotic lesion (Holmes 1938; Takahashi 1956; Dawson 1999) preventing subsequent systemic spread of the virus. It has been reported that one infected seed in a subsample of 500 healthy seeds can be detected with this method (Hadas et al., 2004). However, to increase test sensitivity, the ISHI-Veg comparative test used a subsample size of 250 seeds for validation purposes. The method does not distinguish between TMV and ToMV.

**Validation studies**

Copies are available: by e-mail from ista.office@ista.ch; by mail from the ISTA Secretariat, Zürichstrasse 50, 8303 Bassersdorf, Switzerland.
Safety precautions
Ensure you are familiar with the hazardous nature of the materials being used and take appropriate safety precautions, especially during preparation of buffers, grinding, autoclaving, weighing out of ingredients, and dusting leaves with carborundum powder. It is assumed that persons carrying out this procedure are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Treated seed
This test method is suitable for untreated seed, for seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, trisodium phosphate, etc.) processes with the aim of disinfestation or 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 comparison testing. This test method has not been validated for seed treated with protective chemicals or biological substances.

Sample and subsample size
The sample (total number of seeds tested) and subsample size to be tested depends on the desired tolerance standard (maximum acceptable number of seeds infected) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). In any case the maximum recommended subsample size is 250 seeds. The minimum recommended sample size is 3000 seeds.

Materials
Reference material: known TMV/ToMV-infected seeds, or prepared reference material (e.g. flour of pea seeds mixed with ground Nicotiana leaves infected with TMV/ToMV/pepper mild mottle virus (PMMoV), or liquid extract of TMV/ToMV/PMMoV-infected leaves of solanaceous hosts)
Balance: capable of weighing to the nearest 0.01 g
pH meter: capable of being read to the nearest 0.1 pH unit
Automatic pipettes: check accuracy and precision regularly
Grinder: e.g. Ultra Turrax or Hydraulic press or equivalent
Tobacco plants: resistant to all races of the pathogen for local lesion assay (e.g. Nicotiana tabacum ‘Xanthi NN’)
Carborundum powder: e.g. 320 mesh grit powder, Fisher Scientific or equivalent
Controlled greenhouse/growth chamber: capable of operating/maintaining temperature at 20–25 °C
Environment Room or cabinet (module)
Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands etc. both before and after handling each sample. This can be achieved by swabbing or spraying equipment and gloved hands with an alkaline soap or equivalent and then rinsing with water to remove residues.

Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:

\[ \text{TSW} = \left( \frac{\text{weight of seeds}}{\text{number of seeds}} \right) \times 1000 \]

Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

Method

(Critical control points are indicated by CCP)

1. Extraction of virus from the seed
   1.1 Using a grinder, grind seeds of each subsample in the PBS seed extraction buffer at a rate of (4 mL per 100 seeds), or in an alternative ELISA buffer if the assay is performed after ELISA pre-screening (CCP).
   1.2 Process seed extracts within 4 h after grinding or store them at 4 °C when the assay is performed after ELISA pre-screening (CCP).

2. Positive control (seeds or reference material) (CCP)
   2.1 Choose one of the following listed positive controls and follow its preparation:
      i. follow procedure described in step 1.1 to extract virus from a known infested seed subsample, or
      ii. grind flour of pea seeds mixed with ground TMV/ToMV/PMMoV-infected Nicotiana leaves in seed extraction buffer (5 mL per 5 g), or in an alternative ELISA buffer if the assay is performed after ELISA pre-screening (CCP), or
      iii. use 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 (CCP).

3. Negative control (seeds or seed extraction buffer)
   3.1 Follow procedure described in step 1.1 to extract virus from a known virus-free seed subsample or use seed extraction buffer.

4. Local lesion assay (mechanical inoculation of plants)
   4.1 Grow tobacco plants known to be resistant to all races of TMV or ToMV (e.g. Nicotiana tabacum ‘Xanthi NN’) in small pots, at 20–25 °C and under sufficient light intensity.
      4.1.1 Choose plants at the growth stage with 4–5 true leaves (6–7 weeks after seed sowing). Do not use old or flowering plants (CCP).
      4.1.2 Dust two (nearly) fully expanded consecutive leaves of each of two plants with carborundum powder such that there is a very fine layer on the leaf surface (CCP).
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4.2 Inoculate each seed extract on each dusted leaf of the two plants, going across the entire surface. Do not use the primary leaf (oldest true leaf) (CCP).

4.2.1 Place a drop of inoculum (100 µL) onto the leaf. Smear the drop with fingers, wearing plastic gloves or plastic finger tips, on the leaf surface with constant but slight pressure (CCP).

4.2.2 Inoculate positive and negative controls in a similar manner as the seed subsamples.

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

5. Incubation of plants

5.1 Incubate the plants for 5–7 days, at 20–25 °C with at least 12 h of light (CCP).

6. Examination of plants

6.1 Examine plants for the development of typical necrotic lesions on the inoculated leaves by comparing with the positive and negative control (CCP). Record the number of developed necrotic lesions (CCP).

**General methods**
(common to many test procedures)

**Grinding of seeds**

A grinder that can be cleaned thoroughly (e.g. Ultra Turrax) or extraction bags (e.g. Universal, Art. No. 430100 from Bioreba) in conjunction with a suitable grinder (e.g. Hydraulic press, Research Electronic Control) should be used to avoid cross-contamination between samples during the grinding step.

**Reporting results**

The result of a seed health test should indicate the scientific name of the pathogen and the test method used. When reported on an ISTA Certificate, results are entered under ‘Other determinations’.

In the case of a negative result (pathogen not detected in any of the subsamples), the results should be reported in terms of the tolerance standard and detection limit. The tolerance standard depends on the total number of seeds tested, n, and is approximately 3/n (P = 0.95) (Roberts et al., 1993)

In the case of a positive result, the report should indicate the number of positive subsamples out of the total number tested and the sample size or the maximum likelihood estimate of the proportion of infected seeds.

**Quality assurance**

**Specific training**

This test should be only performed by persons who have been trained in virological methods or under the direct supervision of someone who has.
Critical control points (identified by CCP in the methods)

- If the local lesion assay is performed after ELISA pre-screening, it has to be shown that local lesions can be obtained with the alternative buffer equivalent to PBS (Steps 1.1, 2.1.ii, 2.1.iii). Seed extracts and positive and negative controls must be stored at 4 °C until the assay begins. It is strongly recommended that 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 to results shown by both controls which will have been prepared and stored under the same conditions. For this purpose, it is recommended to prepare seed extracts and controls at the same time (Step 1.2). If a laboratory routinely uses ELISA as a pre-screen, the correlation between ELISA responses and the number of lesions in the 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.

- Depending on the kind of reference material, adjust its quantity to induce the development of countable local lesions on the leaves (Steps 2.1.ii, 2.1.iii).

- The grinding of the flour reference material into fine particles must be done to ensure the efficacy of virus extraction (Step 2.1.ii).

- The optimal growth stage of plants for the mechanical inoculation is the 4–5 true leaves stage (Fig. 1). Old or flowering plants have a strongly reduced sensitivity to virus infection (Padmanabhan et al., 2008) which influences the number of local lesions on inoculated leaves (Takahashi, 1972) and therefore should not be used. Watering of plants the day before inoculation will ensure leaves with high turgor (Step 4.1.1).

- Leaves should be dusted with the appropriate quantity of carborundum powder (Step 4.1.2).

- The oldest true leaf must not be used since it is different in shape, texture and thickness and thus less sensitive (Step 4.2).

- Smearing of the extract on the leaf surface should be performed with gentle finger movements with constant but slight pressure but avoiding leaf damage. Inoculation of leaves should be performed by wearing gloves and/or plastic finger tips which should be changed between subsamples. Hands should be cleaned thoroughly between samples with an alkaline soap or equivalent and then rinsed with water to remove soap residues (Step 4.2.1).

- The sensitivity of the test is reduced significantly under suboptimal incubation conditions of plants: too high temperature (Whitham et al., 1994; Ordog et al., 2002; Dijkstra et al., 1977) or insufficient light (Matthews, 1991) (Step 5.1). Especially the temperature is a critical factor for the validity of the test and should be monitored closely. Hypersensitive reaction of tobacco plants is not expressed at temperatures higher than 28 °C (Samuel, 1931; Kiraly et al., 2008; Takahashi, 1975; Weststeijn, 1981; Padgett et al., 1997; Dawson, 1999). Therefore, the test could be less sensitive in greenhouses during the summer period, because of too high temperatures.

- The sensitivity of the leaves is significantly reduced by the presence of downy mildew in the tobacco plants. Plants must be free of pathogens, and no visible symptoms must be present (Koenraadt H., personal communication).
Figure 1. Overview of *Nicotiana tabacum* ‘Xanthi NN’ plants at different growth stages (CCP) (two pictures per plant; pot diameter: 14 cm).
The use of both positive and negative controls is very important to validate the result. When a relatively high number of local lesions are observed, a comparison with the positive and negative controls will readily confirm that most, if not all, of the virus lesions are authentic (Step 6.1). However, in cases where the number of lesions for a sample is low, it can be confirmed that a lesion was caused by a virus infection and not by an artefact (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 (Hadas, 1999).

**Preparation of seed extraction buffer (phosphate buffered saline (PBS) pH 7.2–7.4)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.15</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled/deionized water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

**Preparation**
1. Weigh out all ingredients into a suitable container.
2. Add 1000 mL of distilled/deionized water and dissolve ingredients.
3. Check the pH with a pH meter and adjust if necessary.
4. Autoclave at 121 °C, 15 psi for 15 min.

**Storage**
Store buffer at 5 ± 4 °C. Use within a month after preparation.
**References**


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