International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

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

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

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

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 or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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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

Host: *Solanum lycopersicum* L.
Pathogen(s): *Tobacco mosaic virus* (TMV); *tomato mosaic virus* (ToMV)

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2011-12-02
Version 1.1, 2014-01-01: Scientific name changed to *Solanum lycopersicum*; improved phrasing of Background; 'indexing' replaced by 'local lesion assay'; amendment of Sample preparation; different buffer permitted in Methods
Version 1.2, 2017-01-01: Reporting results revised
Version 1.3, 2021-01-01: Sample size revised
Version 1.4, 2024-01-01: Sample size revised
Version 1.5, 2027-01-01: Flowchart added 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 & Dawson 1998; Hadas *et al.*, 2004, Huttinga & Rast 1995; Demski 1981). ELISA (= enzyme-linked immunosorbent assay) 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 & Campbell 1984). The local lesion assay is derived from a multi-laboratory comparative test organised by the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg). It is based on the detection of infectious virus by mechanical inoculation of resistant *Nicotiana* assay plants with tomato seed extract (Holmes 1929; Hadas 1999; Hadas *et al.*, 2004). In tobacco plants carrying the N gene such as *Nicotiana tabacum* 'Xanthi NN' (Stange *et al.*, 2004; Diaz-Griffero *et al.*, 2006) resistance to *tobamoviruses* (Holmes 1938; Hammond-Kosack & 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; Taliansky *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.

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 size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 3000 seeds and the maximum subsample size must be 250 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

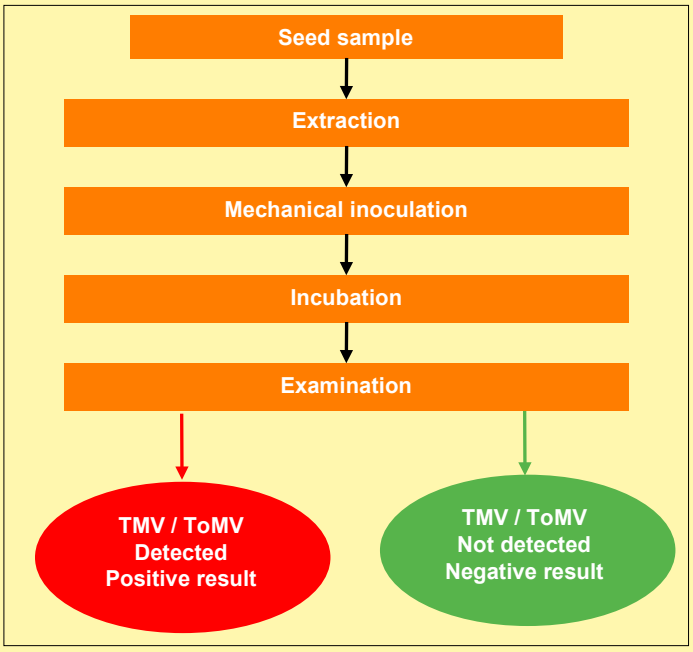
Controlled greenhouse/growth chamber: capable of operating/maintaining temperature at 20–25 °C

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.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.



Methods

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 (phosphate buffered saline) 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 prescreening (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 prescreening (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).

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

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 detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested.

Quality assurance

Specific training

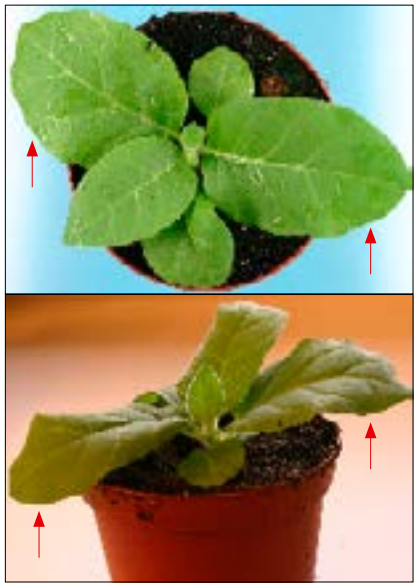
This test should be only performed by persons who have been trained in virological methods or under their direct supervision.

Critical control points (CCP)

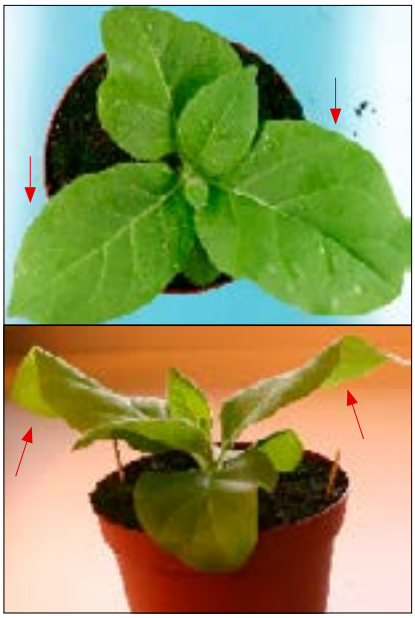
- If the local lesion assay is performed after ELISA pre-screening, it has to be shown that local lesions can be obtained with the alternative buffer equivalent to PBS (Steps 1.1, 2.1.ii, 2.1.iii). Seed extracts and positive and negative controls must be stored at 4 °C until the assay begins. It is strongly 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



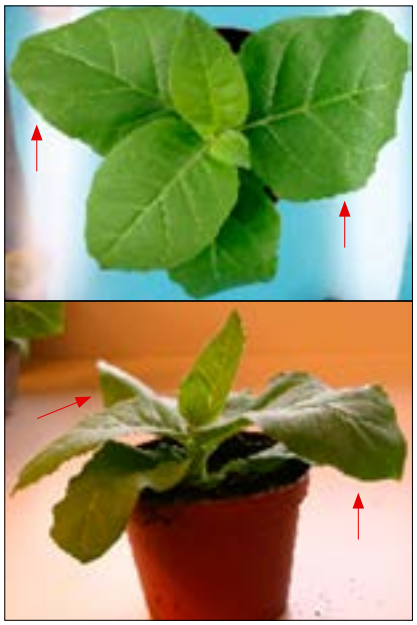
Plant 1. Too small.



Plant 2. Usable but relatively small leaves.



Plant 3. Usable plant.



Plant 4. Usable.



Plant 5. Plant too old.



Plant 6. Plant too old and in regenerative phase.

Figure 1. Overview of *Nicotiana tabacum* 'Xanthi NN' plants at different growth stages **(**CCP**)**(two pictures per plant; pot diameter: 14 cm).



Figure 2. Local lesions on leaves of *Nicotiana tabacum* 'Xanthi NN' plant.

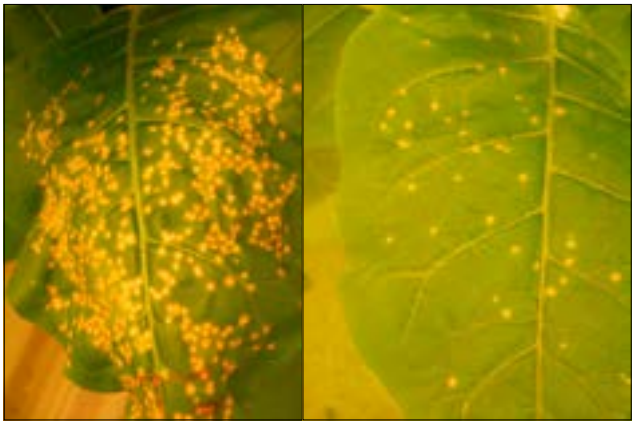


Figure 3. Local lesions on leaves of *Nicotiana tabacum* 'Xanthi NN' plants.

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., pers. comm.).
- 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).

Media and solutions

Seed extraction buffer (phosphate buffered saline [PBS] pH 7.2–7.4)

NaCl: 8.0 g/l
Na_2HPO_4: 1.15 g/l
KH_2PO_4: 0.2 g/l
Distilled/deionised water: 1000 ml

Preparation

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

Storage

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

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