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EPPO STANDARD ON DIAGNOSTICS

PM 7/153 (1) Mechanical inoculation of test plants

Specific scope: This Standard describes how to perform mechanical inoculation of test plants for detection, propagation and characterisation of plant viruses and viroids.[1]
This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: This Standard was first approved in 2022–07.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Mechanical inoculation of test plants is one of the oldest diagnostic methods used in plant virology. This method can be used for detection, propagation and characterisation of plant viruses and viroids (hereafter referred to as viruses). For detection, mechanical inoculation of test plants enables broad screening for plant viruses without prior knowledge of those, which may be present, and provides a single test for generic detection of mechanically transmitted viruses (including unexpected viruses and viruses which are new to science) (Roenhorst et al., 2013). For this reason, mechanical inoculation of test plants has been one of the preferred methods for use in post-entry quarantine testing (EPPO, 2019; Verhoeven & Roenhorst, 2000, 2003). In addition, mechanically inoculated test plants and/or other systemic hosts are used for propagation of virus isolates for maintenance (collection) and production of reference material in which preferably only the virus of interest is present. In the case of mixed infections, test plants only susceptible to the virus of interest can be used to obtain pure isolates. Finally, for viruses new to science, mechanical inoculation of (test) plants may be used to investigate biological properties, such as host range and transmission (characterisation). In the past, mechanical inoculation was also used for virus identification, by selecting test plants that could produce a characteristic/specific symptom profile for the virus isolate. However, nowadays, molecular and/or serological tests are performed for confirmation (Roenhorst et al., 2013).

Over the last decades, serological and molecular methods have replaced the use of mechanical inoculation of test plants for specific detection and identification of plant viruses. In addition, the emerging use of High-Throughput Sequencing analysis (HTS) provides an additional tool for broad detection without prior knowledge being required. Moreover, an advantage of using HTS compared to test plants is the ability of HTS to detect and identify viruses that are not mechanically transmissible (Adams et al., 2009; Villamor et al., 2019). However, the use of HTS poses new challenges, by discovering many viruses, which are new to science. To allow assessment of the impact of these novel viruses, mechanical inoculation of (test) plants often forms the first step for biological characterisation and propagation for research and maintenance (Massart et al., 2017).

2 | GENERAL INSTRUCTIONS

2.1 | Selection and use of test plants

The selection of test plants depends on the intended use of the test. When the aim is a broad screening for plant viruses (detection), a set of test plants known to be susceptible to a large variety of viruses should be used. For example, the combination of *Chenopodium quinoa, Nicotiana occidentalis* P1 and *N. occidentalis* subsp. *hesperis*-67A (previously: *Nicotiana hesperis*- 67A) has been shown to detect all potato-infecting viruses previously detected with a set of 10 test plant species (Verhoeven & Roenhorst, 2000, 2003). In general, 3–5 test plant species will provide a reliable basis for screening (Roenhorst et al., 2013). It is recommended to select test plants based on an assessment of the viruses that can be expected and include at least one plant species from the same family as the sample under investigation, e.g. *Phaseolus vulgaris* for samples from bean or *Ammi majus* for carrot. *C. quinoa*, *N. benthamiana* and *N. occidentalis* have been found to be suitable species for a large variety of viruses (J. W. Roenhorst, personal communication). In order to minimize the chance of not detecting a virus, a more extended list of species has been empirically found suitable (O. Schumpp, personal communication). This list also includes *Catharanthus roseus* cv. Vitesse

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

F1, *Chenopodium amaranticolor*, *Cucumis sativus* cv. Philadelphia, *N. clevelandii*, *N. glutinosa*, *N. tabacum* cv. White Burley, *N. tabacum* cv. Xanthi, *Phaseolus vulgaris* cv. Weinlanderin, and *Solanum lycopersicum* cv. Mt-favet. The Descriptions of Plant Viruses (DPV; https://www.dpvweb.net/) website indicates test plants suitable for detection of individual viruses.

For propagation, plant species that develop a systemic infection should preferably be used. The choice of the species will generally be dependent on the virus and consists of one of the aforementioned species.

For characterisation, the choice of test plants preferably includes a variety of different families and/or genera, and often will be complemented by the crop species considered at risk.

When selecting plants for inoculation, it should be kept in mind that transmission can be affected by components of the plant species as well as the stability of the virus. For example, inoculum prepared from *Alstroemeria* sp. can cause necrosis on the inoculated leaves, thereby preventing systemic infection (J. W. Roenhorst, personal communication). Also, in the case of less-stable viruses, such as tospoviruses, which are easily degraded after grinding of the sample material, the chance of transmission may be reduced, even over a short period of time. To enhance transmission, different buffers are used for specific host species (Appendix 1). Furthermore, it is important to inoculate test plants at their 'optimum' stage. In general virus resistance will increase with the maturity of the plant, implying a decreased susceptibility for infection. The optimum stage for inoculation of the most common test plant species is given in Appendix 2. It is recommended to inoculate a sample onto at least two plants of each test plant species. The exact number of replicates required, depends on the virus, the test plant species, and the purpose of inoculation (see Appendix 2).

2.2 | Inoculum preparation

To prepare an inoculum, symptomatic material should be taken, avoiding necrotic tissue. Preferably, new growth should be taken, since viral accumulation is the highest in this tissue. Other options are using flowers, fruits or roots, especially if components in leaf material are expected to hamper successful transmission. For non-symptomatic plants, actively-growing tissue (e.g. young leaves) should be sampled. If applicable, plant material from different stems or areas of the plant should be combined. To avoid cross contamination, gloves should be worn when collecting plant material. The inoculum is prepared by grinding plant material in inoculation buffer. Generally, approximately 0.5 g of plant material is ground in about 5 mL inoculation buffer (ratio plant material: buffer approximately 1:10), but other amounts of plant material and plant material: buffer ratios are also used (see Appendix 1). The plant material and buffer can be transferred to a mortar and ground using a pestle or put in an extraction bag and ground using a homogeniser. To prevent virus degradation, it is recommended to use a refrigerated inoculation buffer and, if applicable, a pre-cooled mortar and pestle for grinding, and to keep the inoculum on ice until use.

2.3 | Inoculation of test plants

The selected test plants should be placed in a greenhouse, growth room or cabinet, separating the plants inoculated with different sample homogenates to avoid cross contamination by contact. Prior to inoculation, plants may be placed in the dark for 12 h. For some viruses a dark period prior to inoculation has been shown to enhance infection (Agrawal et al., 1979; Kimmins et al., 1967). In addition, the circadian rhythm of plants has been shown to influence the rate of infection for different viruses (Agrawal et al., 1979). Therefore, it is advised to always perform inoculations during the same period of the day, e.g. in the morning or afternoon. To allow and/or enhance transmission of the virus into the plant cells, leaves should be dusted with an abrasive, such as Carborundum or Celite, while wearing a protecting mask. Alternatively, the abrasive can be added to the inoculum provided that the solution is homogeneous. For inoculation, the inoculum can be taken directly from the mortar. When using extraction bags, the contents of the extraction bag should be transferred into a container (e.g. Petri dish) prior to inoculation. Gloves should be used when dipping fingers into the inoculum, followed by gently rubbing the inoculum onto the leaves from the base of the leaves to the top avoiding the mid vein. Alternatively, sterile cotton swabs can be used for inoculation. Rubbing of the leaves should be done with care to avoid damaging the leaves. The type (e.g. cotyledons, first true leaves) and/or number of leaves to be inoculated depends on the (expected) virus, test plant species and preferences of the laboratory. The inoculated leaves can be marked to facilitate recognition of virus symptoms. Gloves/equipment should be changed/cleaned between different samples. After inoculation, plants should be rinsed with tap water, to remove the abrasive.

2.4 | Growing of test plants and recording of symptoms

Plants should be grown in pots in an insect-proof glasshouse/growth chamber between 18 and 25°C and a day length of at least 14 h. It should be noted that for some combinations of viruses and test plant species, temperature may be critical. Plants should be monitored twice a week for at least 3 weeks, recording the local and systemic symptoms. However, some viruses will only express

symptoms after a longer period (e.g. nucleorhabdoviruses, such as physostegia chlorotic mottle virus, may take up to 4 weeks to express symptoms [H. Ziebell, personal communication]). Symptom expression can also take a longer period when the virus concentration in the inoculum is low. Photos of common virus symptoms are provided in Appendix 3. For individual viruses DPV (https://www.dpvweb.net/) provides images of (characteristic) symptoms on a range of common test plants species.

2.5 | Harvesting material

Harvesting of plant material for further testing or inclusion in a virus collection is performed after infection is established. Material should be collected in the same way as described for inoculum preparation (Section 2.2). The optimum stage of harvesting is determined by the severity of the symptoms and/or reduction of growth. If possible, collection of necrotic material should be avoided. It should be noted that in addition to the choice of test plants, other factors can affect the amount of infected material suitable for harvesting. These include the virus concentration in the inoculum, the virulence and/or aggressiveness of the virus isolate, and the stage of the test plant at the time of inoculation. For example, when an inoculum with a high virus concentration or an aggressive isolate is inoculated onto relatively young and small test plants, growth is likely to stop almost immediately and only small amounts of infected material will be produced. In these cases, it is advised to dilute the inoculum, use older plants for inoculation, and/or choose another plant species.

3 | FIRST LINE CONTROLS

3.1 | Positive controls

When aiming to detect a single specific virus, the positive control should be the target virus. However, when the test is used for generic screening for viruses, there are many candidates. Depending on the range of test plants selected, many different virus species could potentially be detected and it is impossible to include them all as controls. In such a case it is recommended to use an 'average' virus with regard to stability and ease of mechanical transmission that produces symptoms in a relative short period, such as cucumber mosaic virus or tobacco ringspot virus (Roenhorst et al., 2013). Viruses that are unstable or difficult to transmit are less suitable as a positive control while a highly-infectious virus, such as a tobamovirus, will increase the risk of cross contamination. The positive control is inoculated preferably on each of two suitable test plant species in duplicate. A positive control should be preferably inoculated in each compartment, growth room or chamber, when the inoculation is performed. However, depending on the situation, the frequency of inoculating controls can be adapted to the needs. Plants should be visually inspected and symptoms recorded for at least 3 weeks (see Section 2.4). If the positive control develops the expected symptoms, the inoculation procedure is considered successful and the growing conditions are considered as being suitable for a successful infection of the test plants. If not, the reason for the failure should be traced, e.g. buffer composition, environmental conditions, viability of the virus or cross contamination (in case non typical symptoms appear) in order to take appropriate measures (e.g. replacing the buffer and/or repeating the inoculation).

3.2 | Negative controls

For the negative control, inoculation buffer or an inoculum prepared from healthy plant material of the tested plant species may be used. The negative control should be inoculated on the same two test plant species as the positive control and treated in the same way. For the negative control, no symptoms are expected within the growing period. If no symptoms are observed on the negative control plants, the symptoms on the positive control plants are likely to result from infection by the control virus. If the same virus-like symptoms appear on the negative control, the cause should be traced to take appropriate measures (e.g. replacing the buffer and/or repeating the inoculation). Virus-like symptoms might be due to e.g. mechanical damage during inoculation or external influences such as the presence of other pests and diseases, nutritional abnormalities, chemical treatments. Such symptoms on the negative control indicate that similar symptoms on other inoculated test plants may have resulted from external factors and are not caused by a virus. Moreover, it also might reveal cross contamination and the need for a critical evaluation of current and previous results to trace the origin and analyse possible consequences.

4 | FEEDBACK ON THIS STANDARD

If you have any feedback concerning this Standard, please send it to diagnostics@eppo.int.

5 | STANDARD REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press this will also be marked on the website.

ACKNOWLEDGEMENTS

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APPENDIX 1 - PROTOCOL FOR MECHANICAL INOCULATION OF TEST PLANTS

1. Buffers and chemicals

Standard phosphate buffers can be used for inoculation of most viruses and plant species. Examples of inoculation buffers used in different laboratories in the EPPO region are given below. Note that the suitability of these buffers, including the use of additives for specific applications, is based on the experience of those laboratories.

For inoculation of viroids, standard buffer B or distilled water can be used.

Store stock solutions and buffers at 4°C for a maximum period decided by the laboratory. Alternatively, aliquots of stock solutions or buffers can be frozen.

A. Standard buffer 0.06 M phosphate buffer pH 7 (Fera, GB) can be prepared as follows:

   a. 9.46 g of Na_2HPO_4 per litre of distilled water
   b. 9.07 g of KH_2PO_4 per litre of distilled water

Mix (a) and (b) in the ratio of 3 parts (a) to 2 parts (b) to give 0.06 M phosphate buffer pH 7.

For plant materials with a high tannin content, such as strawberry and raspberry, add 1 g of polyvinylpyrrolidone 40 000 (PVP40) per 100 mL buffer A. For Pelargonium, add 1 g of polyethylene glycol 6000 (PEG) instead of PVP per 100 mL buffer.

B. Standard buffer 0.05 M phosphate pH 7.4 (NVWA, the Netherlands)

   a. 89.0 g of $Na_2HPO_4 \cdot 2\ H_2O$ per litre of distilled water
   b. 69.0 g of $NaH_2PO_4 \cdot H_2O$ per litre of distilled water

Mix 500 mL of (a) and 200 mL of (b) to prepare a 0.5 M stock and adjust pH to 7.4 if applicable. Take 40 mL of 0.5 M stock, add distilled water up to 800 mL. Add 20 g polyvinylpyrrolidone 10 000 (PVP10), stir and add distilled water up to 1 L.

Buffer has been shown suitable for inoculation of a variety of virus species from different genera (including unstable viruses such as tospoviruses) from both herbaceous and woody hosts.

C. Standard buffer 0.02 M phosphate buffer pH 7.6 (Agroscope)

   a. 31.20 g of $NaH_2PO_4 \cdot 2\ H_2O$ per litre of distilled water (0.2 M)
   b. 71.64 g of $Na_2HPO_4 \cdot 12\ H_2O$ per litre of distilled water (0.2 M)

Add 1.3 mL of (a) plus 0.044 g sodium diethyldithiocarbamate trihydrate ($[C_2H_5]_2NCSSNa \cdot 3H_2O$) to 160 mL of distilled water. Adjust to pH 7.6 with (b) and add water up to a volume of 200 mL.

D. Buffer for woody hosts 0.04 M phosphate buffer pH 7.2 (Agroscope)

   a. 31.20 g of $NaH_2PO_4 .\ 2\ H_2O$ per litre of distilled water (0.2 M)
   b. 71.64 g of $Na_2HPO_4 .\ 12\ H_2O$ per litre of distilled water (0.2 M)

Add 10 mL of (b) plus 0.1125 g of sodium diethyldithiocarbamate trihydrate to 38 mL of distilled water. Adjust to pH to 7.2 with (a). Transfer to fume hood and add 92 µL of thioglycolic acid, and 500 µL of nicotinic acid, and make up to 50 mL with distilled water.

2. Equipment and materials

- Carborundum (400 mesh), e.g. VWR Chemicals 22540.298[1]
- Extraction bags[2], e.g. Bioreba 410100
- Homogeniser,[2] e.g. hand homogenizer Bioreba 400010
- Petri dishes[2]
- Gloves
- Protecting mask
- Labels
- Water resistant marker
- Water bottle/hose for rinsing plants after inoculation

[1]Alternative: Celite (e.g. Sigma Aldrich D5509),
[2]Alternative: Mortar and pestle.

3. Procedure

Mechanical inoculation

- It is advised to perform inoculations during the same period of the day, e.g. in the morning or afternoon.
- Select healthy plants in appropriate developmental stage to be inoculated and place them in in the glass-house/growth chamber.
- Prior to inoculation, plants may be placed in the dark for 12 h.
- Dust abrasive onto leaves of test plants (wear mask). Alternatively, the abrasive can be added to the inoculum.
- Put about 0.5 g[3] plant material into extraction bag or mortar.
- Add about 5 mL[3] refrigerated inoculation buffer.
- Grind the plant material
- When using an extraction bag, avoid leakage while grinding, and pour the inoculum into a petri dish.
- When using a mortar and pestle the inoculum can be used directly.
- Keep inoculum on ice or inoculate immediately.
- Use one or two fingers to rub the inoculum gently from the base of the leaf to the tip and avoid the midrib (use gloves or swabs), while supporting the leaf with the other hand. Note that when the abrasive is added to the inoculum, mix with fingers before applying the inoculum onto the leaves.
- Rinse inoculated plants with tap water (within 2–5 min).
- Grow test plants at 18–25°C for viruses and 26–28°C for viroids with supplementary illumination to ensure a day length of at least 14 h.
- Inspect test plants for symptoms at least twice a week for at least 3 weeks.

[3]Amount of plant material and plant-material: buffer ratios can be adapted to specific needs and/or preferences of the laboratory.

Prevention of cross contamination

- Change gloves between different samples
- Separate test plants of different samples, e.g. by screens
- Clean (potentially contaminated) equipment and surfaces

APPENDIX 2 - OPTIMUM STAGE FOR INOCULATION OF COMMON TEST PLANTS SPECIES

Test plant species	Number of leaves[a]	Remarks
Ammi majus	1–2	
Brassica rapa subsp. *sylvestris* (formerly *Brassica campestris*)	2–3	
Capsicum annuum	2–3	
Chenopodium giganteum (formerly *Chenopodium amaranticolor*)	3–4	
Chenopodium quinoa	3–4	
Cucumis sativus cv Chinese slangen[b]	2 cotyledons + small top leaf	Remove other leaves when present
Datura metel	2–3	
Datura stramonium	2–3	
Gomphrena globosa	About 6	
Nicotiana benthamiana	3–4	
Nicotiana quadrivalvis (formerly *Nicotiana bigelovii*)	3–4	
Nicotiana debneyi	2–3	
Nicotiana glutinosa	3–4	
Nicotiana miersii	2–3	
Nicotiana occidentalis	4–6	Different accessions available, e.g. 37B, P1
Nicotiana occidentalis subsp. *hesperis* (formerly *Nicotiana hesperis*)	4–6	
Nicotiana rustica	1–2	
Nicotiana tabacum cv. Samsun	1–2	
Nicotiana tabacum cv. White Burley	1–2	
Nicotiana tabacum cv. Xanthi	1–2	
Petunia x hybrida (formerly *Petunia hybrida*)	4	
Phaseolus vulgaris cv. Dubbele witte zonder draad	2	Remove stem and leaves when >2 leaves
Physalis floridana	2–3	

Test plant species	Number of leaves[a]	Remarks
Pisum sativum cv. Kelvedon Wonder[b]	4–6	
Solanum lycopersicum cv. Money-maker	1–2	
Solanum lycopersicum cv. Rutgers	1–2	
Vicia faba cv. Witkiem[b]	2–4	

[a] Fully developed/expanded leaves.
[b] Other varieties possible.

APPENDIX 3 - PHOTOS OF SOME COMMONLY OBSERVED VIRUS AND VIROID SYMPTOMS

Symptom description	Figure
Blisters	1, 2
Chlorosis	3
Chlorotic lesions	4
Chlorotic rings	5
Chlorotic patterns	6
Concentric rings or patterns	7
Growth reduction or stunting	8, 9
Interveinal chlorosis	10
Irregular necrotic lesions	11
Leaf curl	12
Leaf malformation	13
Mosaic	14
Mottle	15
Necrosis	16
Necrotic lesions	17
Necrotic rings	18
Necrotic zones	19
Rugosity	20
Shot hole	21
Stem necrosis	22
Top necrosis	23
Vein clearing	24
Vein necrosis	25
Wilting	26

Note that Descriptions of Plant Viruses (DPV: https://www.dpvweb.net/) provides information on suitable test plant species and (characteristic) symptoms for individual viruses.

FIGURE 1 Blisters on *Nicotiana occidentalis* P1 (photo courtesy: NVWA, NL)

FIGURE 2 Blistering on *Datura stramonium* (photo courtesy: NVWA, NL)

FIGURE 3 Chlorosis (yellowing) on *Phaseolus vulgaris* (photo courtesy: NVWA, NL)

FIGURE 4 Chlorotic lesions on *Chenopodium quinoa* (photo courtesy: NVWA, NL)

FIGURE 5 Chlorotic rings on *Nicotiana occidentalis* P1 (photo courtesy: NVWA, NL)

FIGURE 6 Chlorotic patterns on *Nicotiana tabacum* cv. White Burley (photo courtesy: NVWA, NL)

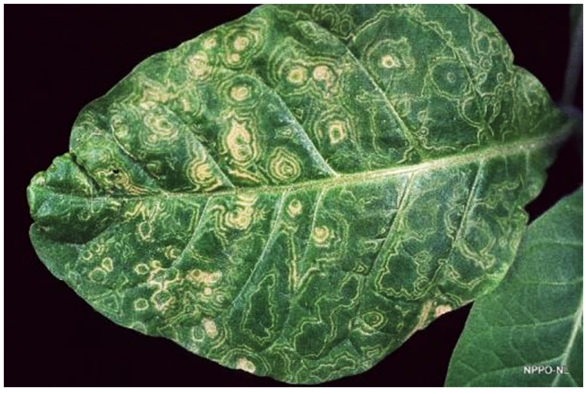

FIGURE 7 Concentric rings (and patterns) on *Nicotiana tabacum* cv. White Burley (photo courtesy: NVWA, NL)

FIGURE 8 Stunting on *Nicotiana occidentalis* P1 (photo courtesy: NVWA, NL)

FIGURE 9 Growth reduction or stunting on *Solanum lycopersicum* (photo courtesy: NVWA, NL)

FIGURE 10 Interveinal chlorosis on *Cucumis sativus* (photo courtesy: NVWA, NL)

FIGURE 11 Irregular necrotic lesions on *Chenopodium quinoa* (photo courtesy: NVWA, NL)

FIGURE 12 Leaf curl on *Nicotiana occidentalis* P1 (photo courtesy: NVWA, NL)

FIGURE 13 Leaf malformation on *Solanum lycopersicum* (photo courtesy: NVWA, NL)

FIGURE 14 Mosaic on *Solanum melongena* (photo courtesy: NVWA, NL)

FIGURE 15 Mottle on *Chenopodium quinoa* (photo courtesy: NVWA, NL)

FIGURE 16 Necrosis on *Datura stramonium* (photo courtesy: NVWA, NL)

FIGURE 19 Necrotic zones on *Nicotiana tabacum* (photo courtesy: NVWA, NL)

FIGURE 17 Necrotic lesions on *Chenopodium quinoa* (photo courtesy: NVWA, NL)

FIGURE 20 Rugosity on *Datura stramonium* (photo courtesy: NVWA, NL)

FIGURE 18 Necrotic rings on *Chenopodium quinoa* (photo courtesy: NVWA, NL)

FIGURE 21 Shot hole on *Nicotiana occidentalis* P1 (photo courtesy: NVWA, NL)

FIGURE 22 Stem necrosis on *Impatiens* sp. (photo courtesy: NVWA, NL)

FIGURE 23 Top necrosis on *Chrysanthemum* sp. (photo courtesy: NVWA, NL)

FIGURE 24 Vein clearing (chlorosis) on *Solanum melongena* (photo courtesy: NVWA, NL)

FIGURE 25 Vein necrosis on *Phaseolus vulgaris* (photo courtesy: NVWA, NL)

FIGURE 26 Wilting on *Capsicum annuum* (photo courtesy: NVWA, NL)