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Diagnostics
Diagnostic

PM 7/113 (1) Pepino mosaic virus

Specific scope

This standard describes a diagnostic protocol for detection and identification of *Pepino mosaic virus* in all plant parts, particularly on tomato seeds[1].

Specific approval and amendment

Approved in 2012-09.

Introduction

Pepino mosaic virus (PepMV) was originally described from pepino (*Solanum muricatum*) in Peru in 1980. Since 1999, when PepMV started infecting tomato crops (*Solanum lycopersicum*) in the Netherlands, UK and Spain, the rapid and worldwide spread of this virus to and in the main production areas of protected (glass and plastic house) tomatoes has attracted considerable attention. The economic importance of PepMV for the tomato industry has been debated as its significance seems to be determined by the marketability and economic value of smaller and discoloured tomato fruits in a given market. Available evidence suggests that fruit yields and fruit quality losses depend on the PepMV isolate present and on the environmental conditions prevailing during the growing season.

Currently, four major genotypes or strain groups sharing complete nucleotide sequence identities ranging from 78% to 95% are distinguished: European (EU), Peru, Ch2 and US1. EU is the PepMV genotype that is genetically most similar (95%) to, but biologically distinct from, the Peruvian strain group and that predominated initially in European tomato crops. Since 2004, however, isolates of strain group EU seem to be replaced by, and/or to occur increasingly in mixed infections with, strain Ch2 in Europe. This latter genotype, first identified from tomato seeds originating from Chile, is genetically very distinct (79% identity) from the EU strain. Isolates of strain group US1 clearly differ genetically (identities of 78–82%) from EU, Peru and Ch2 have as yet been identified only rarely from tomato crops in the USA and Europe. There have also been reports on the occurrence in tomato of recombinant PepMV isolates which have chimeric genomes sharing striking nucleotide sequence identities with isolates of strain groups EU and Ch2. Although a wide range of leaf (e.g. mosaic, yellow angular spots, blistering, nettle heads) and fruit symptoms (fruit marbling or flaming) has been associated with PepMV infections in tomatoes, there is currently no evidence for a causal relationship between severe leaf and/or fruit symptoms and a particular genotype of PepMV. However, there is a report suggesting that mixed infections by two genotypes (EU and Ch2) or the infection with a recombinant PepMV isolate can result in more severe PepMV symptoms (Hanssen *et al.*, 2008).

PepMV is very efficiently transmitted by mechanical means; i.e. fruit harvesting, pruning, and other cultural practices lead to rapid spread in protected tomato crops. In addition, bumblebees have been associated with PepMV transmission in glasshouses. A low seed transmission rate has been demonstrated; however, available evidence suggests that PepMV does not infect the embryo or endosperm but contaminates the seed coat. Long distance spread of PepMV is thought to be through contaminated seeds or infected transplants.

Like most other potexviruses, PepMV has a fairly narrow natural host range that appears to be largely restricted to Solanaceous species. In addition to tomato and the original host, pepino (*S. muricatum*), natural infections by PepMV have been reported not only from the wild tomato species *S. chilense*, *S. chmielewskii*, *S. parviflorum* *S. peruvianum* and potato germplasm, but also from several weeds belonging to various plant families and growing in the vicinity of tomato glasshouses. Since the experimental host range of

[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.

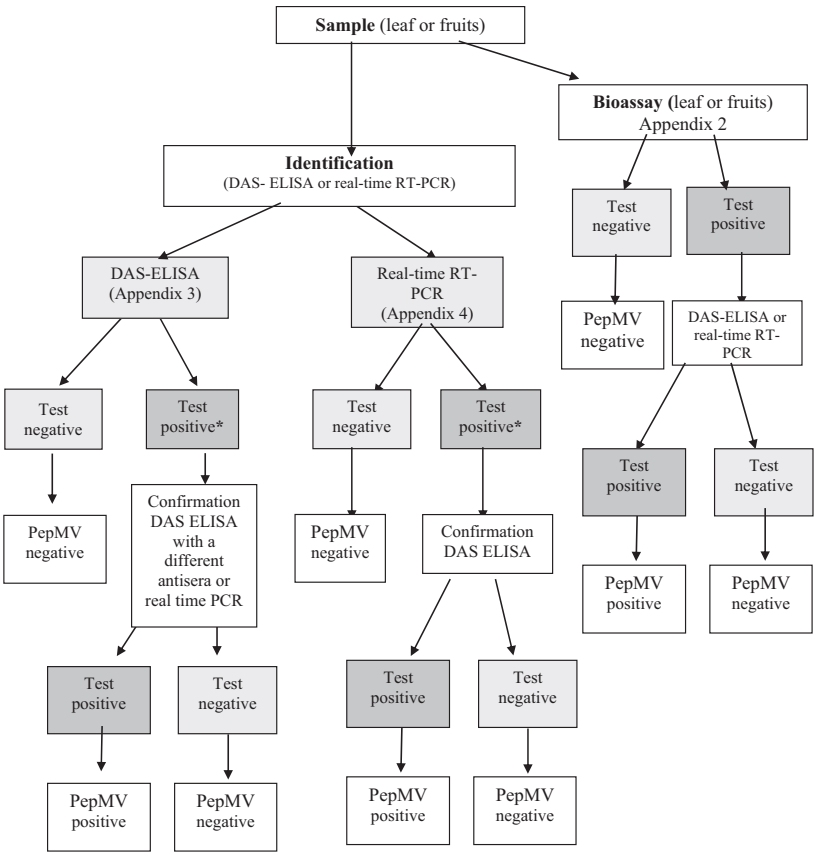

Fig. 1 Flow diagram for the detection and identification of PepMV on fruit or leaf samples. Sample (leaf or fruits). *In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

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PepMV includes solanaceous crop plants such as potato, tobacco, *Capsicum* peppers and eggplant, these crops may also be at risk.

Flow diagrams describing the procedures for detection and identification are given in Figs 1 and 2.

Identity

Name: *Pepino mosaic virus*
Synonyms (including former names): none
Acronym: PepMV
Taxonomic position: Viruses: *Tymovirales*: *Alphaflexiviridae*: *Potexvirus*
EPPO code: PEPMV0
Phytosanitary categorization: EPPO A2 list no. 369; regulated pest in the EU based on emergency decision 2004/200/EC.

Detection

Disease symptoms

PepMV can be detected on growing plants (tomato, pepino), on tomato fruits and on consignments of tomato seeds originating from infected plants. Symptoms of PepMV can be extremely variable, ranging from latent to very severe infections. Fruit discolourations, such as marbling or flaming, are the most typical and economically significant symptoms (Fig. 3). Occasionally, fruit cracking and malformation have been observed. In addition to fruit symptoms, leaf symptoms such as nettle heads, blistering or bubbling, chlorosis, mosaic and yellow angular leaf spots, and leaf or stem necrosis have been associated with PepMV infections (Fig. 4). As plants mature, foliar symptoms generally disappear. Despite the variability in PepMV symptoms, PepMV can be normally detected in almost any above- and below-ground part of an actively growing plant infected about 4 weeks earlier.

Sampling for seed testing

The recommended minimum sample size is 3000 seeds with a maximum sub-sample size of 250 seeds (ISHI-Veg Seed Health Testing Methods Reference manual http://www.worldseed.org/isf/ishi_vegetable.html). For small seed lots, smaller samples size may have to be tested (e.g. in France only 1000 seeds are sampled from lots smaller than 600 g).

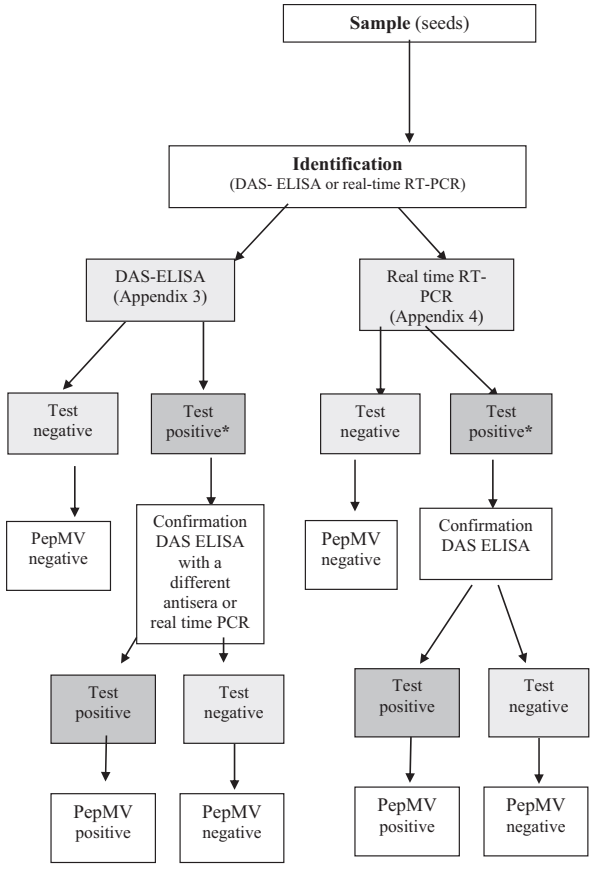

Fig. 2 Flow diagram for the detection and identification of PepMV on seeds. Sample (seeds). *In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

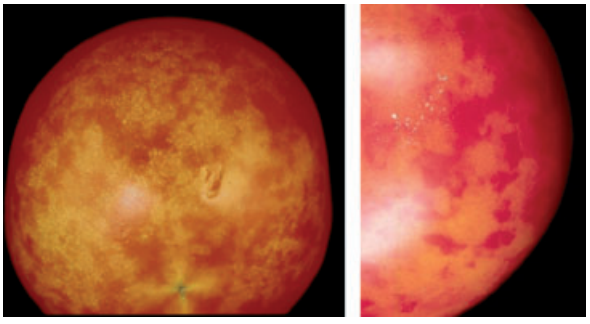

Fig. 3 Fruit symptoms characteristic of PepMV infections.

Bioassay

Mechanical inoculation onto test plants

• Leaf or fruit extracts

Mechanical inoculation from extracts from fresh tomato leaves or fruits to herbaceous test plants is simple, sensitive and reliable. Although it has been a traditional method of virus detection, it does not lead to specific PepMV identification when used alone, as the symptoms produced on test plants are of little diagnostic value. However, test-plant inoculations can be used for virus detection and isolation as well as for increasing PepMV concentrations in plant tissue for subsequent identification methods, such as DAS-ELISA.

Fig. 4 Leaf symptoms consisting of leaf blistering (left) and bright yellow flecks (middle and right), a rare but characteristic symptom of a PepMV infection.

• Seed extracts

A bioassay may be used but its sensitivity is very variable. A positive result with a bioassay will indicate the presence of viable PepMV, whereas a negative result does not allow any conclusion on the presence of the pest to be drawn. Because of this variability in sensitivity bioassay is not recommended as a test for detecting PepMV in seeds.

The most sensitive and recommended biotest plant species for PepMV detection are *N. occidentalis* P1, *N. occidentalis* 37B and *N. benthamiana*. When tomato plant material (leaves, roots, fruits) is used as the source of inoculum the type of extraction buffer to be used for test-plant inoculations is not critical, e.g. 0.02 M Na/K phosphate buffer, pH 7.0. Celite is added to the inoculum as an abrasive or Carborundum is used for dusting the leaves prior to inoculation. This should be washed off after inoculation to avoid both damage to the inoculated leaves and masking of symptoms. Inoculated plants are preferably kept at a temperature range of 20 ± 3°C in a glasshouse or growth chamber with a minimum of 12 h of light. If leaf and fruit symptoms are not conspicuous or appear dubious, PepMV can be readily transmitted by sap inoculation to test plants such as:

- *N. benthamiana* and *N. occidentalis* 37B. Systemic mosaic, leaf chlorosis (sometimes necrosis) and leaf deformations develop with all PepMV strains tested so far.
- *N. occidentalis* P1. Local chlorotic and necrotic lesions, and systemic chlorosis, dwarfing and necrotic lesions (Verhoeven *et al.*, 2003).

No local lesion host suitable for all PepMV strains is known.

For further details on seed extraction and bioassays see Appendices 1 and 2.

Identification

For identification of PepMV different serological and molecular tests or combinations thereof are available.

Serological methods

Available serological tests for PepMV are:
- Double antibody sandwich enzyme-linked immunosorbent (DAS-ELISA).
- Immunoelectron microscopy (IEM).
- Lateral-flow immunoassay (LFIA).

Lateral-flow immunoassays (LFIA) and immunoelectron microscopy (IEM) tests for the identification of PepMV are available or have been described. However, these tests have not been validated and are therefore not recommended nor included in this protocol,

Double antibody sandwich-(DAS-)ELISA

ELISA can readily be used for detecting and identifying PepMV in field- and glasshouse-grown plants, test plants and on tomato seeds. DAS-ELISA kits containing all necessary components needed for the test are widely available commercially and can be used. DAS-ELISA is the preferred and recommended method for analysing large quantities of PepMV-suspected samples and for PepMV detection and identification on tomato seeds. For details on DAS-ELISA on seed extracts and test plants see Appendix 3. The ELISA method described here was evaluated in a test performance study on tomato seeds in 2009 as part of the EU FP6 PEPEIRA project (and shown to perform well in more than 95% of the results of 18 participating laboratories).

Molecular methods

A large range of molecular methods has been used for PepMV detection and identification. The most common nucleic acid-based tests (sometimes combined with serology or restriction fragment length polymorphism [RFLP]) are:
- Conventional Reverse Transcription-PCR (RT-PCR) adapted from Ling *et al.* (2008).
- Real-time Reverse Transcription PCR (real-time RT-PCR) adapted from Ling *et al.* (2007).
- Immunocapture Reverse Transcription-PCR (IC RT-PCR)
- Reverse Transcription-PCR RFLP (RT-PCR RFLP).

IC RT-PCR and RT-PCR RFLP tests for the identification of PepMV are available or have been described. However, these tests have not been validated and are therefore not recommended nor included in this protocol.

Some of the aforementioned tests have also been shown to be suitable for strain differentiation of PepMV (Martínez-Culebras *et al.*, 2002; Hanssen *et al.*, 2008; Alfaro-Fernández *et al.*, 2009; Gutiérrez-Aguirre *et al.*, 2009). As mixed infections with PepMV strains have become more common under natural conditions, multiplex RT-PCR formats or RT-PCR followed by RFLP or DNA sequencing have been developed, allowing the simultaneous detection and identification of different strains of PepMV in less time and at lower cost. For routine diagnosis of PepMV, however, more generic tests should be used to detect isolates of all four known strains of PepMV. Such generic primers and probes have been described for a conventional RT-PCR (Ling *et al.*, 2008) and a real-time RT-PCR (Ling *et al.*, 2007). The real-time RT-PCR is presented in Appendix 4. Both tests were evaluated in a test performance study on tomato seeds in 2009 as part of the PEPEIRA project. While the real-time RT-PCR test gave expected results in the vast majority of the 11 participating laboratories, the conventional RT-PCR gave expected results in only 7 of the 14 laboratories. The reason for the poor performance of the conventional RT-PCR in some laboratories is not known. Since it appears that this RT-PCR is not fully reliable under all circumstances this test is not recommended nor included in this protocol.

Recommended tests

For qualitative detection and identification of PepMV, DAS-ELISA is recommended as the initial test of choice. Real-time reverse transcription PCR (real-time RT-PCR) is recommended as the molecular test of choice. Both tests are described in more detail in Appendices 3 and 4, respectively. For seed testing for the maximum subsample size recommended (250 seeds) the tests are equivalent (both able to detect one infected seed in 250).

A bioassay to detect the presence of viable PepMV is only recommended for leaf or fruit samples of tomato or test plants but not for seed samples.

Confirmation of test results

Positive samples should be confirmed. If the initial test was a DAS-ELISA, a second DAS-ELISA (with a different antiserum) or the real-time RT-PCR should be performed as a confirmatory test. This test should be performed on the initial sample. If the initial test was a real-time RT-PCR, and given that the sensitivity of both ELISA and real-time RT-PCR is sufficient to detect one infected seed in a sub sample of 250, DAS-ELISA can be used to confirm a positive real-time RT-PCR of a particular seed sub-sample. So far, no other real-time RT-PCR test targeting a different viral genome region exists.

Reference material

Leaf samples infected with each of the four major strains of PepMV can be obtained from Dr. R. van der Vlugt, Plant Research International, Wageningen, NL (rene.vandervlugt@wur.nl).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis.*

Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

Further information

Further information on this organism can be obtained from R. van der Vlugt, Plant Research International, Wageningen, NL, and from other members of the PEPEIRA consortium.

Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

Protocol 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

This protocol was originally drafted by H.J. Vetten, R.A. Mumford and R. van der Vlugt.

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Appendix 1 – Seed extraction

Extraction of seed samples

When testing tomato seed samples, care should be taken to avoid contamination e.g. place a paper or plastic sheet on the bench when grinding the seed samples as, in rare cases, bags can leak.

1. Preparing sub-samples can be done on the basis of the mean weight of 5–10 sub-samples of 250 tomato seeds.
2. Each sub-sample is placed in a special extraction bag (e.g., Bioreba cat. no. 480100, shown here) to which 10 mL (±0.1 mL) of 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4 pH 7.2; see below) are added.
3. Close bags by heat-sealing (seal the bag at the upper edge after ensuring that no air bubbles are trapped in the bags and that bags are really air tight at the new heat-sealed seam).
4. Make sure all seeds are immersed in buffer and refrigerate bags (4 ± 2°C) overnight.
5. Place the seed- and buffer-containing extraction bag on a flat, hard surface and (grind the seeds in the bag using a hand or semi-automated homogenizer (e.g., Bioreba art. nos. 400010 and 400005). Grinding is done for about 2 min. After grinding for 1 min, the liquid in the bag should turn milky. Continue grinding for another minute until the milky discoloration of the buffer becomes more intense. [Note that complete maceration of seeds is not accomplished with this method - it is not required]
6. Grind the other sub-samples in a similar fashion.
7. Cut open the bags with a pair of scissors. Make sure to disinfect the equipment between each bag using sodium hypochlorite, flaming off or a commercial viricide. Avoid cross-contamination between samples. It is recommended that healthy controls are processed at the end to evaluate potential cross-contamination.
8. Recover the milky liquid (volume of approximately 7 mL) from the bag (from the space behind the mesh) and place it in a 10-mL vial or tube.
9. When the extract is to be tested the same day, keep it on ice or refrigerated. Aliquots of the extract which are not tested directly should be stored at −20°C (±2°C) for future reference or confirmatory tests.

Extraction buffer

0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4 pH 7.2 Buffer
Solution A Prepare 0.1 M Na_2HPO_4. $2H_2O$
Solution B Prepare 0.1 M $KH_2PO_4.H_2O$
Add solution A to solution B to reach a pH of 7.2.

Appendix 2 – Bioassay

A bioassay may be performed on a sample of tomato leaves or fruits showing symptoms of virus infection. Test plants (*Nicotiana benthamiana* or *N. occidentalis* '37B') are inoculated and a DAS-ELISA (or real-time RT-PCR) test on the inoculated plants is performed in order to confirm PepMV infection. Only 1 mL of each, leaf or fruit extract is used for inoculation of two test plants. Note that validation data is not available for ELISA or real-time RT PCR on test plants.

1. Grind a sample of leaf or fruit of approx. 0.5–1.0 g in a special extraction bag (e.g., Bioreba cat. no. 480100) to which 5 mL (±0.1 mL) of 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4 pH 7.2) is added.
2. Remove the extracts directly for mechanical inoculation of test plants. Process extracts immediately after grinding or store at 4°C (±2°C) for a maximum of 20 h if they are first analysed by ELISA (or RT-PCR). Do not freeze.
3. Inoculate test plants at the right stage, i.e. when they have about 4 fully expanded leaves (see Fig 5). They should have been raised under sufficient light intensity at an average temperature of 20–25°C, not yet have started flowering, and have good turgor at the time of inoculation.
   - 3.1 Inoculate each extract onto three fully expanded leaves of each of two plants, going across the whole surface.
     - 3.1.1 Dust the leaves moderately with carborundum (320 mesh grit powder, Fisher Scientific or equivalent).
     - 3.1.2 It is recommended that healthy controls are inoculated at the end to evaluate potential cross-contamination.
     - 3.1.3 Place 100 µL droplets of leaf or fruit extract (or buffer) onto each leaf to be inoculated. Smear the droplets with fingers (or a glass spatula) across the leaf surface without applying pressure. Repeat this for the other two leaves of each plant. Work with gloves, and change gloves between different samples.
     - 3.1.4 Rinse the plants with tap water a few minutes after inoculation of each sample. Do not wait until you have inoculated all plants.
   - 3.2 In order to allow the test plants to become systemically infected, incubate them for at least 14 days under controlled conditions at 20 ± 3°C and with at least 12 h of light per day.

Fig. 5 *N. occidentalis* '37B' (left) and *N. benthamiana* (right) seedling at the latest stage suitable for inoculation (when it has about 4 fully expanded leaves). Preferably inoculate three fully expanded leaves (marked by 'X').

4. Score test plants for symptom development at regular intervals (e.g., day 6, 10 and 14) after inoculation.
5. After 14 days, determine infection of the test plants by PepMV using ELISA.
   - 5.1 For each sub-sample, sample and pool leaf material from both test plants making sure that the pooled leaves weigh 0.2–0.5 g. Select leaves that are from the top of the plants and have expanded during the preceding week (not the inoculated leaves). [Process samples immediately, store at 4°C (±2°C) for not more than 48 h or freeze until use. If the samples were frozen, process them as soon as they have thawed].
   - 5.2 Grind each pooled leaf sample in 5 mL (± 0.1 mL) 0.1 M phosphate buffer (0.1 M Na_2HPO_4 14.2/KH_2PO_4 6.8, pH 7.2). Pro cess extracts immediately after grinding, store at 4°C (±2°C) for a maximum of 24 h or freeze at −20°C (±2°C) until use.
   - 5.3 Run a DAS-ELISA on the leaf extracts. If infectious PepMV is present, this should result in clearly positive ELISA reactions.

Appendix 3 – DAS-ELISA

The source of antibodies is critical. Several PepMV antisera are available from various suppliers which may differ in reactivity against the individual virus strains (R. van der Vlugt, unpublished data). Based on the current available information (based on unpublished results and the PEPEIRA test performance study) commercial antisera from either Prime Diagnostics (www.primediagnostics.com) or Bioreba (www.bioreba.com) detect all currently known strains of PepMV and are recommended for use in this DAS-ELISA protocol. No cross reaction was observed with other common viruses (e.g. CMV, TSWV, PVY) that may be present in tomato (R. van der Vlugt, pers. comm.). The antiserum from Prime Diagnostics was successfully used in the PEPEIRA evaluation of test performance. The instructions are presented below for both Prime diagnostics and Bioreba antisera (for more details see the instructions of the manufacturer).

The method for testing either seed extracts or plant leaf extracts is basically similar with only some differences in the preparation of the samples.

Preparation of seed extracts and of leaf extracts from plants is described in Appendix 1 and 2.

Buffers and reagents

	Prime diagnostics	Bioreba
Coating buffer	1.59 g Na_2CO_3 2.94 g $NaHCO_3$ Dissolve in 900 mL distilled/demineralized water Adjust pH to 9.6 with HCl Add distilled/demineralized water to 1000 mL total volume	1.59 g Na_2CO_3 2.93 g $NaHCO_3$ 0.20 g NaN_3 Add distilled/demineralized water to 1000 mL total volume Adjust pH to 9.6 with HCl
PBS (10×)	81.8 g NaCl 1.49 g KCl 2.72 g KH_2PO_4 14.2 g $Na_2HPO_4 \times 2\ H_2O$ (or 28.6 g $Na_2HPO_4 \times 12\ H_2O$) Dissolve in 900 mL distilled/demineralized water Add distilled/demineralized water to 1000 mL total volume	(see wash buffer)
PBS (1×)	100 mL 10× PBS 900 mL demineralized water Adjust pH to 7.4 with NaOH Add distilled/demineralized water to 1000 mL total volume	(see wash buffer)
Wash buffer	0.1% Tween-20 in 1× PBS	8.00 g NaCl 0.20 g KH_2PO_4 1.15 g Na_2HPO_4 0.20 g KCl 0.50 g Tween 20 Contains no preservative. Use within 2 days or add 0.2 g/L NaN_3 Add distilled/demineralized water to 1000 mL total volume Adjust pH to 7.4
Conjugate buffer	900 mL 1× PBS 0.5 mL Tween-20 20 g polyvinyl pyrrolidone (PVP-40000, Sigma) 2 g ovalbumine (grade VI) 0.5 g NaN_3 Dissolve well by stirring (PVP and ovalbumine take a long time to disolve and the solution will remain cloudy). Add 1× PBS to 1000 mL total volume and store at 4°C	2.40 g TRIS 8.00 g NaCl 20.00 g PVP K25 (MW 24000) 0.50 g Tween 20 2.00 g BSA (bovine serum albumin) 0.20 g $MgCl_2\ 6\ H_2O$ 0.20 g KCl 0.20 g NaN_3 Adjust pH to 7.4 with HCl; Add distilled water to 1000 mL total volume
Substrate buffer	97 mL diethanolamine 600 mL distilled/demineralized water Adjust to pH 9.8 with HCl Add distilled/demineralized water to 1000 mL total volume	97 mL Diethanolamine 0.20 g NaN_3 Just before use (always prepare fresh): Dissolve pNPP (para-nitrophenyl-phosphate) at 1 mg/mL in substrate buffer. Add distilled water to 1000 mL total volume Adjust pH to 9.8 with HCl
Substrate	5 mg para-nitrophenylphosphate (pNPP) tablets, Sigma104–105 3 tablets in 20 mL substrate buffer (final concentration0.75 mg/mL)	See Bioreba product information
Coating antibody	Purified polyclonal virus-specific antibody (IgG) raised in rabbit	Purified polyclonal virus-specific antibody (IgG) raised in rabbit
AP conjugate	Alkaline phosphatase conjugated polyclonal virus-specific antibody (IgG), raised in rabbit	Alkaline phosphatase conjugated polyclonal virus-specific antibody (IgG), raised in rabbit
Positive control	Partially purified preparation from PepMV-infected test plant (10× concentration) to be diluted 10× in 0.1 M phosphate buffer prior to use.	Lyophylised (freezedried) control prepared from PepMV infected test plant; to be diluted in 2.5 mL H_2O

Equipment and materials

- ELISA plates: 96 wells plates of good quality (medium or high-binding capacity i.e. Greiner cat. no. 655001 or Nunc-Immunoplate maxisorp cat. no. 439454).
- Plate washer: The use of an automated plate washer is recommended. The washing may also be performed by hand. Empty the plates with force and fill them several times with the wash buffer. Finally soak the plates with the wash buffer for 5 min and empty them again with force (on a folded towel).
- Plate reader: Any ELISA plate reader can be used, Multichannel pipettes (8 or 12 rows). Make sure to use different pipettes for dispensing the coating IgG and the conjugate solutions or use filter tips.

Method

The method is described as validated in the Pepeira project.

Coating of plates

- Dilute the coating antibody at a ratio of 1:1000 in coating buffer.
- Load 200 µL of the antibody solution into each well of the ELISA plate. Cover the plate with a lid and place the plate in a humid box (wet tissue on the bottom of the box). Close the box and incubate it overnight in a refrigerator or for 3 h at approximately 37°C[2].

Sample incubation

- Wash the plate in the plate washer with the washing buffer: 3 washings per plate.
- As an alternative for a plate washer, hand washing using a wash bottle can be used. Make sure all wells are filled and empty them by force.
- Load 200 µL of the seed or leaf extracts in duplicates into the wells of the ELISA plate. Use the first column of the plate (#1) for extracts of healthy seeds (in case of seed testing) or healthy plant extract (in case of leaf testing).
- Cover the plate with a lid and place the plate in a humid box. Close the box and incubate the box overnight in a refrigerator.

Conjugate incubation

- Wash the plate in the plate washer with the washing buffer: 3–4 soakings and washings per plate to remove all traces of unbound virus in the sample.
- Alternatively wash the plate by hand.
- Dilute the AP conjugated antibody 1:1000 in conjugate buffer.
- Load 200 µL of the conjugate solution into each well of the ELISA plate.
- Cover the plate with a lid and place the plate in a humid box (put a damp piece of paper on the bottom). Close the box and incubate the box over-night in the refrigerator or 3 h at approximately 37°C.[3]

Substrate incubation

- Wash the plate in the plate washer with the washing buffer: 3–4 soakings and washings per plate to remove all traces of unbound conjugate (this is a critical washing step).
- Alternatively, wash the plate by hand.
- Prepare the substrate fresh. Take care to use clean (= alkaline phosphatase-free) glass and plastic ware (autoclave glass ware prior to use).
- Load 200 µL of the substrate into each well of the ELISA plate.
- Incubate the plate at room temperature until the positive control (PC) and positive samples turn yellow. This may take from less than 5 min (for the PC) up to 1 h depending on the concentration of the virus in the samples.
- Read the plate in a plate reader after 15, 30 and 60 min of substrate incubation. It is recommended that raw data are recorded i.e. do not automatically subtract the values of buffer or healthy control columns).

Data interpretation

Calculate the average A405 readings (AVG) and standard deviation (STD) of all healthy controls after 60 min. Determine the threshold value using the following formula: (AVG + (3 × STD)). A sample should be considered positive if the ELISA values of both duplicates are above the threshold value. If one of the duplicates of a particular samples is above and the other duplicate is below the threshold value it is recommended that that particular seed extract or plant is re-sampled and re-tested. If both values of a sample are below the threshold, the sample can be considered negative. The positive control should meet the standard as supplied by the antiserum manufacturer.

Using twice the average of the healthy controls to calculate the threshold values above which a sample can be considered positive, results in a significant decrease in sensitivity of the DAS-ELISA at the recommended maximum sub-sample size of 250 seeds. This is likely to result in false negative samples and is therefore not recommended for data interpretation.

ELISA Plate design

To ensure direct comparison of results duplicates of samples should be loaded onto the same ELISA plate and samples should only be compared against positive and negative controls present on the same ELISA plate. Column 1

[2]Bioreba recommends 4 h at approximately 30°C.

[3]Bioreba recommends 5 h at approximately 30°C.

should contain healthy seed or plant extracts. Apart from the sample extracts, a positive control (as supplied by the antiserum supplier) must be included on each ELISA plate in duplicate.

Example of the plate design for ELISA analysis. All samples are tested in duplicate including the 10× diluted positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B*	B	B	B	B	B	B	B	B	B	B	B
B	HC	SE1	SE1	SE7	SE7							PC
C	HC	SE2	SE2	SE8	SE8							PC
D	HC	SE3	SE3	etc.								B
E	HC	SE4	SE4									B
F	HC	SE5	SE5									B
G	HC	SE6	SE6									B
H	B	B	B	B	B	B	B	B	B	B	B	B

B = Conjugate buffer; HC = healthy control (seed or plant extract); PC = positive control; SE1 = Sample extract 1; SE2 = Sample extract 2 etc.

Appendix 4 – Real-time RT-PCR

1. General information

1.1 This method is based on the test published by Ling *et al.* (2007), which was modified and evaluated in a test performance study in 2009 as part of the PEPEIRA project (http://www.pepeira.wur.nl). Further validation data has been published by Gutiérrez-Aguirre *et al.* (2009).

1.2 The TaqMan test used was designed against the following 5 strains covered by 11 isolates (with their GenBank accession no. in brackets): EU-tom (AJ438767, AJ606359, AJ606360, AF340024, AF484251); Ch-1 (DQ000984, AY509926); Ch-2 (DQ000985); Peruvian (AJ606361, AM109896); US-2 AY509927).

1.3 The amplicon covers a 107 bp region of the TGB2 gene of PepMV, corresponding to nucleotides 5126–5232 in isolate Ch1 (accession number DQ000984).

1.4 Oligonucleotides:

Primer	Sequence	Isolate specificity
KL05-48 forward 1	5′-ACTCCTAGAGCTG ACCTCAC-3′	Ch1, EU-tom, Peruvian
KL05-49 forward 2	5′-ACTCCTAGAGCTG ATCTTAC-3′	Ch2, US2,
KL05-50 probe	5′-FAM-TGTCAGCTTG CATTTACTTC CAAAA-BHQ-3′	All PepMV isolates
KL05-51 reverse 1	5′-TCTCCAGCAACAG GTTGGTA-3′	Ch1, EU-tom, Peruvian
KL05-52 reverse 2	5′-TCACCTGCAACTG GTTGATA-3′	Ch2, US2
COX F	5′-CGT CGC ATT CCA GAT TAT CCA-3′	Plant endogene (cytochrome oxidase) control
COXSOL 1511T	5′-VIC-AGG GCA TTC CAT CCA GCG TAA GCA-BHQ-3′	Plant endogene (cytochrome oxidase) control
COX RW	5′-CAACTACGGATA TATAAGRR CCRRAACTG-3′	Plant endogene (cytochrome oxidase) control

1.5 The test should be performed using a hot-start Taq DNA polymerase and MMLV reverse transcriptase.

1.6 The test has been successfully performed using reagents from different manufacturers including ABI One-Step RT-PCR Master Mix Reagents Kit, Ambion-ABI Ag-Path ID one step RT-PCR kit and Bio-Rad One Step RT-PCR.

1.7 The test has been successfully performed on a range of different real-time PCR systems including ABI (7900, 7500), Roche (LightCycler 480), Bio-Rad (CFX96).

1.8 All test samples (and preferably all control samples) should be run in duplicate. At all stages while setting up PCR reactions, precautions should be taken to avoid contamination of samples and reagents.

1.9 TaqMan probes: different combinations of dyes can be used to suit specific real-time systems.

2. Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 Leaves of tomato plants or tomato seeds can be tested using these methods (they have not been validated on other plants). For reliability, subsamples of no more than 250 seeds should be used in an individual test (although test performance studies do show that real-time PCR can detect the equivalent of one infected seed in 6250).

2.1.2 RNA can be reliably extracted using Qiagen RNeasy Plant kits, following the manufacturer's instructions. Other extraction methods have been demonstrated as effective for the detection of PepMV and can be used but should be validated against the RNeasy kit to ensure comparability.

2.1.3 Extracted RNA should be stored at −20°C or refrigerated for short-term storage i.e. max. 8 hours.

2.2 Single-step real-time reverse transcription-polymerase chain reaction (real-time RT-PCR).

	Working concentration	Volume per reaction (µL)	Final concentration
Total reaction volume of a single PCR reaction in µL	–	25	–
ABI *Taq* Gold Buffer A buffer	10×	2.5	1×
MMLV reverse transcriptase	4 U/µL (Note: pre-diluted 1:50 from 200 U/µL)	0.125	10 units
$MgCl_2$	25 000 µM	5.5	5500 µM
dNTPs	6250 µM	2.0	500 µM
Taq polymerase	5 U/µL	0.125	0.625 units
KL05-48 forward primer	7.5 µM	1.0	0.3 µM
KL05-49 forward primer	7.5 µM	1.0	0.3 µM
KL05-51 reverse primer	7.5 µM	1.0	0.3 µM
KL05-52 reverse primer	7.5 µM	1.0	0.3 µM
KL05-50 probe	5.0 µM	0.5	0.1 µM
Total RNA	–	1.0	–
Molecular grade water	–	9.25	–

2.2.1 PCR cycling parameters: based on ABI Universal conditions: 30 min at 48°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For ABI machines do NOT run in 9600 emulation mode.

Single-step real-time RT-PCR for extraction inhibition control reaction:

	Working concentration	Volume per reaction (µL)	Final concentration
Total reaction volume of a single PCR reaction in µL	–	25	–
ABI *Taq* Gold Buffer A buffer	10×	2.5	1×
MMLV reverse transcriptase	4 U/µL (Note: pre diluted 1:50 from 200 U/µL)	0.125	10 units
$MgCl_2$	25000 µM	5.5	5500 µM
dNTPs	6250 µM	2.0	500 µM
Taq polymerase	5 U/µL	0.125	0.625 units
COX-F forward primer	7.5 µM	1.0	0.3 µM
COX-RW reverse primer	7.5 µM	1.0	0.3 µM
COXSOL 1511T probe	5.0 µM	0.5	0.1 µM
Total RNA	–	1.0	–
Molecular grade water	–	11.25	–

2.2.2 PCR cycling parameters: see 2.2.1

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene[4]) amplification of samples spiked with exogeneous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid. The internal control should only be run in one of the duplicate to avoid that it affects sensitivity.

3.2. Interpretation of results

The cycle cut off value for PepMV is set at 37, and was obtained using the equipment/materials and chemistry used as described in this appendix. When necessary, the Ct cut off value should be determined for the required internal control. The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of the controls

- The PIC and PAC amplification curves should be exponential.

[4]The plant cytochrome oxidase gene does not monitor the RT step.

- NIC and NAC should be negative (Ct > cut off).
- PIC, PAC and IPC should have a Ct value below the cut off value.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and a Ct value of ≤ 37.
- A sample will be considered negative, if it produces a Ct $\geq$ 40.
- Tests should be repeated if any contradictory or unclear results are obtained (i.e. 37 < Ct value <40).

4. Performance criteria available for the real-time RT-PCR

4.1. Analytical sensitivity data

- Gutiérrez-Aguirre *et al.*, 2009 demonstrated detection down to a theoretical limit of detection equivalent to 10–100 genome copies. The test could detect one naturally infested seed in 5000.
- Ling *et al.*, 2007 could detect 20 pg of infected total RNA and 1 artificially infested seed in 1000 (no lower concentrations were tested).

4.2. Analytical specificity data

Gutiérrez-Aguirre *et al.*, 2009 demonstrated that the test is able to detect 15 isolates covering all major genotypes - Peruvian, European tomato, US1, Ch2/US2.

Ling *et al.*, 2007 demonstrated that the test is able to detect 4 lab isolates (US1, US2, Ch1, Ch2) and 25 field isolates from US and Canada.

Tested against one isolate of *Potato virus X* (PVX), another potexvirus. *In silico* alignments indicate no significant sequence homology between test and any other viruses/pathogens.

4.3. Data on Repeatability

From Pepeira Test Performance Study: 98.9%

4.4. Data on Reproducibility

From Pepeira Test Performance Study 100%