



International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods



7-026: Detection of Squash Mosaic Virus, Cucumber Green Mottle Mosaic Virus and Melon Necrotic Spot Virus in cucurbits

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Crop:	Cucurbits
Pathogen:	Squash Mosaic Virus (SqMV), Cucumber Green Mottle Mosaic Virus (CGMMV) and Melon Necrotic Spot Virus (MNSV)
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Background

SqMV, CGMMV and MNSV are seed-transmissible viruses of cucurbits, and therefore the detection of these viruses in seeds of cucurbits is an important tool in control strategies. Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark and Adams, 1977). ELISA methods have also been described for the detection of PEBV and PSbMV (Hamilton and Nichols, 1978; Van Vuurde and Maat, 1985; Maury et al., 1987).

The method, using ground seed and a DAS-ELISA, can be used to simultaneously detect SqMV, CGMMV and MNSV in a single extract. Note that the extract is tested in three microtiter plates, one each for SqMV, CGMMV and MNSV. The theoretical detection limit is one infested seed in 100 seeds. To ensure a 95% probability that infestations of 0.15% or higher are detected it is necessary to test 20 subsamples of 100 seeds each. ELISA positive seed lots will not necessarily lead to seed transmission. Seed transmission of these viruses can be monitored in a grow-out, but this technique is time consuming and rather laborious.

Validation studies

Koenraadt, H.M.S. and Remeeus, P.M. (2009)

Copies are available by e-mail from ista.office@ista.ch, or by mail from the ISTA Secretariat.

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

Safety precautions

Make sure you are familiar with the hazardous nature of the materials being used and take appropriate safety precautions, especially during preparation of buffers, grinding, autoclaving, and weighing out of ingredients. It is assumed that persons carrying out this procedure are in a microbiological laboratory and are 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

Dry heat is often used for the control of CGMMV in contaminated seed lots. ELISA does not discriminate between infectious and non-infectious CGMMV, and a positive reaction in this test may cause a non-CGMMV-infected seed lot to be unnecessarily discarded.

This method has not been validated for the determination of SqMV, CGMMV or MNSV in seed treated with crop protection products or with heat. Although ELISA is compatible with some seed treatment chemicals (Pataky et al., 2004), seed treatments may affect the performance of this test. This method must only be performed on untreated seed.

Sample and subsample size

The sample (total number of seeds tested) and subsample size to be tested depend on the desired tolerance standard (maximum acceptable number of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). In any case the subsample size should not exceed 100 seeds.

Materials

Reference material: SqMV-, CGMMV- and MNSV-infested seeds or standardized reference material (flour of seeds containing SqMV, CGMMV and MNSV)

Microtiter plates: 96-well plates suitable for ELISA (CCP)

Antisera: suitable for detection of SqMV-, CGMMV- and MNSV-infested seeds (e.g. PRI, Wageningen, the Netherlands)

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: capable of pipetting to the nearest 0.001 mL

Grinder: capable of grinding seeds to fine flour (e.g. Retsch Grindomix GM 200)

Incubator: capable of maintaining a temperature of 4 ± 2 °C

Incubator: capable of maintaining a temperature of 37 ± 2 °C

ELISA plate reader

Tubes: 10 ml (LDPE)

Vortex: suitable for vortexing 10 ml tubes

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 clean all equipment, surfaces, containers, hands, etc. both before and after handling each sample.

Count the number of seeds in a known weight. Calculate the estimated thousand-seed weight as:

$$\text{Estimated thousand-seed weight} = \frac{\text{Weight of seed}}{\text{Number of seed}} \times 1000$$

Based on the estimated thousand-seed weight, weigh out subsamples of the required size into new and clean bags or containers.

Method

[Critical control points are indicated by CCP]

1. Coating of ELISA plates
 - 1.1 Add appropriate (as defined by supplier) dilution of SqMV, CGMMV and MNSV-coating serum to coating buffer to obtain coating solution. Be sure that the antisera are not only suitable for diagnostics but also for the detection of viruses in seed extracts (CCP).
 - 1.2 Coat one plate with 100 µl of SqMV-coating solution per well. Coat a second plate with 100 µL of CGMMV-coating solution per well. Coat a third plate with 100 µL of MNSV-coating solution per well.
 - 1.3 Cover ELISA plates with lid or wrap with plastic to minimize evaporation.
 - 1.4 Incubate plates overnight at 4 ± 2 °C or as defined by the supplier.
2. Extraction of virus from the seed and incubation of extracts
 - 2.1 Count or weigh 20×100 seeds per subsample.
 - 2.2 Grind each subsample to fine flour in a grinder (CCP).
 - 2.3 From each subsample, weigh out 0.5 g of flour and transfer to a 10 mL tube.
 - 2.4 Add 5 mL of extraction buffer to each tube.
 - 2.5 Vortex each tube for 15 s. Allow extract to settle for at least 5 min on the bench to facilitate pipetting.
 - 2.6 Remove coating solution from ELISA plates and immediately rinse plates thoroughly, three times, using PBS/Tween 20 to remove residues (CCP).

- 2.7 Immediately after rinsing, pipette 100 µL of each seed extract into a well. Use two wells per subsample.
- 2.8 Add positive and negative controls to each ELISA plate. Use at least two dilutions for the positive control: one “low” dilution that gives a high extinction and a “high” dilution that gives an extinction just above the detection threshold (CCP). Negative controls must include a healthy seeds extract.
- 2.9 Cover plates with lid or wrap with plastic to minimize evaporation and incubate overnight at 4 ± 2 °C or as defined by the supplier.
3. Incubation of conjugate
 - 3.1 Prepare appropriate dilution of SqMV-, CGMMV- and MNSV-conjugated antiserum using conjugate buffer as defined by the supplier.
 - 3.2 Remove seed extracts from ELISA plates and rinse plates three times with washing buffer PBS/Tween 20 to remove residues of seed extract (CCP).
 - 3.3 Immediately after rinsing, add 100 µL of diluted conjugate to each well of the ELISA plate.
 - 3.4 Cover plates with lid or wrap with plastic to minimize evaporation and incubate for 3 h at 37 ± 2 °C or as defined by the supplier.
4. Addition of substrate to ELISA plates
 - 4.1 Prepare substrate solution (10 mg *para*-nitrophenyl phosphate in 20 mL of substrate buffer).
 - 4.2 Remove conjugate from ELISA plates and rinse thoroughly 3 times by hand using washing buffer PBS/Tween 20. Alternatively use a reliable washing device (CCP).
 - 4.3 Add 100 µL of substrate solution to each well.
 - 4.4 Incubate in the dark for 2 h at 20 ± 2 °C or as defined by the supplier.
 - 4.5 Measure extinction value (A405) with ELISA plate reader. (See General methods, point 2.)

General methods (common to many test procedures)

1. Grinding seeds

Grind each subsample of 100 seeds to give a fine flour. Be sure to use a grinder that can be cleaned thoroughly, since cross-contamination is likely during the grinding step.

2. Recording of ELISA extinction

Record the results for all wells in the microtitre plate. Check first whether the positive and negative controls meet the expectations, since otherwise the results of the test are invalid and the test must be repeated.

It is recommended to use a negative-positive threshold of 2.5 times the background of healthy samples.

3. 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 infested seeds.

Quality assurance

Critical Control Points

[Identified by CCP in the methods]

Using different types of microtitre plates can influence sensitivity.

The quality of antisera from different sources is known to be variable. Therefore, be sure that the antisera are not only suitable for diagnostics but also for the detection of viruses in seed extracts (Step 1.1).

The use of fine flour will improve the efficacy of extraction. Therefore, grind seeds for 20 s at 10 000 r.p.m. to get a fine flour. Note that some blades easily get blunt and therefore grind less efficiently with time (Step 2.2).

Coated microtitre plates will lose activity rapidly when they are left to dry on the bench for some time. Therefore, limit the time as much as possible that empty microtitre plates are left on the bench (Step 2.6).

The use of appropriate positive and negative controls is very important to validate the result. Be sure that, apart from a "high" positive control, there is always a "low" positive control in each plate (Step 2.8).

Poorly washed microtitre plates between the different incubation steps often cause high backgrounds in ELISA. Washing can be done by hand using PBS/Tween 20 or with a washing device. Thoroughly washing microtitre plates is very critical in several steps (2.6, 3.2 and 4.2) in the ELISA, particularly after the incubation with the conjugated antiserum (Step 4.2).

Coating buffer (pH 9.6)**Na₂CO₃:** 1.59 g**NaHCO₃:** 2.93 g**Extraction buffer (0.05 M, pH 7.4)****NaCl:** 8.0 g**KH₂PO₄:** 1.0 g**Na₂HPO₄ · 12H₂O:** 14.5 g**Ovalbumine (Grade II):** 2.0 g**Tween 20:** 10.0 mL**PVP (ELISA grade, mol. wt. 10 000):** 20.0 g**Conjugate buffer (0.05 M, pH 7.4)****NaCl:** 8.0 g**KH₂PO₄:** 1.0 g**Na₂HPO₄ · 12H₂O:** 14.5 g**Tween 20:** 0.5 mL**PVP (ELISA grade, mol. wt. 10 000):** 20.0 g**BSA (ELISA grade, e.g. BSA fraction 5):** 5.0 g**Substrate buffer (pH 9.6)****Diethanolamine:** 97 mL**HCl (32%):** 15 mL**Washing buffer PBS/Tween 20 (0.05 M, pH 7.4)****NaCl:** 8.0 g**KH₂PO₄:** 1.0 g**Na₂HPO₄ · 12H₂O:** 14.5 g**Tween 20:** 1.5 mL**Preparation of individual buffers**

1. Weigh or measure out all ingredients into a suitable container.
2. Dissolve/mix ingredients and adjust volume to 1000 mL with distilled/de-ionized water.
3. Check the pH with a pH meter and adjust if necessary.

Storage of buffers

Store buffers as mentioned above at 4 ± 2 °C. Use them within a month after preparation.

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