International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-026: Detection of *squash mosaic virus*, *cucumber green mottle mosaic virus* and *melon necrotic spot virus* in Cucurbitaceae seed

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.

Published by
The International Seed Testing Association (ISTA)
Richtiarkade 18, CH-8304 Wallisellen, Switzerland

© 2027 International Seed Testing Association (ISTA)

Online ISSN 2310-3655

All rights reserved. No part of this publication may be reproduced, stored in any retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without prior permission in writing from ISTA.

7-026: Detection of *squash mosaic virus*, *cucumber green mottle mosaic virus* and *melon necrotic spot virus* in Cucurbitaceae seed

Host: Cucurbitaceae
Pathogen(s): *Squash mosaic virus* (SqMV); *cucumber green mottle mosaic virus* (CGMMV); *melon necrotic spot virus* (MNSV)

Authors: Koenraadt, H.M.S.[1] & Remeeus, P.M.[2]
Naktuinbouw, P.O. Box 40, 2370 AA
Roelofarendsveen, Netherlands
[1]E-mail: h.koenraadt@naktuinbouw.nl
[2]E-mail: bert.petra@freeler.nl

Revision history

Version 1.0, 2007-09-01
Version 1.1, 2013-01-01: Definition of sample size
Version 1.2, 2016-01-01: Addition of grow-out confirmation method for *squash mosaic virus*
Version 1.3, 2017-01-01: Reporting results revised; Grow-out confirmation method steps 1.2.4, 1.2.5 modified; CCP (g) modified
Version 1.4, 2021-01-01: Sample size revised
Version 1.5, 2024-01-01: Sample size revised
Version 1.6, 2025-01-01: Host and Background revised; Host, Background and Materials revised in Grow-out confirmation method
Version 1.7, 2027-01-01: Flowcharts added in Methods (including in Grow-out confirmation method)

Background

Squash mosaic virus (SqMV), *cucumber green mottle mosaic virus* (CGMMV) and *melon necrotic spot virus* (MNSV) are seed-transmissible viruses of Cucurbitaceae, and therefore the detection of these viruses in seeds of Cucurbitaceae is an important tool in control strategies. Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark & Adams, 1977). ELISA methods have also been described for the detection of PEBV and PSbMV (Hamilton & Nichols, 1978; Van Vuurde & 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 microtitre 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 or confirmed by using a grow-out method. See page 7-026-7 for the grow-out confirmation method for SqMV.

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 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 2000 seeds and the maximum subsample size must be 100 seeds.

Materials

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

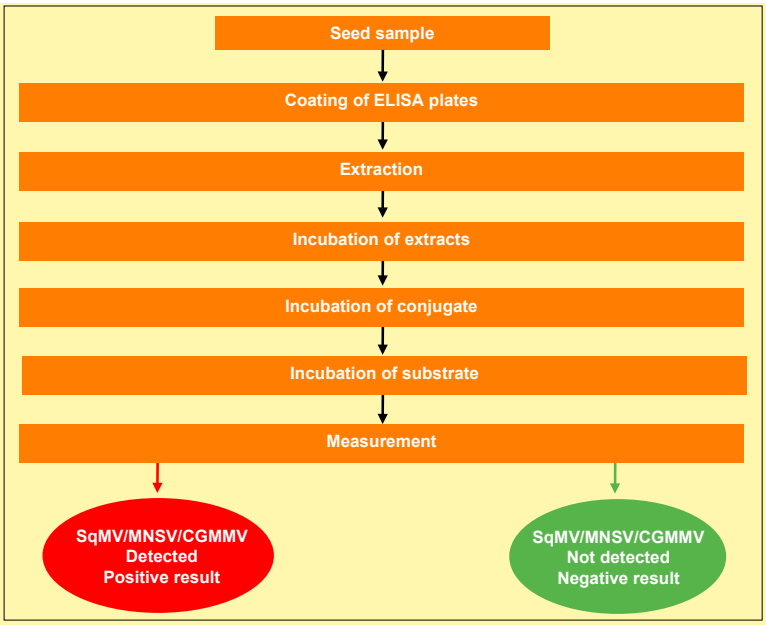
Automatic pipettes: capable of pipetting to the nearest 0.001 ml
Grinder: capable of grinding seeds to fine flour (e.g. Retsch Grindomix GM 200)
Incubator: capable of maintaining a temperature of 4 ±2 °C
Incubator: capable of maintaining a temperature of 37 ±2 °C
ELISA plate reader
Tubes: 10 ml (LDPE)
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.

1. Count the number of seeds in a known weight. Calculate the estimated 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. 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 minimise 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 minimise 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 minimise 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 (A_{405}) with ELISA plate reader. (See General methods, Recording of ELISA extinction.)

General methods

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.

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.

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

Critical control points (CCP)

- 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 rpm. 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).

Media and solutions

Coating buffer (pH 9.6)

Na_2CO_3: 1.59 g/l
$NaHCO_3$: 2.93 g/l

Extraction buffer (0.05 M, pH 7.4)

NaCl: 8.0 g/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Ovalbumine (Grade II): 2.0 g
Tween™ 20: 10.0 ml
PVP (ELISA grade, mol. wt. 10 000 Da): 20.0 g

Conjugate buffer (0.05 M, pH 7.4)

NaCl: 8.0 g/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Tween™ 20: 0.5 ml
PVP (ELISA grade, mol. wt. 10 000 Da): 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/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Tween™ 20: 1.5 ml

All buffers

Preparation

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

Storage

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

References

Clark, M. F. & Adams, A. N. (1977). Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology,* **34**, 475–483.

Hamilton, R. I. & Nichols, C. (1978). Serological methods for detection of *pea seed-borne mosaic virus* in leaves and seeds of *Pisum sativum*. *Phytopathology,* **68,** 539–543.

Maury, Y., Bossennec, J. M., Boudazin, G., Hampton, R. O., Pietersen, G. & Maguire, J. (1987). Factors influencing ELISA evaluation of transmission of *pea seed-borne mosaic virus* in infected pea seed: seed-group size and seed decortication. *Agronomie,* **7,** 225–230.

Pataky, J. K., Block, C. C., Michener, P. M., Shepherd, L. M., McGee, D. C. & White, D. G. (2004). Ability of an ELISA-based seed health test to detect *Erwinia stewartii* in maize seed treated with fungicides and insecticides. *Plant Disease,* **88,** 633–640.

Roberts, S. J., Phelps, K., Taylor, J. D. & Ridout, M. S. (1993). Design and interpretation of seed health assays. In *Proceedings of the First ISTA Plant Disease Committee Symposium on Seed Health Testing, Ottawa, Canada* (ed. J. W. Sheppard), pp. 115–125, Agriculture Canada, Ottawa, Canada.

Van Vuurde, J. W. L. & Maat, D. Z. (1985). Enzyme-linked immunosorbent assay (ELISA) and disperse-dye immuno assay (DIA): comparison of simultaneous and separate incubation of samples and conjugate for the routine detection of *lettuce mosaic virus* and *pea early-browning virus* in seeds. Netherlands *Journal of Plant Pathology,* **91,** 3–13.

Validation references

ISTA (2009). Proposal for a new method for the detection of *cucumber green mottle mosaic virus* (CGMMV), *melon necrotic spot virus* (MNSV) and *squash mosaic virus* (SqMV) in Cucurbitaceae with DAS ELISA. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.

Grow-out confirmation method for *squash mosaic virus*

Host: Cucurbitaceae
Pathogen: *Squash mosaic virus* (SqMV)

Authors: Lybeert, H.[1], Cucuzza, J.[2] & Politikou, L.[3]
[1]HM Clause S.A., rue Louis Saillant, BP 83, 26802 Portes-lès-Valences CEDEX, France
E-mail: hubert.lybeert@hmclause.com
[2]Monsanto Vegetable Seeds, 37437 State Highway 16, Woodland, CA, USA
E-mail: jim.cucuzza@monsanto.com
[3]ISF, 7 chemin du Reposoir, 1260 Nyon, Switzerland
E-mail: liana.politikou@ufs-asso.com

Background

The ISTA Seed Health Method 7-026 provides the possibility of simultaneous detection of *squash mosaic virus* (SqMV), *melon necrotic spot virus* (MNSV) and *cucumber green mottle mosaic virus* (CGMMV) on seed of Cucurbitaceae from a single seed extract with a DAS-ELISA test. However, the SqMV transmission from seed to seedling is only due to the embryonic infection despite its possible location in the seed coat and papery layer (Alvarez & Campbell, 1978; Nolan & Campbell, 1984). The DAS-ELISA test detects both infectious and non infectious virus particles in a test sample and it can lead to a false positive result (Nolan & Campbell 1984; Maury *et al.*, 1987; Koenraadt & Remeeus, 2009) and to an overestimation of the virus transmission rate in the sample. Therefore, the DAS-ELISA test can serve as a pre-screening step (Hamilton & Nichols, 1978) of seed lots carrying SqMV, and its positive results can be further confirmed by a grow-out test with seeds from the same lot.

Seeds, from a DAS-ELISA pre-screened SqMV-positive lot, are sown in a suitable substrate and are grown under defined greenhouse conditions until plants reach the growth stage of 3–4 true leaves (Powell & Shlegel 1970; Alvarez & Campbell, 1978; Nolan & Campbell, 1984). Symptoms are compared to symptoms developed on mechanically inoculated control plants following the inoculation method described by Alvarez & Campbell (1978). Tissue of plants showing typical and atypical SqMV symptoms is collected individually and tested by DAS-ELISA for confirmation of visual symptoms. If no typical/atypical symptoms appear, or if they are not confirmed caused by SqMV in the DAS-ELISA test, plant tissue of symptom-free plants is collected and tested in pools by DAS-ELISA test for the presence or absence of SqMV. This is done because preliminary tests showed that the SqMV can be transmitted to plants without expressing any symptoms (H. Lybeert, personal communication).

The grow-out test method was derived from a peer validation study between four laboratories organized by the International Seed Health Initiative for Vegetables, ISF (ISHI-Veg). In that study, melon seed was evaluated, but Cucurbitaceae in general can be evaluated. However, the ELISA confirmation test on plant tissue samples should be evaluated and verified for the various Cucurbitaceae being evaluated in the grow-out test before the results are accepted.

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 sample size is 2000 seeds.

Materials

SqMV-infected and dehydrated melon leaves: Approx. 1 g fresh weight (equivalent) (for preparation of SqMV inoculum). Melon is used for SqMV inoculum because the titre of the virus is highest and more consistent in melon than in other Cucurbitaceae.

Known SqMV-free Cucurbitaceae seeds: For growing healthy plants; one part will be used as the negative control and the other part will be mechanically inoculated to be used as the positive control. The species of Cucurbitaceae controls must match the species of Cucurbitaceae being evaluated in the grow-out test.

Greenhouse: capable of maintaining a temperature of 16–35 °C

Insect-proof installation: If not available, monitoring of insects is required.

Plastic planting trays

Potting soil and vermiculite

Carborundum powder: e.g. 320 mesh grit powder, Fischer Scientific or equivalent

Latex gloves, latex finger cots

Scalpel, cork borer, forceps

Alkaline soap or equivalent: for disinfection of surfaces, equipment etc.

Balance: capable of weighing to the nearest 0.01 g

Plastic extraction bags: e.g. Art. No. 430100 from BioReba, Switzerland

Grinder for plant tissue samples: e.g. hand tissue homogenizer

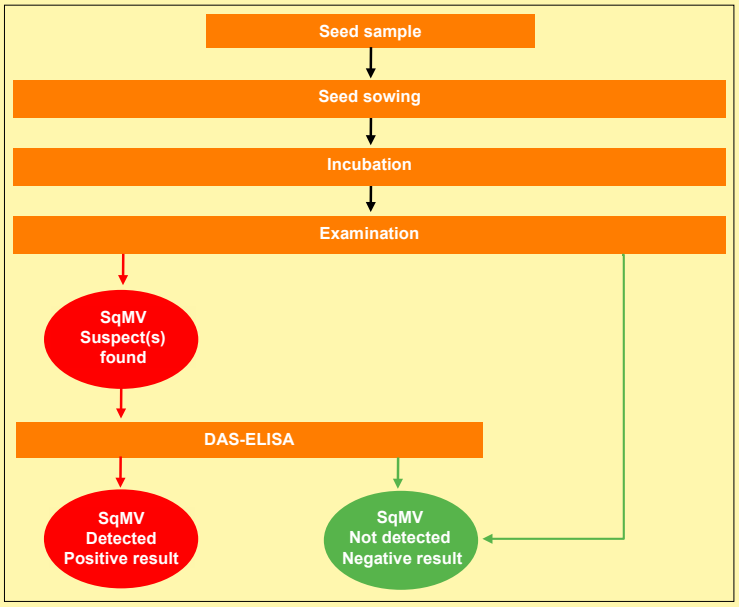
For the DAS-ELISA test, materials specified on page 7-026-03 regarding SqMV must be used.

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 equipment, 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 / numbers of seeds) × 1000
2. Based on the TSW, weigh out samples of the required size into new and clean bags or containers.



Methods

Critical control points are indicated by CCP.

1. Greenhouse grow-out

1.1 Seed sowing and greenhouse incubation

1.1.1 Clean and disinfect thoroughly all planting trays that will be used.

1.1.2 Fill the trays with well-watered potting soil and make holes of approx. 2 cm depth in each tray using a planting tag or equivalent.

1.1.3 Sow approximately 10 seeds from the known SqMV-free sample in one separate tray. The grown plants will be used as negative control plants (CCP).

1.1.4 In another separate tray sow approximately 10 seeds from the known SqMV-free sample. The grown plants will be mechanically inoculated and serve as positive control plants (CCP).

1.1.5 Continue by sowing the seeds of the sample under evaluation into the rest of the trays (CCP).

1.1.6 Cover the seeds in all trays with a thin layer of vermiculite and place the trays by keeping adequate space between each other in a greenhouse with insect-proof installation. (CCP).

1.1.7 Maintain the greenhouse temperature at 24–35 °C during the day and 16–22 °C during the night until seedlings emerge (CCP).

1.1.8 From seedlings emergence to final reading, maintain the greenhouse temperature at 24–35 °C (CCP).

1.2 Mechanical inoculation of positive control plants

1.2.1 When the 1st true leaf of each plant begins to emerge, (approximately 10 days after sowing) prepare the virus inoculum as follows and inoculate the plants mechanically with it (CCP).

1.2.2 Grind the dehydrated SqMV-infected melon leaves (approx. 1 g fresh weight) into 4 ml of virus extraction buffer in a mortar with a pestle.

1.2.3 Add 0.075 g of carborundum powder and mix well.

1.2.4 Place a drop(s) of the virus inoculum on the surface of both cotyledons and 1st leaf of each emerged

plant and rub it with fingers using latex gloves and/or finger cots. Use enough liquid so that all surface areas are wetted. Apply light pressure, such that the leaf tissue is not damaged.

1.2.5 Rinse the plants with tap water within 5 min of completing the inoculation and continue their greenhouse incubation with the rest of the plants until the final reading.

1.3 SqMV symptoms and collection of plant tissue samples for DAS-ELISA testing (CCP)

1.3.1 Monitor all plants in greenhouse every 3–5 days upon emergence without handling (CCP).

1.3.2 When the majority of plants reach the stage of 3–4 true leaves (approximately 18–24 days after sowing) inspect for typical and atypical SqMV symptoms on their leaves by comparison with the positive and negative control plants (CCP). Typical SqMV symptoms are the systemic mosaic or vein banding in leaves and sometimes the leaf deformation (ICTVdB Management, 2006)(Fig. 1). Atypical SqMV symptoms are the discoloration of leaves and spots developing on them (Lecoq *et al.*, 1998).

1.3.3 If typical or atypical SqMV symptoms appear, count the number of plants with each kind of symptoms (optional).

1.3.4 Continue with the collection of plant tissue samples for DAS-ELISA testing as follows: Start collecting plant tissue samples from the negative control plants, continue with the plants from the evaluated sample and end with the positive control plants (CCP).

1.3.5 For the plants of the negative control, cut with a cork borer a plant tissue piece from one of the younger leaves of each plant. Pool all pieces together in a plastic extraction bag (e.g. Art. No. 430100 from BioReba, Switzerland)(10 plant tissue pieces in 1 bag).

1.3.6 For plants of the evaluated sample with SqMV typical symptoms cut with a cork borer a plant tissue sample of the younger symptomatic leaf of each plant individually. Place each piece separately in a plastic extraction bag (e.g. Art. No. 430100 from BioReba, Switzerland. (1 plant tissue piece in 1 bag). For plants with SqMV atypical symptoms repeat procedure to collect individual plant tissue pieces (1 plant tissue piece in 1 bag).

1.3.7 For the positive control plants follow the procedure described in 1.3.5 (pool all 10 plant tissue pieces in 1 bag).

2. Virus extraction from plant tissue samples and DAS-ELISA confirmation

2.1 Depending on the total number of plant tissue samples that will be collected, calculate the number of ELISA plates that will be needed.



Figure 1. Typical SqMV symptoms on leaves of melon plants at the 3–4 true leaves stage.

2.2 Follow the process to coat ELISA plates (Methods, step 1) and the supplier's instructions on their incubation conditions.
2.3 Grind all plant tissue samples with a suitable grinding device (e.g. hand homogenizer).
2.4 Add 10 ml of ELISA extraction buffer (as described by the supplier) per gram of plant tissue into each container.
2.5 Proceed with the DAS-ELISA test as described in Steps 3.1 to 4.5 to confirm SqMV-positive plant tissue samples (CCP).
2.6 Compare results of plant tissue samples from the evaluated sample to results of positive and negative control plants (CCP). If positive results are given in the DAS-ELISA test either on the typical or the atypical SqMV symptomatic plant tissue samples, terminate the grow-out and record the seed sample as being SqMV-positive.
2.7 If negative results are given in the DAS-ELISA test or no typical or atypical symptoms appear, test all symptom-free plants in a DAS-ELISA test for the presence or absence of SqMV.
2.8 Repeat procedure in Step 1.3.4 to collect plant tissue samples from control plants and plants from evaluated sample (CCP).
2.9 Cut with a cork borer a plant tissue piece from one of the younger leaf of each symptom-free plant in the evaluated sample and make pools of 20 plant tissue pieces maximum in plastic extraction bags (e.g. Art. No. 430100 from BioReba, Switzerland).
2.10 Repeat Steps 2.1 to 2.5.
2.11 Compare results of plant tissue samples from the evaluated sample to results of positive and negative control plants (CCP). In this case, the final result of the seed sample will be given by the DAS-ELISA test result of symptom-free plants (negative or positive confirmation).

General methods

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.

Using the values of the negative controls, establish a cut-off value for healthy versus positive responses. Use supplier recommended levels if received from the serum supplier (for example, 2 times the average negative control value). Alternatively, use the mean of the negative control value plus three times (3×) the standard deviation of the negative control values. For example, the mean of all negative controls is 0.050 and the standard deviation is 0.004. The negative cut-off = 0.050 + 3(0.004) = 0.062. This will account for 95 % of healthy observations. Above this value are considered a positive response. All negative controls from a batch of plates run together can be used for this calculation.

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

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

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

Quality assurance

Critical control points (CCP)

- It is important to start sowing the healthy seeds that will serve as negative and positive (after mechanical inoculation) control plants and then continue with the sowing of the seeds of the evaluated sample (Steps 1.1.3 and 1.1.4) to avoid cross-contamination of the virus at this point.
- The percentage of seed germination should be taken into consideration when sowing seeds, in order to make sure that the minimum recommended sample size to analyse is obtained (Step 1.1.5).
- Cucurbitaceae plants can be tall enough to bend and touch each other. To reduce the chances of cross-contamination between plants, it is important to keep adequate space between the trays. Also, the greenhouse must have an insect-proof installation to reduce the risk of virus transmission by beetles (Step 1.1.6).
- The ability to detect SqMV could be influenced by variations in environmental conditions in the greenhouse (Steps 1.1.7 and 1.1.8). Therefore, it is recommended not to perform the grow-out test during the winter period unless artificial light and heating can compensate for the lack of natural light and temperatures.
- It is recommended to place the virus inoculum on ice and proceed with the inoculation procedure as soon as possible (Step 1.2.1).

- Do not handle the plants throughout the monitoring period to avoid cross-contamination (Step 1.3.1). If plants are damped-off or other disease symptoms are present, then the grow-out test should be considered invalid and redone (Step 1.3.1).
- The use of negative and positive control plants is very important to compare the visually observed SqMV symptoms on evaluated Cucurbitaceae plants in the grow-out test (Step 1.3.2) and to validate the DAS-ELISA results (Steps 2.6 and 2.11). If control plants do not perform as expected, the test is considered as invalid and discarded, and a retest must be done.
- The DAS-ELISA confirmation test on plant tissue samples should be evaluated and verified for the various Cucurbitaceae being evaluated in the grow-out test before the results are accepted. (Step 2.5).
- It is important to start the collection of plant tissues with the negative control plants then continue with the plants of the evaluated sample and end with the plants of the positive control to avoid cross contamination of the virus at this point (Steps 1.3.4 and 2.8).
- Gloves should be changed and the cork borer should be cleaned with alkaline soap or virus disinfectant between each plant tissue sample during their collection to avoid cross-contamination (Steps 1.3.4 and 2.8).
- If symptom-free plants need to be confirmed by DAS-ELISA, then freshly collected plant tissue samples from the positive and negative control plants must be included in the test (Step 2.8)
- For the DAS-ELISA test, the critical control points are described in Step 2.5.

Media and solutions

Virus extraction buffer

For the preparation of the virus inoculum.

Na_2HPO_4 $2H_2O$: 0.53 g
Sodium diethyldithiocarbamate trihydrate $(C_2H_5)_2$ $NCS_2Na \cdot 3H_2O$: 0.2 g
Distilled/deionised water: 100 ml

Preparation

1. Weigh all ingredients into a suitable container.
2. Dissolve/mix ingredients and adjust volume to 100 ml with distilled/deionised water.

Storage

Store at 4 ±2 °C. Use within a month after preparation.

References

Alvarez, M. & Campbell, R. N. (1978). Transmission and distribution of *squash mosaic virus* in seeds of cantaloupe. *Phytopathology*, **68**, 257–263.

Hamilton, R. I. & Nichols, C. (1978). Serological methods for detection of *pea seed-borne mosaic virus* in leaves and seeds of *Pisum sativum*. *Phytopathology* **68**, 539–543.

ICTVdB Management (2006). *Squash mosaic virus*. In: ICTVdB – The Universal Virus Database, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA. (http://ictvdb.bio-mirror.cn/ICTVdB/00.018.0.01.015.htm).

Koenraadt, H. M. S. & Remeeus, P. M. (2009). Proposal for a new method for the detection of *cucumber green mottle mosaic virus* (CGMMV), *melon necrotic spot virus* (MNSV) and *squash mosaic virus* (SqMV) in Cucurbits with DAS ELISA. *ISTA Method Validation Report MVR-02-006*, 64–81.

Lecoq, H., Piquemal, J.-P., Michel, M. J. & Blancard, D. (1988). Virus de la mosaique de la courge: une nouvelle menace pour les cultures de melon en France? [*Squash mosaic virus* (SqMV): a new threat to melon crops in France?]. *PHM Revue Horticole, INRA: Paris*, **289**, 25–30.

Maury, Y., Bossennec, J. M., Boudazin, G., Hampton, R. O., Pietersen, G. & Maguire, J. (1987). Factors influencing ELISA evaluation of transmission of *pea seed-borne mosaic virus* in infected pea seed: seed-group size and seed decortication. *Agronomie* **7**, 225–230.

Nolan, P. A. & Campbell, R. N. (1984). *Squash mosaic virus* detection in individual seeds and seed lots of Cucurbits by Enzyme-Linked Immunosorbent Assay. *Plant Disease* **68**, 971–975.

Powell, C. C. Jr. & Shlegel, D. E. (1970). Factors influencing seed transmission of *squash mosaic virus* in cantaloupe, *Phytopathology* **60**, 1466–1469.

Validation references

ISTA (2015). Proposal for a confirmation method of seed transmitted *squash mosaic virus* (SqMV) on DAS-ELISA positive Cucurbitaceae seeds. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.