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Detection of Cucumber green mottle mosaic virus (CGMMV), Melon necrotic spot virus (MNSV) and Squash mosaic virus (SqMV) in Cucurbit Seed

JUNE 2025

Developed by ISHI

Detection of Cucumber green mottle mosaic virus (CGMMV), Melon necrotic spot virus (MNSV) and Squash mosaic virus (SqMV) in Cucurbit Seed

Crop: Cucumber (*Cucumis sativus*), Melon (*Cucumis melo*), Pumpkin (*Cucurbita maxima* and *Cucurbita moschata*), Squash (*Cucurbita pepo*), Watermelon (*Citrullus lanatus*)

Pathogen(s): Cucumber green mottle mosaic virus (CGMMV, now *Tobamovirus viridimaculae*), Melon necrotic spot virus (MNSV, now *Gammacarmovirus melonis*) and Squash mosaic virus (SqMV, now *Comovirus cucurbitae*).

Version: 2 (June 2025)

PRINCIPLE

Detection of Cucumber green mottle mosaic virus (CGMMV, now *Tobamovirus viridimaculae*), Melon necrotic spot virus (MNSV, now *Gammacarmovirus melonis*) and Squash mosaic virus (SqMV, now *Comovirus cucurbitae*) in seed of cucurbits is done with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The test is complete if no virus is detected, and the seed lot considered healthy. A positive DAS-ELISA is only indicative of the presence of virus particles and can detect both infectious virions and non-infectious virus particles. For SqMV, a positive pre-screen ELISA is followed by a confirmation method based on a grow-out and DAS-ELISA assay. See Figure 1 for the method process workflow for CGMMV, MNSV and Figure 2 for the method process workflow for SqMV.

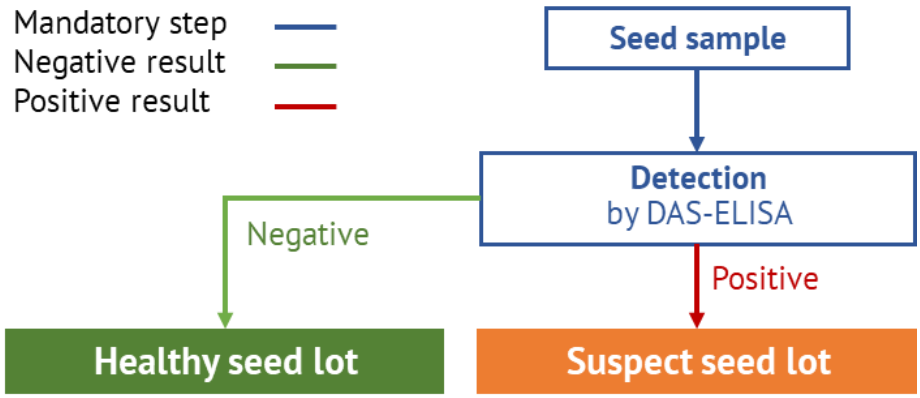

Figure 1. Method process workflow CGMMV, MNSV.

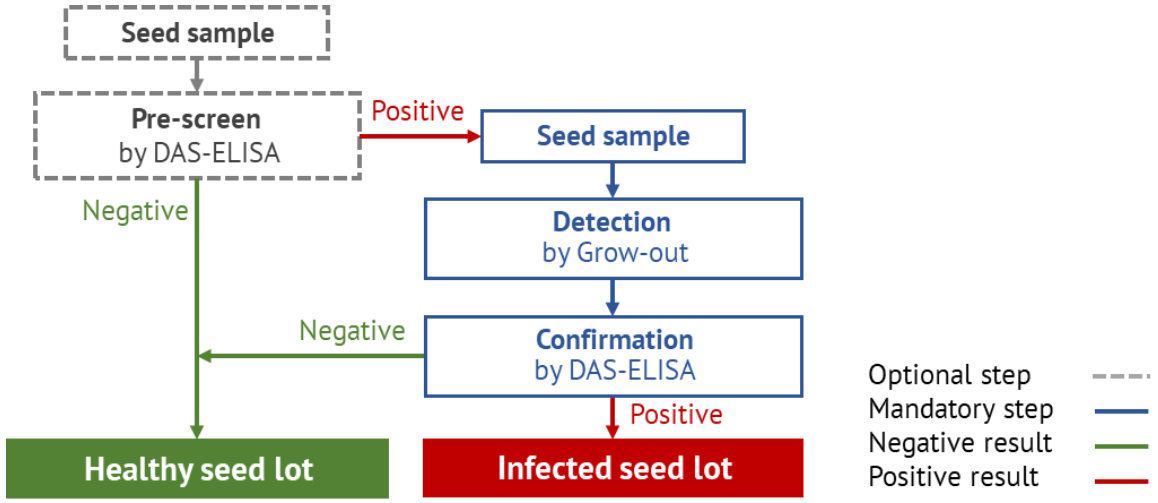

Figure 2. Method process workflow SqMV.

METHOD VALIDATION

Version 1 of this method for detection of SqMV, CGMMV and MNSV in cucurbit seed is an ISTA Rule (7-026). The DAS-ELISA was validated in a comparative test using melon and cucumber seed (Koenraadt and Remeeus, 2009). The grow-out confirmation for SqMV was validated in a comparative test using melon seed (Lybeert *et al.*, 2015).

The DAS-ELISA described in version 2 has been validated according to the ISHI guidelines for the validation of seed health tests using cucumber, melon, squash, pumpkin and watermelon seed (Ettedgui *et al.*, 2025).

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report.

For the pre-screen DAS-ELISA assay:

This test method is suitable for untreated seed.

This test method may be suitable for seed that has been treated using physical (e.g., hot water) or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for inhibition by experimental comparisons or other means.

Although ELISA is compatible with some seed treatment chemicals (Pataky *et al.*, 2004), seed treatments may affect the performance of this test. It is the responsibility of the user to check for such antagonism by analysis, sample spiking, or experimental comparisons.

For the confirmation grow-out method:

This test method is suitable for untreated seeds and for seed that has been treated using physical (e.g., hot water) or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, as well as seed treated with protective chemicals or biological substances.

The confirmation grow-out method is only suitable for SqMV. It has not been validated for MNSV and CGMMV.

It has been validated on melon seed. In principle, all cucurbits can be evaluated with this method, however, the ELISA confirmation assay on plant tissue samples should be evaluated and verified for the various cucurbits being evaluated in the grow-out method before accepting any results.

METHOD EXECUTION

To ensure process standardisation and valid results, it is strongly recommended to follow the Best Practices in Seed Health Tests developed by ISHI.

The ability to detect SqMV could be influenced by variation in environment conditions in the greenhouse. It is recommended not to perform the grow-out during the winter period unless artificial light and heating can compensate for the reduced natural light and temperature.

SAMPLE AND SUBSAMPLE SIZE

For the DAS-ELISA assay on seeds, the recommended minimum sample size is 2,000 seeds with a maximum subsample size of 100 seeds.

For the confirmation method for SqMV in melon, the recommended minimum sample size is 2,000 seeds.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	September 2007	First version of the protocol (ISTA Rule 7-026).
1.1-1.6		See revision history ISTA Rule 7-026
2.0	June 2025	ELISA validation for all crop species mentioned according to the ISHI guidelines for the validation of seed health tests included. Protocol presented in accordance with ISHI protocol guidelines, no longer ISTA Rule 7-026.

Protocol for Detecting Squash mosaic virus (SqMV), Cucumber green mottle mosaic virus (CGMMV) and Melon necrotic spot virus (MNSV) in Cucurbit Seed.

An older version of this protocol is available on ISTA's website: ISTA 7-026: Detection of squash mosaic virus, Cucumber green mottle mosaic virus and Melon necrotic spot virus in cucurbit seed

I. PRE-SCREEN BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

- Coating and conjugated antisera for the target pathogen
- ELISA buffers (Table I.1 to I.5). Note: other buffer compounds can be used according to the antisera supplier recommendations.
- para-nitrophenyl phosphate (pNPP) for substrate solution
- Controls (Table I.6)
- Grinder
- ELISA necessities
- Lab disposables

Table I.1. Coating buffer, pH 9.6.

Compound	Amount/L
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Table I.2. Seed extraction buffer, pH 7.4.

Compound	Amount/L
NaCl	8.0 g
$Na_2HPO_4 \cdot 12H_2O$	14.5 g
KH_2PO_4	1.0 g
Ovalbumin (grade II)	2.0 g
Tween™ 20	10.0 mL
PVP (ELISA grade, mol. wt. 10,000 Da)	20.0 g

Table I.3. Washing buffer PBS/Tween™ 20, pH 7.4.

Compound	Amount/L
NaCl	8.0 g
$Na_2HPO_4 \cdot 12H_2O$	14.5 g
KH_2PO_4	1.0 g
Tween™ 20	1.5 mL

Table I.4. Conjugate buffer, pH 7.4.

Compound	Amount/L
NaCl	8.0 g
$Na_2HPO_4 \cdot 12H_2O$	14.5 g
KH_2PO_4	1.0 g
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

Table I.5. Substrate buffer, pH 9.6**.**

Compound	Amount/L
$C_4H_{11}NO_2$	97 mL
HCl (32 %)	15 mL

Table I.6. Types of controls used.

Control type	Description
Positive process control (PPC)	Cucurbit seed with infectious SqMV, CGMMV and MNSV or standardised reference material (flour of seeds or leaves containing SqMV, CGMMV and MNSV)
Negative process control (NPC)	Cucurbit seed free of SqMV, CGMMV and MNSV
Buffer control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen

1. Coating of ELISA plates

1.1. Add an appropriate dilution of coating serum specific for the tested virus, according to the manufacturer's instructions, to the coating buffer (Table I.1.) to obtain coating solution.

1.2. Add coating solution, as recommended by the manufacturer, to each well in the ELISA plates.

Note: Using different types of microtiter plates may influence sensitivity.

1.3. Cover the plates with a suitable cover or film.

1.4. Incubate plates for 3 hours at 37 ± 2 °C, or as defined by the supplier.

2. Seed extraction

2.1. Divide each sample into subsamples of a maximum of 100 seeds each. Prepare positive and negative controls (Table I.6). Grind each subsample, the negative process control (NPC) and the positive process control (PPC), to fine flour in a grinder.

Note: Be sure to use a grinder that can be cleaned thoroughly, since cross-contamination is likely during the grinding step.

2.2. From each subsample, weigh out 0.5 g of ground seeds and transfer to a suitable container.

2.3. Add 5 mL of extraction buffer (Table I.2) to each container (ratio of 1:10).

Note: Use the same buffers also for commercial controls.

2.4. Mix/vortex thoroughly each container. Allow extract to settle for at least 5 min on the bench to facilitate pipetting.

2.5. After plate incubation (step 1.4), wash the plates at least three times using washing buffer (Table I.3) to remove residues.

2.6. Immediately after washing of the plates, transfer 100 µL seed extract from each subsample (step 2.4) to two wells to create a duplicate.

2.7. Load 100 µL of each control, in duplicate. Use at least two dilutions, high and low for the positive controls.

2.8. Cover the plates with a suitable lid or film and incubate overnight at 4 ± 2 °C, or as defined by the supplier.

3. Incubation of conjugate

3.1. Add an appropriate dilution of conjugate serum of the tested virus, according to the manufacturer's instructions to the conjugate buffer (Table I.4) to obtain conjugate solution.

3.2. Remove the seed extract from ELISA plates and wash at least three times using washing buffer (Table I.3) to remove residues.

3.3. Immediately after washing the plates, add 100 µL of conjugate solution to each well of the ELISA plates.

3.4. Cover the plates with a suitable cover or film and incubate for 3 hours at 37 ± 2 °C, or as defined by the supplier.

4. Addition of substrate to ELISA plates

4.1. Prepare substrate solution by adding 10 mg para-nitrophenyl phosphate (pNPP) to 20 mL of substrate buffer (Table I.5).

4.2. Remove the conjugate solution from ELISA plates and wash at least three times using washing buffer (Table I.3).

4.3. Add 100 µL of substrate solution to each well.

4.4. Incubate in the dark for 1 hour at room temperature, or as defined by the supplier.

Note: Light influences results and exposure to light should be avoided as much as possible.

4.5. Measure the extinction value (A_{405}), optical density (OD), with ELISA plate reader.

Note: The source of antiserum is critical. In the comparative test study, the antiserum supplied by PRIME Diagnostic was used. If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

5. Validity of test results

Test results are only valid when all included controls presented in Table I.6 give the expected results, as defined by the internal laboratory validation or by the supplier. It is recommended to use a negative-positive threshold of two-times the background of healthy negative controls.

A subsample is positive when the extinction value (A_{405}), OD, is equal or above the calculated threshold. A subsample is negative when the extinction value (A_{405}), OD, is lower than the calculated threshold.

An SqMV positive test result should be followed by a grow-out (described in section II) to determine the presence of infectious SqMV.

II. DETECTION BY GROW-OUT AND CONFIRMATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

- Controls (Table II.1)
- Greenhouse capable of maintaining a temperature of 16–35 °C
- Insect-proof installation
- Plastic planting trays
- Potting soil and vermiculite
- Planting tag or equivalent
- Mortar and pestle
- Virus extraction buffer (Table II.2)
- Carborundum powder e.g., 320 mesh grit powder, Fisher Scientific or equivalent.
- Latex gloves, latex finger cots
- Scalpel, cork borer, forceps
- Alkaline soap or equivalent
- 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

Table II.1. Types of controls used.

Control type	Description
Positive control (PC)	Dehydrated melon leaves infected with SqMV (approximately 1 g fresh weight)
Negative process control (NPC)	Cucurbit seed from the same *Cucurbitaceae* sp. being evaluated in the grow-out test known to be free from SqMV

Table II.2. Virus extraction buffer.

Compound	Amount/100 mL
$Na_2HPO_42H_2O$	0.53 g
$(C_2H_5)_2NCS_2Na\ 3H_2O$	0.2 g

1. Seed sowing and greenhouse incubation

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

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

1.3. Sow approximately 10 seeds from the negative process control (NPC, Table II.1) in one separate tray. The grown plants will be used as negative control plants.

1.4. In another separate tray, sow approximately 10 seeds from the NPC. The grown plants will be mechanically inoculated with the Positive Control and serve as positive control plants.

1.5. Continue by sowing the seeds of the sample under evaluation onto the rest of the trays.

1.6. Cover all the seeds with a thin layer of vermiculite and place the trays by keeping adequate space between each other, in a greenhouse with insect-proof installation. This to reduce cross-contamination by touching plants, or virus transmission by beetles.

1.7. Maintain the greenhouse temperature at 24-35 °C during the day and 16-22 °C during the night until seedlings emerge.

1.8. From seedlings emergence to final reading, maintain the greenhouse temperature at 24-35 °C.

2. Mechanical inoculation of positive control plants

2.1. When the first 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.

2.2. Grind in a mortar with a pestle the dehydrated SqMV-infected melon leaves (approximately 1 g fresh weight) into 4 mL of virus extraction buffer (Table II.2).

2.3. Add 0.075 g of carborundum powder and mix well.

Note: It is recommended to place the virus inoculum on ice and proceed with the inoculation procedure as soon as possible.

2.4. Place a drop(s) of the virus inoculum on the surface of both cotyledons and first leaf of each emerged plants and rub it with fingers using latex gloves and/or finger cots. Use enough liquid so that all surface areas are wet. Apply light pressure, to avoid damaging the leaf tissue.

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.

3. SqMV symptoms and collection of plant tissue samples for confirmation DAS-ELISA testing

3.1. Monitor all plants in the greenhouse every 3-5 days upon emergence without handling.

3.2. When most 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.

Typical SqMV symptoms are the systemic mosaic or vein banding in leaves and sometimes the leaf deformation, see Figure II.1. Atypical SqMV symptoms are the discoloration of leaves and spots developing on them (Lecoq *et al.* 1998).

Note: If plants are damped-off or other disease symptoms are present, then the grow-out test should be considered invalid and repeated.

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

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

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.

Note: 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.

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 (10 plant tissue pieces per bag).

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 (one plant tissue piece per bag). For plants with SqMV atypical symptoms repeat the procedure to collect individual plant tissue pieces (one plant tissue piece per bag).

3.7. For the positive control plants, follow the procedure described in 3.5 (pool all 10 plant tissue pieces per bag).

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

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

4.2. Follow the process to coat ELISA plates described above at section I.1 and the supplier's instructions on their incubation conditions.

4.3. Grind all plant tissue samples with a suitable grinding device (e.g., hand homogenizer).

4.4. Add 10 mL of ELISA extraction buffer, as described by the supplier, per gram of plant tissue into each container.

4.5. Proceed with the DAS-ELISA assay as described above at sections I.3 to I.5 to confirm SqMV-positive plant tissue samples.

4.6. Compare results of plant tissue samples from the evaluated sample to results of positive and negative control plants. If positive results are given in the DAS-ELISA assay 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.

4.7. 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.

4.8. Repeat the procedure described in step 3.4 to collect plant tissue samples from control plants and plants from evaluated sample.

4.9. Cut with a cork borer a plant tissue piece from one of the younger leaves of each symptom-free plant in the evaluated sample and make pools of 20 plant tissue pieces maximum in plastic extraction bags.

4.10 Repeat steps 4.1 to 4.5.

4.11 Compare results of plant tissue samples from the evaluated sample to results of positive and negative control plants. 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).

5. Validity of test results

Test results are only valid when all included controls presented in Tables II.1 and I.6 give the expected result.

REFERENCES

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