Method for the Detection of *Lettuce mosaic virus*
on Lettuce Seed and Seedlings

Crop:	*Lactuca sativa*
Pathogen:	*Lettuce mosaic virus* (LMV)
Revision history:	Version 4.3, July 2017

Sample and sub-sample size

The minimum sample size of the seedling assay is 2,000 seedlings and the maximum sub-sample size is 100 seedlings. For the seed assay the minimum sample size is 10,000 seeds and the maximum sub-sample is 500 seeds.

Principle

Lettuce seeds or seedlings (according to the choice of the laboratory) are ground in a buffer solution to extract the virus. The extract is tested using DAS ELISA for the detection of LMV.

Sensitivity and Restrictions on Use

- This test method is suitable for untreated seed.
- This test method is suitable for seed that has been treated using physical processes for disinfestation or seed that has been treated using chemicals for disinfestation provided any residue, if present, does not influence the assay. It is the responsibility of the user to check for such antagonism and/or inhibition by analysis, sample spiking, or experimental comparisons.
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a user chooses to test treated seed using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.
- In the seed assay, one infected seed can be detected in a sub-sample of 500 seeds.
- Comparison of antibodies is recommended (1).
- In the seedling assay, one infected seedling can be detected in sub-sample of 100 seeds.

Method Execution

To ensure process standardization and valid results, it is strongly recommended to follow the best practices developed by ISHI-Veg for *ELISA Assays in Seed Health Tests* (see http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/).

Note: The section **Validation** has been updated. Information on the sections **Sensitivity** and **Restrictions on Use** have been modified and a section on **Method Execution** has been added.

Validation

These methods have been used in the seed industry since 1990 (2, 3, 4, 5 and 6). Validation was done in an ISHI–Veg comparative test (7).

The method has been approved by the US National Seed Health System (NSHS) as a Standard A (see http://seedhealth.org/seed-health-testing-methods/).

Method description

1. Coating of ELISA plates

1.1. Add appropriate (as defined by supplier) dilution of LMV coating serum to coating buffer. Ensure that the antisera are not only suitable for diagnostics but as well for the detection of viruses in extracts of seeds and seedlings.

1.2. Coat plates with 180 µl of coating buffer/well.

1.3. Cover ELISA plates with lid or wrap with plastic wrap to minimise evaporation.

1.4. Incubate ELISA plates overnight with coating buffer at 4 °C.

2. Extraction of virus from seedlings or seeds

2.1. Virus extraction from seedlings

2.1.1. Weigh or count the number of sub-samples needed. For each test use a positive and negative seed lot as a control to validate the results of the test.

2.1.2. Place a filter paper (20 x 14 cm) in the seedling germination box(es) (20 x 15 x 3 cm) (Schleicher & Schuell No. 3014). Alternative sizes of filter paper and box can also be used.

2.1.3. Transfer each sub-sample (100 seeds) onto the pleated filter paper. Spread the seeds throughout the pleat avoiding the edges (ca. 1-2 cm) to allow them to germinate uniformly.

2.1.4. Saturate the filter paper of each seedling germination box with water.

2.1.5. Close the germination box(es) with a transparent lid and incubate for 2 days at 15 °C in darkness.

2.1.6. Add 10 ml of water to each box. Note that the amount of water depends on the type of filter paper used.

2.1.7. Transfer the germination box(es) to an incubator (18-20 °C) with 8 hours of light (ca. 900 lux) per day and incubate the boxes for 4 days.

2.1.8. Remove seedlings from the germination box(es) and transfer them to a tube or other suitable container. Add 1.0 ml extraction buffer and grind each sub-sample using a power driven crusher (e.g. Pollahne, Germany). An alternative extraction device (such as a roller or pneumatic press) could be used if the results are comparable to those obtained using the crusher described above

2.2. Virus extraction from seed

2.2.1. Weigh or count 20 x 500 seeds per sample. For each test use a positive and negative seed lot as a control to validate the results of the test.

2.2.2. Grind the seeds of each sub-sample in at least 5.0 ml extraction buffer using a power driven crusher (e.g. Pollahne, Germany). An alternative extraction device could be used if the results are comparable to those obtained using the crusher described above.

2.2.3. Collect at least 1.5 ml of the seed extract into a tube. Process extracts within 4 hours after crushing. Store extracts at 4 °C if not processed immediately. Centrifugation can be used for clarification of seed extracts.

3. Incubation of extract on ELISA plates

3.1. Remove coating from ELISA plates and rinse them well (e.g. three times) with PBS-Tween to remove residues. Alternatively use a suitable washing device.

3.2. Immediately after rinsing, transfer 180 µl of each seedling or seed extract into a well. Use at least two wells per sub sample.

3.3. Add to each ELISA plate the appropriate positive, negative (healthy seed extract) and buffer controls. For the positive control it is recommended to use at least two dilutions: one 'low' dilution that gives a high extinction (D0 higher than 1.00 after one-hour incubation time) and a 'high' dilution that gives extinction just above the detection threshold (usually D0 around 0.20 after one-hour incubation time).

3.4. Cover plates with a lid or wrap with foil to minimise evaporation and incubate ELISA plates overnight at 4 °C.

4. Incubation of conjugate on ELISA plates

4.1. Prepare the appropriate dilution of LMV-conjugated antiserum using conjugate buffer.

4.2. Remove seed extracts from the ELISA plates and rinse them well (e.g. three times) with washing buffer PBS-Tween to remove residues of the seed extract. Alternatively use a suitable washing device.

4.3. Immediately after rinsing, add 180 µl of diluted conjugate to each well of the ELISA plate.

4.4. Cover plates with a lid or wrap with plastic foil to minimise evaporation and incubate plates for three hours at 37 °C.

5. Addition of substrate to ELISA plates

5.1. Prepare the substrate solution (10 mg para-nitrophenol phosphate in 20 ml of substrate buffer). Use an alternative substrate when the antiserum is conjugated with peroxidase.

5.2. Remove the conjugate from ELISA plates and rinse them thoroughly (e.g. three times by hand) using washing buffer PBS/Tween20 or alternatively use a reliable washing device.

- Note that this step is especially critical for reducing the background.

5.3. Add 180 µl of substrate buffer to each well and incubate for 2 hours at 20 °C. Cover plates with lid or wrap with plastic foil.

5.4. Measure extinction (A_{405}) with spectrophotometer. First check whether the positive and negative controls react according to expectations.

6. Evaluation

For the interpretation of ELISA data different threshold calculations are in use (8). One method to establish a negative cut-off value is 3.25 times the standard deviation plus the healthy sub-samples average. An alternative is to use a threshold of 2 times the average of healthy sub-samples.

Buffers

Coating buffer per liter

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
PH	9.6

PBS Tween buffer per liter

NaCl	8.0 g
KH_2PO_4	1.0 g
Na_2HPO_4 . 12 H_2O	14.5 g
Tween 20	0.5 ml
Deionized water	950 ml*

Extraction buffer

polyvinyl pyrrolidone (PVP 10.000-15.000)	20.0 g
PBS tween	950 ml*
pH	7.4

*Adjust the volume to 1 liter

Conjugation buffer

bovine serum albumin (or ovalbumine)	5.0 g
PBS tween	950 ml*
pH	7.4

*Adjust the volume to 1 liter

Substrate buffer

Diethanolamine	97 ml
Deionized water	850 ml*
pH (adjust with HCl, e.g. 36.8%)	9.8

*Adjust the volume to 1 liter

References

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