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

7-024: Detection of Pea Early-Browning Virus and Pea Seed-borne Mosaic Virus on *Pisum sativum*

Published by the International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2008

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Crop: *Pisum sativum* L.

Pathogen: Pea Early-Browning Virus and Pea Seed-borne Mosaic Virus

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Sponsored by: ISF, ISHI-Vegetables Committee

Revision history: Version 1.0, 1 February 2007.

Background

Pea Early-Browning Virus (PEBV) and Pea Seed-borne Mosaic Virus (PSbMV) are seed-transmissible viruses of the pea, and therefore the detection of these viruses in pea seeds is an important tool in control strategies. The enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark and Adams, 1977), and ELISA methods have been described for the detection of PEBV and PSbMV (Hamilton and Nichols, 1978; Van Vuurde and Maat, 1985, Maury et al., 1987). The test method described here was designed using this information and twenty years of laboratory experience, and evaluated in a comparative test. The method, using ground seed and a DAS-ELISA, can be used to simultaneously detect PSbMV and PEBV in a single extract. Note that the extract is tested in two microtitre plates, one for PEBV and one for PSbMV. The theoretical detection limit is one seed in 100. To ensure a 95% probability that infestations of 0.15% or higher are detected, it is necessary to test 20 subsamples of 100 seeds.

Validation studies

Koenraadt, H.M.S. and Remeeus, P.M. (2007)
Copies are available by e-mail from ista.office@ista.ch, or by mail from the ISTA Secretariat.

Safety Precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of buffers, grinding, autoclaving, and weighing out of ingredients. It is assumed that this procedure is carried out in a microbiological laboratory by persons 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. autoclaving or disinfection) and in accordance with local health, environmental and safety regulations.

Treated Seed

This method has not been validated for the determination of Pea Early-Browning Virus (PEBV) or Pea Seed-borne Mosaic Virus (PSbMV) in treated seed. Seed treatments may affect the performance of this test.
(Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3)

Sample and subsample size

The sample (total number of seeds tested) or subsample size to be tested depends on the desired tolerance standard (maximum acceptable percentage 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: PSbMV- and PEBV-infested seeds or standardized reference material (flour of peas containing PSbMV and/or PEBV).
Microtitre plates: 96-well plates suitable for ELISA (CCP).
Antisera: Suitable for detection of PSbMV- and PEBV-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 peas to fine flour (e.g. Retsch grindomix GM 200).
Incubator: capable of operating at 4 ± 2 °C.
Incubator: capable of operating at 37 ± 2 °C.
ELISA plate reader.
Tubes: 10 mL.

Sample Preparation

This can be done in advance of the assay.

1. It is vital to exclude any possibility of cross-contamination between seed samples, it is therefore essential to clean all surfaces, containers, hands, etc. both before and after handling each sample.

2. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:

   TSW = (weight of seeds/number of seeds) × 1000

4. Based on the estimated TSW, weigh out subsamples of the required size into new, 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 PSbMV- and PEBV-coating serum to coating buffer. 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 180 µl of PSbMV-coating buffer per well. Coat another plate with 180 µl of PEBV-coating buffer per well.
1.3 Cover ELISA plates with lid or wrap with plastic wrap to minimize evaporation.
1.4 Incubate plates overnight at 4 ± 2 °C.
2. Extraction of virus from the seed and incubation of extracts.
2.1 Count or weigh 100 seed subsamples.
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 buffer from ELISA plates and immediately rinse plates three times thoroughly, using PBS/Tween 20 to remove residues. Alternatively, use a suitable washing device (CCP).
2.7 Immediately after rinsing, pipette 180 µl of each seed extract into a well. Use 2 wells per subsample.
2.8 Add positive and negative controls to each ELISA plate. Use at least 2 dilutions for the positive controls: a "low" dilution, which gives a high extinction, and a "high" dilution, which gives an extinction just above the detection threshold (CCP). Negative controls must include a healthy seed extract.
2.9 Cover plates with lid or wrap with plastic wrap to minimize evaporation and incubate overnight at 4 ± 2 °C.
3. Incubation of conjugate
3.1 Prepare appropriate dilution of PSbMV- and PEBV-conjugated antiserum using conjugate buffer as defined by the supplier.
3.2 Remove seed extracts from ELISA plates and rinse plates 3 times with washing buffer PBS/Tween 20 to remove residues of seed extract. Alternatively, use a suitable washing device (CCP).
3.3 Immediately after rinsing, add 180 µl of diluted conjugate to each well of the ELISA plate.

3.4 Cover plates with lid or wrap with plastic wrap to minimize evaporation, and incubate for 3 h at 37 ± 2 °C.
4. Addition of substrate to ELISA plates
4.1 Prepare substrate solution (10 mg *para*-nitrophenol phosphate in 20 mL of substrate buffer).
4.2 Remove conjugate from ELISA plates and rinse thoroughly, either 3 times by hand using washing buffer PBS/Tween 20, or, alternatively, using a reliable washing device (CCP).
4.3 Add 180 µl of substrate solution to each well.
4.4 Incubate for 2 h at 20 ± 2 °C.
4.5 Measure extinction (A_{405}) with ELISA plate reader (see General methods, point 2).

General methods

(common to many test procedures)

1. Grinding of seeds
   Grind each subsample of 100 seeds to 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 should 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 detected and the test method used. When reported on an *ISTA International Seed Analysis 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 in the methods by “CCP”)

– Sensitivity can be influenced by using different types of microtitre plates or materials.
– The quality of antisera from different sources is known to be variable. Therefore ensure that the antisera are suitable not only for diagnostics but also for the detection of viruses in seed extracts. Step 1.1.

– A fine flour will improve the extraction efficacy. Therefore, grind seeds for 20 s at 10 000 r.p.m. to get a fine flour. Note that some knives easily become 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 time that empty microtitre plates sit on bench as much as possible. 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.
– High backgrounds in ELISA are often caused by poor washing of the microtitre plates between the different incubation steps. Washing can be done by hand using PBS/Tween 20 or with a washing device. The thorough washing of microtitre plates is highly 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)
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.
Tween 20: 1.5 mL.
PVP (ELISA grade, e.g. molecular weight 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: 1.5 mL.
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: 8.0g/L.
KH_2PO_4: 1.0 g/L.
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/L.
Tween 20: 1.5 mL.

Preparation of individual buffers

1. Weigh or measure out all ingredients into a suitable container.
2. Adjust volume to 1000 mL with distilled/de-ionized water and dissolve or mix ingredients as appropriate.
3. Check the pH with a pH meter.

Storage of buffers

Store buffers as mentioned above at 4 ± 2 °C for a week.

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