Bulletin OEPP/EPPO Bulletin (2015) **45** (3), 450–453 ISSN 0250-8052. DOI: 10.1111/epp.12260

Diagnostics
Diagnostic

PM 7/126 (1) Electron microscopy in diagnosis of plant viruses

Specific scope

This Standard describes the use of transmission electron microscopy (TEM) for the detection of virus particles in plant sap[1].

Specific approval and amendment

Approved as an EPPO Standard in 2015-09.

1. Introduction

Electron microscopy (EM) can be used for the detection or identification of viruses in tissue extracts of infected plant samples. The principle of detection of plant viruses in sap preparations by transmission electron microscopy (TEM) is based on the adsorption of virus particles onto carbon-coated formvar- or pioloform-supported copper or nickel grids followed by contrasting with a suitable heavy metal solution. This technique is often combined with the use of antisera, referred to as immuno electron microscopy (IEM). The purpose of using an antiserum can be twofold:

(A) to coat EM grids with antibodies for 'trapping' virus particles. This technique is called immunosorbent electron microscopy (ISEM);
(B) to 'decorate' virus particles by attachment of homologous antibodies, resulting in the observation of a visible layer of antibody molecules around the perimeter of the virus particles.

Crude antisera or purified IgGs are suitable for both uses, provided that they are diluted properly.

The preparation of EM grids from plant sap involves simple techniques that can be performed in any plant pest diagnostic laboratory. However, the preparation of carbon-coated formvar- or pioloform-supported grids and the visualization of particles, require specialized facilities and expertise in viewing and evaluating images. Although prepared grids can be stored and shipped for TEM analysis, this technique is not suitable for large-scale testing as samples have to be observed individually and on average a minimum of 5–10 min is required per sample. However, EM can be very useful when no information on virus identity is available. Furthermore, EM may be used in characterization of new viruses and also allows the detection of mixed viral infections.

The most commonly used TEM tests are:

I) Dip preparation for a first screening of virus presence and particle morphology (A);
II) Dip preparation for ISEM and/or decoration for virus enrichment and identification (B, C, D).

Further background information and details not included in this Standard, have been described in various research papers and review articles (Derrick, 1973; Van Regenmortel, 1982; Martelli & Russo, 1984; Milne & Lesemann, 1984; Hampton *et al.*, 1990; Milne, 1993; Hari *et al.*, 2011). Examination of sections of embedded virus infected plant tissue for research purposes is the most commonly known application of EM. As this Standard focuses on use in diagnostics applications, this application is not included.

2. General instructions

An example for the preparation of the grids for different types of EM is presented in Appendix 1. It should be noted that other procedures and variation within procedures exist in the laboratories performing such tests. Due to the direct visualization of virus particles the tests do not require negative and positive controls, however, for identification of viruses e.g. by decoration, the inclusion of a positive control is recommended. As viruses are analyzed by visualization of particle morphology, an observation with EM may allow identification at family level. Identification at species level is only possible when virus particles are 'decorated' by homologous antibodies which have been validated in

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

particular to identify possible cross reactions between related species.

TEM for diagnostic purposes is generally performed as a direct method (Hari *et al.*, 2011). Leaf dip preparations (Hitchborn & Hills, 1965) or clarified crude leaf extracts are placed on top of carbon coated formvar- or pioloform grids and the excess liquid is removed by capillary action after touching the liquid with a filter paper. After washing, the grid is stained with either 0.5–1% ammonium molybdate, phosphotungstic acid or uranyl acetate (Milne, 1993) and dried so that it can be analyzed under high vacuum in the EM.

To improve the attachment of virus particles, grids can be coated by an antiserum prior to incubation with the leaf extract. This 'trapping' of particles results in a selective immune-enrichment of virus particles on the grid (Milne, 1991). Another advantage of the layer of antibodies and other serum constituents is the inhibition of non-specific binding of structures from the sample preparation. The sensitivity of this immunosorbent electron microscopy (ISEM) is generally comparable to that of ELISA. This method requires some knowledge of the sample and which virus(es) is (are) likely to be present.

Following incubation and washing, the grid can either be negatively stained and observed or a secondary antibody (which may be conjugated to gold particles) can be used prior to negative staining. In this case, the sample is incubated with a drop of diluted anti-virus antiserum, so that antibodies can attach to the virus particles (Milne, 1991). Decoration with homologous antibodies (IEM) followed by staining will usually allow virus identification, provided that the analytical specificity of the antibodies is known. When only some virus particles are decorated this may suggest the presence of a mixed infection. Further testing may be performed using an extended selection of antibodies.

The selection of antisera for trapping and decoration depends on the information available on the sample. For samples showing specific virus symptoms, or samples to be tested for a given virus, the appropriate virus-specific antisera should be selected. If no information can be derived based on symptomatology, a range of antisera for 'all' viruses possibly occurring on the plant species may be selected. In this case, it is recommended to prepare a grid without antibodies as a control.

3. Feedback on this standard

If you have any feedback concerning this Standard, please send it to diagnostics@eppo.int.

4. Standard revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press this will also be marked on the website.

5. Acknowledgements

This protocol was originally drafted by: W Jelkmann, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Schwabenheimer Strasse 101, 69221 Dossenheim (Germany). JW Roenhorst, National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen (Netherlands).

6. References

Derrick KS (1973) Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* **56**, 652–653.

Hampton R, Ball E & De Boer S (1990). *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. A Laboratory Manual*, pp. 389. American Phytopathological Society. Press, St Paul, MN.

Hari V, Ghaffar MHA & Undyala V (2011). Fluorescent and ultramicroscopic methods for virus diagnostics and study of virus-plant cell interactions. In: *Virus and Virus-like Disease of Pome and Stone Fruits* (Ed. Hadidi A, Barba M, Candresse T & Jelkmann W), pp. 315–322. APS Press, St. Paul, MN.

Hitchborn JH & Hills GJ (1965) The use of negative staining in the electron microscopic examination of plant viruses in crude extract. *Virology* **27**, 528–540.

Martelli GP & Russo M (1984). Use of thin sectioning for visualization and identification of plant viruses. In: *Methods in Virology*, Vol III (Ed. Maramorosch K & Kaprowski H), pp. 143–216. Academic Press, New York, NY.

Milne RG (1991). Immunoelectron Microscopy for Virus Identification. Chapter 7, In: *Electron Microscopy of Plant Pathogens* (Ed. Mendgen K & Lesemann DE), pp. 87–102. Springer-Verlag, Berlin Heidelberg, New York, NY.

Milne RG (1993). Electron microscopy of in vitro preparations. In: *Diagnosis of Plant Virus Diseases* (Ed. Matthews REF), pp. 215–252. CRC Press, Baton Rouge.

Milne RG & Lesemann DE (1984) Immunosorbent electron microscopy in plant virus studies. *Methods in Virology* **8**, 85–101.

Van Regenmortel MHV (1982). *Serology and Immunochemistry of Plant Viruses*, pp. 302. Academic Press, New York, NY.

Appendix 1

Example of various procedures for (immuno-) preparations for Electron Microscopy (primarily from Q-bank Plant Viruses and Viroids; www.q-bank.eu; other buffers and equipment in use in different laboratories are included as options where possible).

For preparation of samples sterile buffers and instruments (razor blades, tweezers) should be used, thereby using different instruments for each sample.

Buffers and chemicals

PBS (10x)*	81.80 g 1.49 g 2.72 g 14.20 g 800 mL	NaCl KCl KH_2PO_4 $Na_2HPO_4 \times 2H_2O$ Sterile Aqua bidest or demineralised water set pH to 7.4 with NaOH
Uranyl acetate† (UAc) solution, pH 4.9 (freshly prepared or stored in a dark bottle and filtered before use)	0.5–1% (w/v)	In Milli-Q water (or ultra clean water) Note: UAc is highly poisonous and weakly radioactive
Na_2SO_3 solution (fresh)	0.25% (w/v)	In Milli-Q water (or ultra clean water)
Crude antiserum‡		For immuno electron microscopy and decoration [for ISEM IgG fraction (coating) can be used as well]

*For some virus and/or host material other homogenisation buffers are recommended, e.g. addition with 2% (w/v) of Polyvinyl pyrrolidone (MWT 10,000) or 2% (w/v) polyethylene glycol (MWT 10,000) is recommended when working with plants having a high levels of polyphenols. In addition, 2% (w/v) Na_2SO_3 and 0.05% NaN_3 might be added to the homogenisation buffer. If applicable specific information is provided in pest specific diagnostic protocols.

†Other heavy metal solutions used for staining are 0.5–1% (w/v) Ammonium molybdate and 0.5–1% (w/v) Phospotungstic acid. Depending on the virus species and host material either one or the other might give the best results.

‡ELISA dilutions can be taken as initial dilutions.

Equipment and materials

- carbon-coated formvar- or pioloform-supported copper or nickel grids
- filter paper wicks
- glass slides
- parafilm
- plastic petri dishes
- razor blades or other tools to cut small pieces of plant material
- tweezers or glass pistil with bent tips

Procedures

A. Dip preparation for Transmission Electron Microscopy (TEM)

1 Put a droplet Na_2SO_3 solution (or alternative homogenisation buffer) on a glass slide
2 Grind a small piece of plant tissue in the droplet
3 [Optional: Put a droplet of the sample solution on parafilm mounted on a glass petri dish]
4 Dip a grid onto the prepared sample solution (do not submerge, but allow a droplet to lay on top of the carbon-coated side of the grid)
5 Incubate for 1–5 min(s)
6 [Optional: Wash the grid on several droplets of Milli-Q water]
7 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid (prevent the grid from drying)
8 Put a droplet UAc solution (or alternative) on parafilm or plastic petri dish
9 Lay the grid with carbon-coated side on a droplet of the UAc solution*, incubate 1 min
10 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid
11 Let the grid air dry
12 Examine the grid in a Transmission Electron Microscope

*Dispose of UAc waste correctly!

B. Dip preparation for Immunosorbent Electron Microscopy (decoration)

1 Start the procedure as described for A and continue until dipping the grid in the sample solution (Step 1–4)
2 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid (prevent the grid from drying)
3 Wash the grids on several droplets of PBS for 2 min
4 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid (prevent the grid from drying)
5 Incubate grid on a droplet of specific crude antiserum, diluted 1:1000–1:3000 in PBS for 15–60 min [or 'coating' antiserum diluted 1:500–1:1000 in PBS]
6 Wash the grids on several droplets of Milli-Q water (UAc precipitates in the presence of phosphate ions)
7 Continue with staining as described for A (Step 7–12)

C. Dip preparation for Immunosorbent Electron Microscopy (trapping)

1 Put droplets of appropriate solutions on parafilm or petri dish
2 Incubate grid on a droplet of specific crude antiserum, diluted 1:1000–1:3000 in PBS [or 'coating' diluted 1:500–1:1000 in PBS] for 1 h
3 Wash the grid on several droplets of PBS for 2 min
4 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid (prevent the grid from drying)
5 Incubate the grid on a droplet of prepared sample solution for 5–10 min

6 Wash the grids on a several droplets of Milli-Q (UAc precipitates in the presence of phosphate ions) for 2 min
7 Continue with staining as described for A (step 7–12)

D. Dip preparation for Immunosorbent Electron Microscopy (trapping and decoration)

1 Start the procedure as described for C and continue until dipping the grid in the sample solution (Step 1–5)
2 Wash the grid on several droplets of PBS for 2 min
3 Remove excess liquid from the grid carefully with a filter paper wick, only touching the edge of the grid (prevent the grid from drying)
4 Incubate the grid on a droplet of specific crude antiserum, diluted 1:10–1:100 in PBS for 10–15 min
5 Wash the grids on several droplets of Milli-Q (UAc precipitates in the presence of phosphate ions) for 2 min
6 Continue with staining as described for A (steps 7–12)

Note that incubation times on sample and antiserum solutions as well as washing procedures may very between laboratories.

After contrasting and drying the grids are ready for examination in the Electron Microscope. Grids can be stored (e.g. in a grid box in a dry place at room temperature) if not analysed directly.