Schemes for the production of healthy plants for planting
Schémas pour la production de végétaux sains destinés à la plantation

Soil test for virus–vector nematodes in the framework of EPPO Standard PM 4 Schemes for the production of healthy plants for planting of fruit crops, grapevine, *Populus* and *Salix*

Specific scope

This standard describes the sampling and testing of soil in which propagation and certified stocks of the following crops are to be planted: PM 4/8 (*Vitis* spp.), PM 4/9 (*Ribes* spp.), PM 4/10 (*Rubus* spp.), PM 4/11 (*Fragaria x ananassa*), PM 4/16 (*Humulus lupulus*), PM 4/29 (*Prunus avium, Prunus cerasus* and their rootstocks), PM 4/30 (*Prunus armeniaca*, *Prunus domestica*, *Prunus dulcis*, *Prunus persica*, *Prunus salicina* and their rootstocks), PM 4/17 (*Olea europea*), PM 4/32 (*Sambuscus* spp.), PM 4/33 (*Populus* spp. and *Salix* spp.).

Specific approval and amendment

First approved in 2009-09.

Introduction

Schemes for the production of healthy plants for planting of the above crops include testing requirements regarding soil for virus–vector nematodes (nematodes covered in this standard are listed in Table 1). Requirements in these certification schemes differ depending on the plant concerned; three types of requirements exist.

- The soil should be tested and found free from virus–vector nematodes.
- The soil should be tested and found substantially free from virus–vector nematodes or if found these are shown to be free from virus by a slash or bait test.
- The soil should be found substantially free from virus–vector nematodes.

Refer to individual standards to identify which requirement applies to which plants.

Guidance on soil test and on testing viruses in nematodes is given in this Standard.

Soil sampling and extraction of virus–vector nematodes

The methods described here are appropriate for sampling and testing field soil for the presence of longidorid nematodes of the genera *Longidorus, Paralongidorus* and *Xiphinema*, vectors of nepoviruses and sadwaviruses, and for nematode vectors of tobraviruses (i.e. *Trichodorus* and *Paratrichodorus* spp.).

Soil sampling

Soil sampling should normally be performed in spring or autumn when the soil is neither too wet nor too dry, i.e. when there are moist soil conditions. Soil samples should be taken from the top 30–60 cm depth layer, using a semi-cylindrical auger. Because longidorid nematodes are considerably longer than most other plant-parasitic nematode groups (between 3 and 10 mm compared to <1 mm), they are more likely to suffer physical damage during the soil sampling procedures; therefore, corers diameter should be at least 3 cm. A suitable method is to dig out a wedge of soil down to 15 cm with a spade and then, using a hand-trowel, remove a core of soil from the base of the hole, down to at least 30 cm.

The area to be sampled may be subdivided into appropriately-sized smaller areas. In each of these subdivisions, sampling points should be evenly spaced throughout and subsamples should be taken, as described below, in order to provide a sample of at least 500 mL of soil (1500 mL may be preferable when knowledge about production sites indicates that the concentration of nematodes is likely to be low). An example of

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Table 1 Nematodes vectors of viruses and associated virus mentioned in PM 4/8 (*Vitis* spp.), PM 4/9 (*Ribes* spp.), PM 4/10 (*Rubus* spp.), PM 4/11 (*Fragaria x ananassa*), PM 4/16 (*Humulus lupulus*), PM 4/29 (*Prunus avium, Prunus cerasus* and their rootstocks), PM 4/30 (*Prunus armeniaca*, *Prunus domestica*, *Prunus dulcis*, *Prunus persica*, *Prunus salicina* and their rootstocks), PM 4/17 (*Olea europea*), PM 4/32 (*Sambuscus* spp.), PM 4/33 (*Populus* spp. and *Salix* spp.)

Nematode	Associated viruses	PM 4 Standard concerned
Longidorus attenuatus	*Tomato black ring virus (Nepovirus)*	*Vitis* spp., *Rubus* spp., *Fragaria x ananassa*, *Prunus avium, Prunus cerasus* and their rootstocks, *Prunus armeniaca*, *Prunus domestica*, *Prunus dulcis*, *Prunus persica*, *Prunus salicina* and their rootstocks, *Populus* spp.
Longidorus elongatus	*Raspberry ringspot virus (Nepovirus)* *Tomato black ring virus (Nepovirus)*	*Vitis* spp., *Ribes* spp., *Rubus* spp., *Fragaria x ananassa*, *Prunus avium, Prunus cerasus* and their rootstocks, *Prunus armeniaca*, *Prunus domestica*, *Prunus dulcis*, *Prunus persica*, *Prunus salicina* and their rootstocks, *Populus* spp.
Longidorus macrosoma	*Raspberry ringspot virus (Nepovirus)*	*Vitis* spp., *Ribes* spp., *Rubus* spp., *Fragaria x ananassa*, *Prunus avium, Prunus cerasus* and their rootstocks
Paralongidorus maximus	*Raspberry ringspot virus (Nepovirus)*	*Vitis* spp.
Xiphinema diversicaudatum	*Arabis mosaic virus (Nepovirus)* *Cherry leaf roll virus (Nepovirus)** *Strawberry latent ringspot (Sadwavirus)*	*Vitis* spp., *Ribes* spp., *Rubus* spp *Fragaria x ananassa, Humulus lupulus*, *Prunus avium, Prunus cerasus* and their rootstocks, *Prunus armeniaca*, *Prunus domestica*, *Prunus dulcis*, *Prunus persica*, *Prunus salicina* and their rootstocks, *Olea europea*, *Sambuscus* spp., *Populus* spp.
Xiphinema index	*Grapevine fanleaf virus (Nepovirus)*	*Vitis* spp.
Xiphinema italiae	*Grapevine fanleaf virus (Nepovirus)*	*Vitis* spp.
Xiphinema vuittenezi	*Grapevine fanleaf virus (Nepovirus)**	*Vitis* spp.
Xiphinema americanum sensu lato	*Tobacco ringspot virus (Nepovirus)*	*Salix* spp.
Paratrichodorus spp.†	*Tobacco rattle virus (Tobravirus)*	*Populus* spp.
Trichodorus spp.‡	*Tobacco rattle virus (Tobravirus)*	*Populus* spp.

*Although this virus–vector combination is mentioned in the relevant PM 4 standard, vection has not been confirmed.

†It should be noted that the following species of *Paratrychodorus* are recognised vectors of *Tobacco rattle virus (Tobravirus): Paratrichodorus allius, P. anemones, P. christiei, P. nanus, P. pachydermus*, *P. teres.*

‡It should be noted that the following species of *Trichodorus* spp. are vectors of *Tobacco rattle virus (Tobravirus)*: *Trichodorus minor, T. primitivus, T. viruliferus.*

a widely used method is to take samples on a grid pattern (see Fig. 1) over the site with, for example, 20 sub-samples for sites up to 0.2 ha and up to 50–60 for sites between 0.2 and 4 ha. Another possible sampling pattern (more intensive but used in some countries) is to divide the site into units of 0.2 ha and take 60 sub-samples in each of these sample units. Additional samples should be taken from any hedges which surround the site. When the distribution pattern of the nematodes within the field is better known other methods of sampling may be used (see Fig. 1).

The soil should be placed in polythene bags, closed and clearly labelled. Until the samples can be processed, they should be maintained out of direct sunlight and in a cool place (less than 10°C). Care should be taken whilst samples are handled or being moved in transit. Besides being kept cool care should be taken that the samples do not receive any violent treatment (such as being dropped), or stacked for too long on top of one another otherwise the weight might compress and kill the nematodes. Rapid transport to the laboratory is advised. Large stones should be removed from the samples. Soil samples are best placed inside another plastic bag so that a label for the sample can be inserted between the two bags.

Nematode extraction

Samples should be processed as quickly as possible. Each sample should be carefully mixed and crumbled by hand, to break apart the larger clods. Mixing and crumbling of soil should be performed with care if the soil is to be analysed for longidorid nematodes, which may be killed during such activities. Methods for nematode extraction are presented in the Appendix.

Identification of nematodes

For most nematodes identification is required up to species level. This requires that the samples are analysed in a nematology laboratory.

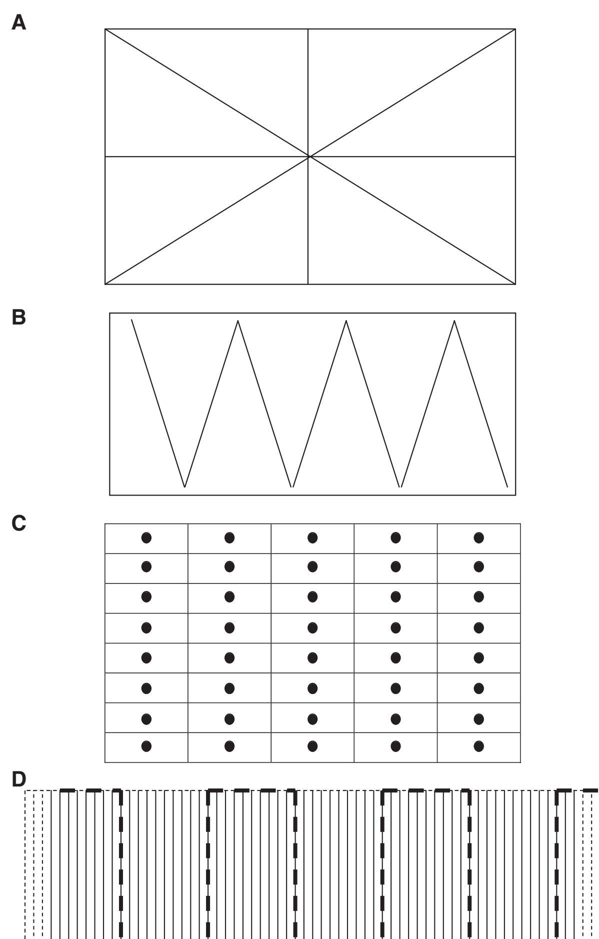

Fig. 1 Examples for sampling soil in bare fields: (A) Sampling along diagonal lines of the field and collection of soil cores following a star scheme; (B) sampling and collection of soil cores in a zigzag, aiming to cover the field homogeneously; (C) sampling and collection of soil cores in a grid; (D) soil sampling following a grid.

Testing nematodes for the presence of viruses

Slash test

The nematodes can be tested directly for the presence of virus by a 'slash test', i.e. breaking up small numbers of nematodes in phosphate buffer (pH 6.9) and inoculating virus-indicator plants with the suspension (Taylor, 1964).

Bait test

Soil bait tests are used as a method of testing soil for nepoviruses often after virus–vector nematodes have been detected. Virus transmission can be tested by growing bait plants in two 600 g sub-samples from a 2 kg soil sample (Taylor & Brown, 1976). The soil sample is placed into a seed tray and virus susceptible plants of *Petunia* sp. and *Cucumis sativus* L. (cucumber) are grown in the soil in a glasshouse at a temperature of 20°C with 12 h additional lighting. The seed trays are placed in shallow trays without holes to allow watering from below. After at least 4 weeks the bait plants are removed and all soil washed off the roots before a sub-sample is ground up in buffer. The exudates are then poured into sample cups and tested by the direct ELISA method for the relevant nepoviruses.

Molecular tests

Alternatively, some viruses can be detected in the nematodes by a PCR test using specific primers. Some publications describing molecular detection of viruses in nematodes are presented in Table 2 (many other publications describing PCR for detection in planta are available, that will be useful for detection in nematodes as well). Nevertheless no method is specifically recommended so far.

References

See also Table 2 for molecular references.

Flegg JJM (1967) Extraction of *Xiphinema* and *Longidorus* spp. from soil by a modification of Cobb's decanting and sieving technique. *Annals of Applied Biology* **60**, 429–437.

Hooper DJ (1986) Extraction of free-living stages from soil. In: *Laboratory Methods for Work with Plant and Soil Nematodes* (Ed. Southey JF), pp. 5–30. Her Majesty's Stationery Office, London (GB).

Seinhorst JW (1955) [A method for the extraction of nematodes from soil]. *Tijdschrift voor Plantenziekten* **61**, 188–190, (in Dutch).

Taylor CE (1964) Transmission. In: *Report of the Scottish Horticultural Research Institute for 1963/1964*, 65 pp. SCRI, Dundee (GB).

Taylor CE & Brown DJF (1976) The geographical distribution of *Xiphinema* and *Longidorus* nematodes in the British Isles and Ireland. *Annals of applied Biology* **84**, 383–402.

Appendix 1 – methods for nematode extraction

Extraction of *Xiphinema* and *Longidorus*

Flegg Modified Cobb technique (as described in Hooper, 1986)

Devised by Flegg (1967) for extracting large Dorylaimid nematodes from soil, the method combines a modified Cobb decanting and sieving method with a Baermann funnel extraction. The final Baermann extraction depends on the ability of active nematodes to move through a fine mesh sieve. Soil type will affect some aspects of this method (e.g. sieving or suspension); adaptation may then be necessary.

Preparing the soil sample and doing the modified Cobb extraction takes about 1 h. The extract has to be left on a Baermann funnel for a further 18–24 h. Collecting the final extract takes 2 min with a further 2 h to let the nematodes settle in order to concentrate the extract. The following equipment is required: a 1-L plastic beaker, three 150 μm aperture sieves and one 4 mm aperture sieve and a 5-L plastic bucket with a diameter such that the 4 mm aperture sieve will just fit into the rim.

The final Baermann funnel extraction requires the following: a 150-mm diameter glass funnel with plastic tubing attached to the

Table 2 Bibliographic references dealing with the molecular detection of viruses within virus–vector nematodes

Viruses	Nematode species	Publications	Test	Crops
Tobacco rattle virus	*Paratrichodorus anemones*	Boutsika K, Phillips MS, MacFarlane SA, Brown DJF, Holeva RC & Blok VC (2004) Molecular diagnostics of some trichodorid nematodes and associated *Tobacco rattle virus. Plant Pathology* **53**, 110–116	RT-PCR (with isolate-specific primers)	No crop mentioned
Grapevine fanleaf virus	*Xiphinema index*	Demangeat G, Komar V, Cornuet P, Esmenjaud D & Fuchs M (2004) Sensitive and reliable detection of grapevine fanleaf virus in a single *Xiphinema index* nematode vector. *Journal of Virological Methods* **122,** 79–86	RT-PCR	Grapevine
Grapevine fanleaf virus	*Xiphinema index*	Esmenjaud D, Abad P, Pinck L & Walter B (1994) Detection of a region of the coat protein gene of Grapevine fanleaf virus by RT-PCR in the nematode vector *Xiphinema index. Plant Disease* **78,** 1087–1090	RT-PCR	Grapevine
Grapevine fanleaf virus	*Xiphinema index*	Finetti-Sialer MM & Ciancio A (2005) Isolate-Specific Detection of *Grapevine fanleaf virus* from *Xiphinema index* through DNA-based molecular probes. *Phytopathology* **95,** 262–268	Real-time RT-PCR	Grapevine
Tobacco rattle virus	*Paratrichodorus pachydermus* and *Trichodorus similis*	Holeva R, Phillips MS, Neilson R, Brown DJF, Young V, Boutsika K & Blok VC (2006) Real-time PCR detection and quantification of vector trichodorid nematodes and *Tobacco rattle virus. Molecular and Cellular Probes* **20,** 203–211	Real-time PCR (to detect at the same time virus and nematode species)	
Strawberry latent virus, Arabis mosaic virus	*Xiphinema diversicaudatum* and *Longidorus macrosoma*	Kulshrestha S, Hallan V, Raikhy G, Adekunle OK, Verma N,. Haq QMR & Zaidi AA (2005) Reverse transcription polymerase chain reaction-based detection of *Arabis mosaic virus* and *Strawberry latent ringspot virus* in vector nematodes. *Current Science* **89,** 1759–1762	RT-PCR	Rose and lily
Tobacco rattle virus	*Trichodorus primitivus*	van der Wilk F, Korsman M & Zoon F (1994) Detection of tobacco rattle virus in nematodes by reverse transcription and polymerase chain reaction. *European Journal of Plant Pathology* **100,** 109–122	RT-PCR	No crop mentioned

stem; a spring burette clip to keep the tubing closed except during release of the sample; a stainless steel stand to hold the funnel; a plastic sieve made from a 125 mm diameter polyethylene ring with nylon mesh of 90 μm aperture.

This technique will handle soil samples of about 200 mL (approximately 200 g of mineral soil). Add about 250 mL of water to the litre beaker. Add the prepared soil sample and soak for about 30 min, stirring intermittently. Fit the burette clip to the Baermann funnel, place the plastic sieve in the funnel and add tap water until it just covers the bottom of the sieve. Ensure that there are no leaks from the funnel. Place the 4 mm aperture sieve on the plastic bucket. Wash the soil/water mixture through the sieve to remove any course debris. Remove the sieve and top up the bucket with water. Stack the bank of three, clean 150 μm aperture sieves in the trough and pre-wet them. Place the edge of the stack on the small piece of rubber tubing so they are tilted towards the operator to allow water to drain away easily. Stir the soil/water mixture in the bucket forcefully by hand (10 turns or more depending on soil type) to suspend the soil. Let the mixture stand for 25 s to allow heavy particles to settle, then quickly and smoothly decant the supernatant through the bank of three 150 μm aperture sieves. Ensure that the sediment bank is not poured through the sieves. Gently wash the residue on the top sieve with a gentle stream of tap water via a flexible hose to wash through any fine particles and foam. Take the top sieve and tilt it 45°. Starting at the apex of the sieve, use the gentle stream of water from the flexible rubber hose to slowly wash the residue to the base. Transfer this residue using a wash bottle to a clean plastic 1 L beaker. For samples with a particularly high organic content, it may be necessary at this point to also gently loosen any debris trapped in the sieve by washing behind whilst keeping the sieve tilted at the same angle. Turn the top sieve over and position it on the bank of sieves. Squeeze the end of the rubber tubing slightly to increase the pressure of the water. Backwash the total area of the top sieve for a few seconds to dislodge any nematodes that have become trapped. Repeat steps this process for the remaining two sieves, but omitting the backwash from the third sieve. Be careful not to overfill the beaker when transferring the solution. Fill the bucket with water again and repeat the process allowing the soil particles to settle for 15 s before decanting into the sieves.

Take the wet plastic sieve from the Baermann funnel to the sink and gently pour the contents of the litre beaker onto the sieve. Wash the beaker and add the washings to the sieve. Carefully replace the plastic sieve in the Baermann funnel and if necessary add more water until the bottom of the sieve and the debris are just covered. After 18–24 h, run about 40 mL of water from the funnel into a suitable container for examination.

Oostenbrink elutriator

A sample of 100 mL or more of moist soil is placed in a 3–4 mm-aperture sieve and then washed into the apparatus via the funnel by means of a nozzle delivering water at about 1000–1500 mL min^{-1}. At the same time water enters the bottom of the apparatus at about 1000 mL min^{-1} through a pipe with a perforated tip, giving an upward current preventing nematodes and fine soil particles entering the bottom of the apparatus, where only heavier particles settle. Washing of sample takes 10–15 min. When the apparatus is almost full, the water with nematodes is drawn off into collection sieves. For collecting *Xiphinema* and *Longidorus* specimens a set of two 20 cm-diameter sieves, with a maximum aperture of 180 µm. The catch from all sieves is placed into a plastic bowl with a little tapwater. The suspension should be decanted onto a nylon sieve of 100 µm aperture which is placed into the extraction dish or a Baermann funnel for at least 20 h. The nematodes in the water can be observed with a stereoscopic microscope (×25).

Extraction *of Trichodorus* spp. and *Paratrichodorus* spp.

For extraction of the smaller Trichodorus spp. and *Paratrichodorus* spp., numerous methods are described (see Hooper, 1986).

The following two-flask technique of Seinhorst (1955) simple but efficient is described below: prepare a subsample of 200 mL, by displacement of water, together with 800 mL of water. Leave the subsample to soak in water for about 1 h, then mix gently. Wash the soil/water mixture through a 4-mm-pore sieve into a large wide-stem funnel fitted with a plug. When all the soil has passed through the sieve, pull out the plug allowing the slurry to run into a wide-neck, 2-L Erlenmeyer (conical) flask. Wash the funnel clean with a little water and top up the flask with water, removing any froth that accumulates. If a flask with standard ground-glass joint, 35-mm diameter, is available the appropriate funnel is used; otherwise a short plastic funnel may be attached with a rubber sleeve. The funnel aperture should be about 12 mm in diameter to obtain a suitable rate of sedimentation/elutriation. With a finger-tip closing the funnel orifice, shake the flask to mix the contents thoroughly and invert it over a similar flask filled with water. The funnel orifice should be just immersed and the finger-tip quickly removed; the soil particles and nematodes then sediment out differentially. After 10 min, the two flasks are separated and the lower one is inverted over a beaker of water for another 10 min. The contents of both flasks are then poured through a set of three sieves of 50-µm pore size. The second stage of the Flegg Modified Cobb technique described above for *Xiphinema* and *Longidorus* is then followed, but using a nylon sieve of 50 µm pore size instead of 100 µm.

Note that *Trichodoridae* can also be extracted with an Oostenbrink elutriator the nematode suspension is then collected with a 40–45 μm aperture sieve.

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Corrigendum

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Since the publication of the EPPO Standard PM 4/35 (1) Soil test for virus–vector nematodes in the framework of EPPO Standard PM 4 Schemes for the production of healthy plants for planting of fruit crops, grapevine, *Populus* and *Salix* (EPPO, 2009) an EPPO Standard on Nematode extraction has been developed and published. Therefore the Appendix 1 which was on methods for nematode extraction should be replaced by a cross reference to the Nematode Extraction Standard (EPPO, 2013).

References

EPPO (2009) PM 4/35 (1) Soil test for virus–vector nematodes in the framework of EPPO Standard PM 4 Schemes for the production of healthy plants for planting of fruit crops, grapevine, *Populus* and *Salix*. *Bulletin OEPP/EPPO Bulletin* **39**, 284–288.

EPPO (2013) PM 7/119 (1) Nematode extraction. *Bulletin OEPP/EPPO Bulletin*, **43**, 471–495.

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