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EPPO STANDARD - DIAGNOSTICS

PM 7/148 (1) Guidelines for the management of nematode collections used for the production and maintenance of reference material

Specific scope: This Standard describes procedures for the management of plant-parasitic nematode collections used for the production and maintenance of reference materials[1].

This Standard should be used in conjunction with PM 7/76 *Use of EPPO Diagnostic Protocols*.

Specific approval and amendment: Approved in 2021–06.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Nematode collections are widely used for taxonomic descriptions and revisions. They are also used for the production of reference material needed for the development and validation of diagnostic tests, interlaboratory comparisons (test performance studies and proficiency tests), equipment calibration and the production of positive controls used in routine testing. Finally, nematode collections can also be used for training and educational purposes.

Reference material is usually produced for quality assurance purposes. 'Reference material' and 'certified reference material' are defined in PM 7/76 (EPPO, 2018a). Only laboratories certified ISO 17034:2016 *General Requirements For The Competence Of Reference Material Producers* (ISO, 2016) can produce certified reference material, but none of the producers of reference material for plant parasitic nematodes are certified against this Standard. Therefore, the information provided in these guidelines has been prepared by non-certified producers of reference material such as research and reference nematology laboratories. These laboratories have established and maintained collections that they use as, or to produce, reference material. They may also provide material for reference, training and research to other laboratories.

[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.

The need for specific guidelines on the management of nematode collections has been identified by the EPPO Panel on Diagnostics in Nematology, based on discussions on requirements for general guidance for nematology collections following the Q-collect Project and a Euphresco funded project 2016-F-186 'Inventory of living collections of cyst and root knot nematodes in Europe and their maintenance techniques (Cyst and Melo Collect)' (den Nijs et al., 2018). It was produced based on the discussions and conclusions of the Workshop on Maintenance of Nematode Collections that was held at NVWA (Wageningen, NL, 2017).

These guidelines cover the most important elements to consider for the management of nematode collections that are used for the production and maintenance of reference material. They also describe several procedures which are being used in diagnostic laboratories within the EPPO region. This Standard aims to provide the information needed either to improve existing collections or to assist diagnostic laboratories that wish to build their own collections. It can also provide guidelines for research laboratories.

2 | THE DIFFERENT TYPES OF COLLECTIONS

Different types of nematode collections can be distinguished according to their end use and maintenance methods: live, dead, nucleic acids and *in silico* collections.

2.1 | Use of terminology in this Standard

In the next sections, a population is considered as a group of individuals of the same species found in a given habitat. For the purpose of this Standard, an isolate is a group of several individuals collected from a population and a specimen is an individual nematode from an isolate or a population. Genomic DNA (gDNA) refers to chromosomal and extra-chromosomal (e.g. mitochondrial) DNA.

2.2 | Collections of live nematodes

When a regular supply of plant parasitic nematodes is required, establishing live collections is essential. Alternatively, when the original infested plant material or soil is not available in sufficient quantities, or the initial level of infestation is low, isolates need to be multiplied to get enough material for identification and other purposes.

Isolates can be maintained and multiplied on suitable host plants or plant parts in greenhouses, growth chambers or micro-plots. Isolates of some nematode species can be maintained *in vitro* on suitable growing media. Live collections can also consist of isolates stored under prescribed conditions in the form of dry cysts, infested materials (soil, growing media, etc.) or plant parts.

The main advantage of live collections is the availability of live isolates that can be further multiplied and used for diagnostic purposes (e.g. as reference, for quality control, development of new identification methods and pathogenicity tests). The main disadvantages are the costs of the infrastructures and staff required to manage such collections and the quite complicated management of overall processes, which needs to be well planned and organized.

Table 1 lists examples of live collections for nematodes on the lists of pests that are recommended for regulation by EPPO and on the EPPO Alert List (in 2021).

2.3 | Collections of dead materials

Dead collections consist of killed and preserved specimens of certain nematode species or groups of species stored on microscopic slides or of plants exhibiting symptoms of nematode infestation.

Collections of slide-mounted nematodes represent an essential and permanent record of the species that occur in a particular country or region and provide a basis for taxonomical studies, for maintaining regulatory control, for developing identification and advisory services, and for educational purposes (Kleynhans, 1999). As permanent slides of killed and fixed specimens can deteriorate after a certain periond of time, they have to be maintained and restored regularly. This should be done by well-trained and experienced laboratory personnel. Together with restoration and maintenance, permanent slide preparation of nematodes is time consuming.

Nematode Collection Europe (NCE) is an online database (see Table 2) of (type) slide collections from

TABLE 1 Examples of live collections for nematodes on the lists of pests that are recommended for regulation by EPPO and on the EPPO Alert List (in 2021)

Regulated nematode species	EPPO list	EPPO code	Type of live collection
Aphelenchoides besseyi	A2	APLOBE	*In vitro* (*Botrytis cinerea Botryotinia fuckeliana*)
Bursaphelenchus xylophilus	A2	BURSXY	*In vitro* (*Botrytis cinerea*/*Botryotinia fuckeliana*)
Ditylenchus dipsaci	A2	DITYDI	Plants (Faba bean)/plant parts (onion, carrot)
Globodera pallida	A2	HETDPA	Plants (potato)/cysts
Globodera rostochiensis	A2	HETDRO	Plants (potato)/cysts
Heterodera glycines	A2	HETDGL	Plants (soybean)
Meloidogyne chitwoodi	A2	MELGCH	Plants (tomato)/plant parts (potato)
Meloidogyne enterolobii	A2	MELGMY	Plants (tomato)
Meloidogyne fallax	A2	MELGFA	Plants (tomato)/plant parts (potato)
Meloidogyne mali	A2	MELGMA	Plants (elm)
Nacobbus aberrans	A1	NACOBA	Plants (tomato)
Radopholus similis	A2	RADOSI	Plants (banana)/plant parts (carrot)
Radopholus similis citrus race	A1	RADOCI	Plants (citrus hybrids)
Xiphinema americanum sensu stricto	A1	XIPHAA	Limited experience in the region so far *Vitis vinifera* is a good host (Cho & Robbin, 1991)
Xiphinema bricolense	A1	XIPHBC	Limited experience in the region so far *Vitis vinifera* is a good host (Graham et al., 1988)
Xiphinema californicum	A1	XIPHCA	Limited experience in the region so far *Vitis vinifera* is a good host (Ferris, 2020)
Xiphinema rivesi	A2	XIPHRI	Limited experience in the region so far *Vitis vinifera* is a good host (Stobbs & Van Schagen, 1996)
Meloidogyne ethiopica	Alert	MELGET	Plants (tomato)
Meloidogyne graminicola	Alert	MELGGC	Plants (rice, *Echinochloa crus-galli*)
Meloidogyne luci	Alert	MELGLC	Plants (tomato)

TABLE 2 List of useful databases for nematologists

Database name and link	Description
EPPO-Q-bank https://qbank.eppo.int/	EPPO-Q-bank is a database on quarantine pests. EPPO-Q-bank comprises sequences data of properly documented species and strains present in collections from which items can be obtained for further studies or used as controls in identification and detection tests. The entries in EPPO-Q-bank are updated by a team of curators with taxonomic, phytosanitary and diagnostic expertise from national plant protection organizations world-wide and institutes with connections to relevant phytosanitary collections.
EPPO Global Database https://gd.eppo.int/	The EPPO Global Database aims to provide all pest-specific information that has been produced or collected by the European and Mediterranean Plant Protection Organization (EPPO). The database is maintained by the EPPO Secretariat and contains: basic information for more than 88,000 species detailed information for more than 1,700 pest species EPPO datasheets, Standards and PRA reports pictures of plants and pests articles of the EPPO reporting service
CABI Crop Protection Compendium (CPC) https://www.cabi.org/cpc	The CABI CPC is an online database that comprises detailed datasheets on pests, diseases, weeds, host crops and natural enemies.
NCBI https://www.ncbi.nlm.nih.gov/	NCBI hosts several databases and bioinformatic tools that are relevant to biotechnology, in particular the Genbank database. This database is not curated so information should be treated with caution.
Nematode Collection Europe (NCE) www.nce.nu	Database of (type) slide collections from Belgium, Germany, the Netherlands and the United Kingdom with a curated nomenclatorial database for Nematoda (excl. animal parasites).
WormBase ParaSite https://parasite.wormbase.org/index.html	A subportal of the WormBase database with over 100 draft genomes of helminths assembled and annotated. It also contains additional information such as protein domains and gene ontology. The data is freely available and the search is supported by different mining tools to select target species. Note that only a small fraction of the information is on plant parasitic nematodes.
BOLD Systems https://www.boldsystems.org/	The Barcode of Life Data System (BOLD) is an online workbench and database that supports the assembly and use of DNA barcode data. It is a collaborative hub for the scientific community and a public resource for citizens at large. End-users have to be aware that this database contains misidentified or not yet published sequences, and sequences from non-vouchered materials.
The NEMAtode-Plant EXpert information system http://Nemaplex.ucdavies.edu	This repository of information on individual nematode species includes information on classification, biology/ecology and management.

Belgium, Germany, the Netherlands and the United Kingdom with a curated nomenclatorial database for Nematoda (excluding animal parasites) and includes an overview of identifications keys.

Plant parts exhibiting damage symptoms of nematode infestation can be stored, dried and pressed in herbariums or in jars in formalin (see details in Section 4.2.6).

2.4 | Nucleic acid collections

Nematode nucleic acid material (e.g. genomic DNA, DNA constructs, whole-genome amplification products) has been increasingly used in research and for diagnostics purposes as an alternative to live nematode material. Nucleic acid material is ready to use. It is prepared from an authenticated source which ensures that the origin of each material is known. The extraction, preparation and quality control is monitored. Gathered in the form of a collection, these materials save on the expense and time needed for culturing live nematodes and can be stored in non-quarantine facilities.

2.5 | *In silico* collections

In silico collections are computer-based repositories of pictures of nematode specimens (usually highlighting morphological features) or of plant disease symptoms caused by plant parasitic nematodes. They can also consist of repositories of genomics, proteomics or metabolomics data. Repositories are now usually in the form of web-based databases, but can also be maintained at the scale of a laboratory. Examples of useful databases in the field of nematode diagnostics are listed in Table 2. The management of *in silico* collections is not covered in these guidelines.

3 | GENERAL REQUIREMENTS FOR THE MANAGEMENT OF NEMATODE COLLECTIONS USED FOR THE PRODUCTION OF REFERENCE MATERIAL

3.1 | Quality management systems

The EPPO Standard PM 7/84 (EPPO, 2018b) describes basic requirements for quality management in plant pest diagnosis. The general management and technical requirements described in this Standard also apply to the management of nematode collections used as (or for the production of) reference material.

3.2 | Minimum quality standards to be achieved for nematological collections used for the production of reference material

Collections should have a reliable inventory of the isolates used for the direct production of reference material with essential associated metadata. The next subsections summarize the metadata that are required, recommended or optional for nematode collections used for the production of reference material.

3.2.1 | General information required to be documented on accessions

Collections should have an inventory of the isolates/specimens used for the production of reference material with essential associated metadata. Traceability of these isolates is essential as the reference material derived is used, for example, for equipment calibration, validation or as material for proficiency tests.

Information to be held	Documentation (on each accession)	Exception
Specimen/isolate scientific name	**Required**	–
Geographic origin of specimen/isolate (at least country or region of origin)	**Required**	–
Geographic place or locality of isolation of specimen/isolate (especially import consignments)	Optional (when known)	–
Host plant from which it was collected or plants associated with	**Required***	–
Plant material, substrate or other source (e.g. commodity) from which it was collected	**Required***	–
Date (at least year) of sampling	**Required**	–
Sampler/collector	Optional	–
Original specimen/isolate number or name given by the original collector/institute	**Required**	–
Reference to accession numbers in other (reference) collections	Recommended	–
Unique accession number in the collection	**Required**	–
Date of deposit in collection	**Required**	–
Preservation conditions (long term) (e.g. liquid nitrogen, −80°C, freeze dried, under oil, slide preparations etc.) and date preserved	Recommended	–
Type of material (nucleic acid, slides, live specimen etc.)	**Required**	–
Intended use and scope of the material	**Required for nucleic acid**	–
Traceable history (over years) of identification (e.g. references, persons)	Recommended	Required for type material
Depositor	**Required**	–
Current quarantine status in the country	**Required**	–
Authors of the current scientific name	Optional	–
Year of publication of scientific name	Optional	–
Links or references to sequence data from the accession	Optional	Recommended for nucleic acid
Images of the accession	Optional	–
Literature references related to the accession	Optional	–
Morphological/morphometric data	Optional	–
Sequence data	Recommended	–

* At least one of these two metadata should be known and is required.

3.2.2 | Identification of a specimen/isolate

Several specimens of a reference isolate (at least 10 specimens, ideally 20) should be identified, preferably with two methods based on different biological principles; morphological, molecular, isozyme or another method. Morphological determination should always be part of the identification process. The date and name of the expert having performed the identification should be stored as metadata.

Issues	Information to be held	Nematode reference collection	Exception
Identification methods*	**Identification performed by, method used and date of identification**	**Required for at least two identification methods**	**-**
	Supporting information for morphological identification	**Required**	**-**
Updating taxonomy	**Information on any update of the taxonomic status of the species (e.g. database, publication)**	**Required**	**-**

* Any specific method should be archived separately.

3.2.3 | Data storage and maintenance

Nematode collections should document procedures on database maintenance and ideally also on data back-up and data-sharing. Staff responsible for data storage and maintenance should be clearly identified, and handling and review of the data should be restricted to them.

In addition to data specific to each accession, data including contact details for all persons responsible for maintaining the collection, contact details for persons donating accessions and contact details for customers to whom accessions are provided or loaned require secure storage (General Data Protection Regulation, GDPR).

Issues	Operating procedures/ competences	Nematode reference collection	Exceptions
Data storage	**Database maintenance**	**Required**	**-**
Data back-up	Data back-up process	Recommended	-
Access to data	Accessibility and visibility of selected data (e.g. via website or paper inventory/ catalogue)	Recommended	-
Contact details	**Contact details for persons responsible for the collection**	**Required**	**-**

3.2.4 | Authentication

The collection should authenticate the material it holds, in particular when it is intended to be used as reference material. For traceability of the chain of accession, it is recommended to keep a record of the movement of the material entering or leaving the collection, including the relevant dates and persons accepting/providing the materials together with the documentation as Material Transfer Agreements (MTA). A standard procedure for labelling or barcoding new accessions with a unique identification number, which is specific to the collection, should be available for use by competent persons approved to authenticate the incoming material. Where appropriate, the label should also indicate the preparation date of the specific batch. Identity should always be checked (see Section 3.2.2) and, where appropriate, verification of purity, viability and/or pathogenicity should also be considered.

In addition to authentication of new accessions, it might be necessary to check batch-to-batch variation when live isolates are maintained. Further checking is required to ensure that material does not become mixed, contaminated or deteriorated, e.g. during storage or when returned to the collection after loan.

Issues	Operating procedures/ competences	Reference collection	Exceptions
Labelling	**Unique number assignment/ barcode labelling**	**Required**	–
Storage facilities	**Where applicable, containment/ isolation measures**	**Required**	–

Issues	Operating procedures/ competences	Reference collection	Exceptions
Purity	**Measures to avoid cross-contamination or mixing**	**Required**	–
Purity check	Method, date of last purity checks and frequency of assessment	**Required**	Not needed for dead material
Chain of accession	Record keeping for movement of accessions in and out of the collection	Recommended	–
Quality control/ comparison with original accession	**Methods to check batch to batch variation**	**Required**	–
	Assessment of quality after storage/ exchange (e.g. after lending the material)	**Required**	–
Viability	Method, date of last viability tests and frequency of assessment	Optional	Not needed for dead material or nucleic acid
Pathogenicity	Method, date of last pathogenicity tests and frequency of assessment	Optional	Not needed for dead material or nucleic acid
Other relevant data	Documentation related to the isolate [MTA, prior informed consent (PIC)/ mutually agreed terms (MAT)]	Recommended	–

3.2.5 | Isolates maintenance and storage

Procedures for maintenance and storage of the materials should be accurately documented. A maintenance plan should be developed for each type of material and should include specific storage conditions and locations. The type and location of all storage facilities and all containment and biosecurity measures for quarantine organisms should be detailed.

Standard operating procedures should be developed to cover specific preservation methods and approaches used to determine short-term and long-term stability of the materials in collection during transport and storage. As an additional security measure, especially for safeguarding live isolates, the duplication of collections at more than one site is possible.

Issues	Operating procedures/ competences	Reference collection	Exceptions
Storage facilities	**Location and maintenance of stores**	**Required**	–
Storage conditions	**Temperature, humidity etc**	**Required**	–
Protection from loss	Duplication of collections	Optional	–
Conservation	Effective conservation methods*	Recommended	–
	Determination of long-term stability	**Required**	–
	Determination of short-term stability (e.g. for transport)	**Required**	–
Containment	**Biosecurity for live quarantine organisms (EPPO PM 3/64)**	**Required**	–

* Methods have been shown to be effective over a known storage period.

3.2.6 | Packaging and dispatching material

Procedures for packing and shipment should be well documented and should conform to national and international shipping and quarantine regulations. Having clear ordering procedures, including customer communication and feedback, and procedures for dealing with non-conforming work (when the activities do not conform to own procedures or customer requirements) are recommended.

Issues	Operating procedures/ competences	Nematode reference collection	Exceptions
Access to the collection	Procedures for ordering or loan of material	Recommended	–
	Packing and transport procedures	**Required**	–
	Customer communications and feedback	Recommended	–
	Customer data	Recommended	–
	Procedures for dealing with nonconforming work	Recommended	–

Issues	Operating procedures/ competences	Nematode reference collection	Exceptions
Legislation	**Adherence to local plant health regulations**	**Required**	–
	Adherence to international quarantine regulations	**Required**	–

4 | SPECIFIC REQUIREMENTS FOR THE MANAGEMENT OF DIFFERENT TYPES OF COLLECTIONS

4.1 | Live collections

4.1.1 | General requirements

4.1.1.1 | Choosing the appropriate host (plant) or growth media

The appropriate host plant, plant parts or growth media should be chosen to maintain and/or multiply the nematode isolates. Good host plants for some nematode species are listed in Table 1 and in the EPPO Global database.

4.1.1.2 | Ensuring optimal culturing conditions of the host plants

- Climate conditions

Climate conditions (e.g. temperature, light and humidity regime) should be controlled and adapted to the nematode species. Regimes might vary depending on where the culturing is performed and the season.

- Water supply

The moisture content of the soil is very important for the plants as well as for the nematodes that are present in the soil. Too much water causes poor growth of the plants, making them vulnerable to plant pathogens. Nematodes cannot move well in soil with an unsuitable moisture content. In too much water they cannot push themselves forward and in too little water they adhere to soil particles and cannot move. It is therefore very important to ensure optimal watering of the plants. Regimes might vary depending on where the culturing is performed.

- Fertilization

The type of fertilizer depends on the plant species, the growth stage and the soil mixture used.

Any green deposit (growth of algae) on the soil surface should be removed to prevent toxicity and to monitor if the soil is drying out. After some time, the pots should be topped up with new soil to prevent green deposit.

- Growing media

The appropriate sanitized growing media should be chosen depending on the nematode species to be maintained or multiplied. If soil is used, it should preferably not contain too much organic matter. Sandy soil is suitable. See Appendices 4–9 for more details.

4.1.1.3 | Preventing contamination

To prevent contamination, a polyethylene sheet should be used to cover the culture table. Screens should be placed between plants with different isolates (to avoid cross-contamination).

In addition, the following recommendations should be followed when handling or pruning the plants (e.g. pruning to prevent the development of flowers or fruit):

- Wear gloves.
- Use a knife for the thicker stems, thinner parts can be pruned by hand.
- Between plants with different isolates change the gloves and disinfect the blade of the knife.
- Always prune in the same direction following the same route as when watering.
- Handle the plants carefully and pay attention to hygiene.

4.1.1.4 | Labelling

During the whole cultivation period, pots or Petri dishes should be appropriately labelled with at least the following information:

- ID number or name of the isolate or specimen
- Genus and species name
- Date of inoculation
- Host plant (when relevant)
- Culture medium (when relevant)

The label should be added immediately after the inoculation and the date of inoculation should be clearly visible. Additional information can also be added on the label.

4.1.1.5 | Pest control

Only non-systemic plant protection products and/or biological agents that are not harmful for nematodes should be used, if needed. To monitor insect pests, yellow and blue sticky traps can be hung above or between the plants in the greenhouse.

In case of *in vitro* culturing, prevention of mites is very important. This can be done by putting the plates on sticky foil or in a layer of water. Using parafilm around the plates alone is not effective, as the mites migrate through tiny holes in the parafilm.

4.1.1.6 | Biohazard/safety procedures

Laboratory procedures should be adequate for the handling of quarantine pests, with particular reference to waste disposal facilities, and should respect the conditions of appropriate licences issued by the NPPO. Quality

control standards should be applied to minimize administrative and other errors, especially concerning labelling and documentation. Laboratory tests may involve the use of, or exposure to, chemicals, biological agents or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed (EPPO, 2018a).

4.1.1.7 | Information to record during cultivation

It is important to keep a record of the following information during maintenance of live isolates:

- Host plant
- Inoculation date
- Inoculum density
- Number of pots
- Location in the greenhouse or growth chamber
- Details that stand out during growth of the plant (e.g. deviation in temperature, use of additional fertilizers)
- Date of the purity check and method
- Date of removal and the reason for disposal

4.1.2 | Specific Standard Operating Procedures (SOPs)

Specific SOPs for the maintenance of different nematode species are described in appendices.

4.1.2.1 | Maintenance and multiplication in vitro

- Appendix 1 Maintenance of nematodes species on carrot discs
- Appendix 2 Maintenance of *Bursaphelenchus* spp. and other Aphelenchina on fungi in Petri dishes
- Appendix 3 Maintenance of *Bursaphelenchus* spp. on barley seeds

4.1.2.2 | Maintenance and multiplication on plants and plant parts (= in vivo)

- Appendix 4 Maintenance of *Ditylenchus dipsaci* on onion
- Appendix 5 Maintenance of *Ditylenchus dipsaci* and *D. gigas* on Faba beans (*Vicia faba*)
- Appendix 6 Maintenance and multiplication of cyst nematodes (Heteroderinea spp.)
- Appendix 7 Maintenance and multiplication of cyst nematodes (*G. rostochiensis*, *G. pallida*)
- Appendix 8 Maintenance of root knot nematodes (*Meloidogyne* spp.)
- Appendix 9 Maintenance of *Meloidogyne* and *Globodera* on potato

4.1.3 | Limiting genetic drift and change in isolates

Materials should be stored appropriately (specific conditions and period).

The identity and purity of all isolates used as reference material in the collection should be checked regularly. When an isolate with a high taxonomic value is found to be non-pure an attempt should be made to purify the isolate (e.g. by checking the identity of individual cysts or *Meloidogyne* females and propagate the related eggs masses or by propagating on a specific crop/variety). It may also be necessary to check the viability of the material before inoculation (e.g. for cyst nematodes).

Assessing the pathogenicity of isolates or cultures is not systematically performed. The pathogenicity of isolates should be checked for reference materials used as positive controls in inoculation studies.

More details on storage and on checking the identity, purity, viability and pathogenicity of isolates are provided in specific SOPs when relevant (see appendices).

4.2 | Dead collections

These collections consist of fixed and preserved nematodes on microscope slides, stored in vials containing glycerol or preserved symptomatic plant material.

4.2.1 | Fixing of live nematode specimens

There is not one method, or combination of methods, which is appropriate for fixing all nematode genera, and the effects on different structures within nematodes can vary. Best results are obtained when nematodes are killed and fixed rapidly at medium temperatures (55–70°C). Boiling fixative should not be used. In addition, live nematodes placed in cold fixative may result in specimens becoming distorted. Protrusion of nematode stylets and male nematode spicules can be induced by the addition of 1 vol of saturated aqueous ammonia (density 0.88 g per mL) in 50 vol of water (i.e. 0.3–0.4 normal ammonia) before fixation.

Detailed procedures for fixing nematodes are presented in Appendix 10.

4.2.2 | Preparation of fixed nematodes for slide mounting or storage

In fixed nematodes, internal body structures, such as gonads, may be obscured by lipids or the intestine. Specimens can be cleared prior to morphological examination by processing to glycerol, which is also suitable as a microscopic slide mountant and as long-term storage liquid.

Well-prepared nematode specimens infused with glycerol will remain in good condition almost indefinitely and their optical quality is better than that of lactophenol-mounted specimens. Processing to glycerol

may take longer. Currently, most permanent slide collections consist of glycerol-mounted nematodes and glycerol methods should preferably be used.

Detailed procedures for preparing nematodes in glycerol are available in Appendix 11.

4.2.3 | Slide mounting nematodes

Nematode specimens can be mounted in glycerol (see Section 4.2.2.) on glass slides with one coverslip, or between two coverslips and secured on an aluminium Cobb slide. The Cobb method summarized below is recommended by numerous nematologists.

- Coverslips should be supported by paraffin wax, with optional use of glass supports providing addional stability. A clean coverslip is placed on the Cobb slide and a heated copper tube is used to produce a wax ring (Figure 1). The thickness of the wax ring may differ depending on the specimens to be slide mounted, i.e. vermiform nematodes, perineal patterns or cyst posteriors.

FIGURE 1 Paraffin wax ring formed on coverslip. (Courtesy: NRC-NPPO, NL.)

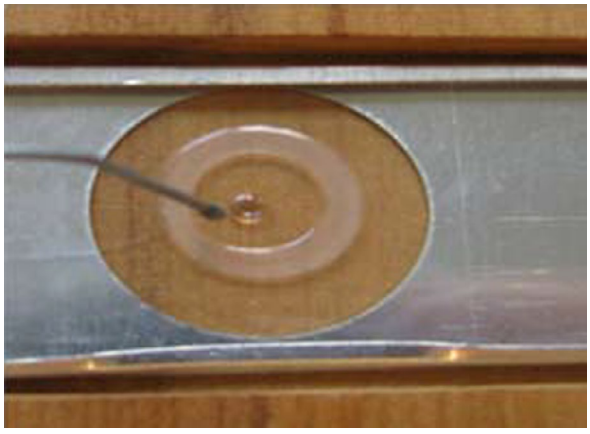


FIGURE 2 A small drop of anhydrous glycerol placed in the centre of the wax ring. (Courtesy: NRC-NPPO, NL.)

- A small drop of anhydrous glycerol is placed in the centre of the wax ring (Figure 2). When used, three glass supports (e.g. appropriately sized glass beads or rods) should be positioned around the edges of the droplet (Figure 3).
- Nematodes (of the same species) are transferred to the glycerol droplet, placed closely together and preferably aligned in the same direction, no more than five nematodes per slide depending on their size. A second, clean coverslip is placed on the wax and the slide heated to ca. 65°C until the wax has melted. The slide is immediately placed on a level surface, at room temperature, to allow the wax to solidify.
- The coverslips should be sealed with either Glyceel (if available), Pertex (the most suitable alternative at present) or another sealant; nail varnish is a cost-effective alternative, but not all available products are suitable for long-term storage.
- The following information should be indicated on the label (Figure 4): unique accession number, specimen/isolate scientific name, number of specimens and lifestages, host plant, collection locality, date of preparation and preservation method. Other required information (see Section 3.2) has to be stored in the management system.

4.2.4 | Slide storage

Microscope slides containing nematodes mounted in an aqueous medium should be stored horizontally in slide cabinets or collection boxes (Figures 5 and 6). Slides are recommended to be stored at an ambient temperature between 18–21°C, ideally at 18°C (Carter & Walker, 1999); damage to slides and specimens stored at inappropriate temperature is most frequently caused by abrupt fluctuations. Direct sunlight can often cause localized hotspots even in cool areas, and uneven temperatures may result in localized condensation and mould formation. Relative humidity (RH) can also adversely affect slide collections. Conditions above 65% RH promote growth of mould and should be avoided as these can cause significant damage to wooden cabinets and specimens. Very low RH (below 25%) can cause brittleness of coverslip sealants and exacerbate evaporation (Carter & Walker, 1999). Slides are recommended to be stored at a RH between 40% and 50%. (G. Karssen, pers. comm., NRC-NPPO, NL). In closed, well-sealed boxes or cabinets, the exposure to fluctuations in humidity are minimized. Silica gel is widely used in museums to control RH in the microenvironments of storage cases/boxes, and this demonstrates its suitability for use in collections. In addition, humidifiers and dehumidifiers can also be used to control RH. Heaters and air conditioning can also be used to prevent temperature fluctuations. Preferably,

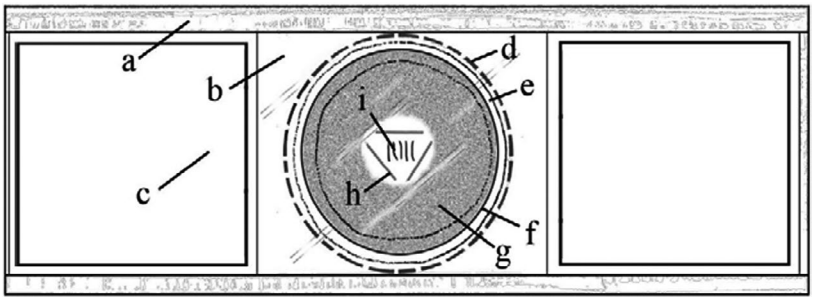


FIGURE 3 Line drawing of Cobb slide mount. (a) Aluminium Cobb slide; (b) square coverslip; (c) label; (d) slide opening; (e) sealant; (f) edge of round coverslip; (g) paraffin wax; (h) glass rod; (i) nematodes. (Courtesy: A. Troccoli, IT.)

FIGURE 4 Labels added and sides of aluminium slide used to secure sealed coverslips. (Courtesy: NRC-NPPO, NL.)

FIGURE 5 WaNeCo type slide collection, stored in wooden boxes and housed in fireproof cabinets at NRC-NPPO, NL. (Courtesy: Fera Science Ltd; GB.)

FIGURE 6 WaNeCo type slide collection, labelled Cobb slides stored horizontally at NRC-NPPO, NL. (Courtesy: Fera Science Ltd, GB.)

nematode collections should be housed in custom-designed storerooms where the environmental conditions can be maintained. This is often not practically or economically feasible, but monitoring allows important environmental fluctuations to be detected before damage occurs.

4.2.5 | Procedures for restoring slide-mounted type specimens

Slides from which the mounting medium has evaporated can be re-infused to restore nematodes as closely as possible to their original state. This method also releases nematodes that have adhered to the slide or coverslip,

allowing specimens to be remounted without incurring further damage.

A binocular microscope is used at all stages to check the progress of the procedure. Great care is needed in handling type material or delicate specimens. Fixed nematodes can be brittle and easily broken; care should be taken not to bend specimens whilst moving them.

On occasion, the glass coverslip or slide can crack; if this occurs, continue to restore the slide. Following re-infusion with glycerol, nematodes can be safely re-mounted. Attempting to remount specimens which have not been re-infused could damage nematodes that have adhered to the slide and/or coverslip. The techniques utilized at Fera Science Ltd (GB) during slide curation are presented in Appendix 12.

4.2.6 | Preservation of symptomatic plant material

Dried and pressed plant specimens, exhibiting damage symptoms of nematode infestation, can be stored in a herbarium (Figure 7). Formalin fixed plant collections (Figures 8 and 9) are suitable for whole plants, root systems, leaf/stem material, bulbs and tubers. Maintenance is necessary due to evaporation of the formalin solution (the plants or plant parts must be completely submerged), discolouration of the formalin solution or any other contamination. Glass vials or jars of varying sizes are widely used in such collections, closed with various types of stoppers or lids. The preferred choice for most curators is ground-glass lids. Plastic and polythene stoppers need to be replaced periodically. Rubber seals disintegrate over time and chemicals from the rubber are known to leach out and turn solutions cloudy. Cork stoppers are cheap but may crumble. A preservation method developed for use at the NRC-NPPO, Wageningen (NL) is presented in Appendix 13.

FIGURE 7 Herbarium specimens exhibiting nematode damage. (Courtesy: Fera Science Ltd, GB.)

FIGURE 8 Formalin fixed plant collection housed in ventilated cabinets in NRC-NPPO, NL. (Courtesy: Fera Science Ltd, GB.)

FIGURE 9 Glass vials with a glass lid and sealed with plastic to reduce evaporation of formalin solution at NRC-NPPO, NL. (Courtesy: Fera Science Ltd, GB.)

4.3 | Nucleic acid collections

Nucleic acid materials are separated into several categories and can be produced and stored in different ways.

The most common nucleic acid categories for nematode material placed in collections are genomic DNA (gDNA), whole-genome amplification (WGA) products and DNA constructs based on nematode target sequences or engineered sequences (e.g. gBlocks).

Depending on the nucleic acid category and its intended use (e.g. to demonstrate the accuracy of diagnostic test results, to calibrate or verify equipment, to monitor laboratory performance, to validate or verify tests, or to enable comparison of tests), different quality descriptors should be assessed during the production and after storage of nucleic acid reference material. Those descriptors are detailed in Section 4.3.4 and were defined based on international standards (e.g. ISO and EPPO Standards), deliverables of a relevant project (i.e. VALITEST project) and the experience of national reference laboratories.

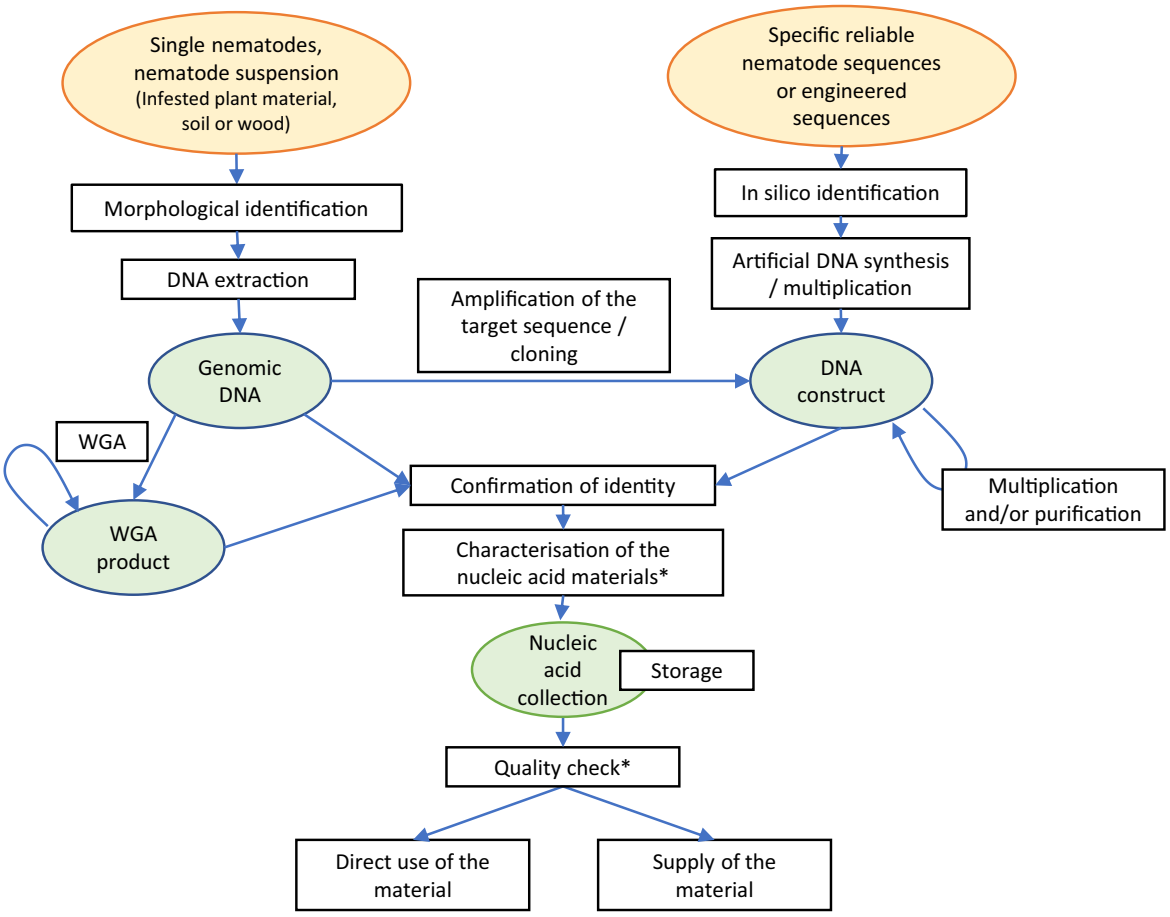
The recommended workflow for the production of nematode reference nucleic acid materials is summarized in Figure 10.

4.3.1 | Production of nucleic acid collection composed of gDNA

4.3.1.1 | Identification and characterization of the material

gDNA reference material is usually extracted from multiple specimen from one isolate (can include different nematode life stages) or from a single specimen derived from non-contaminated nematode suspensions which were extracted from cultures maintained in the laboratory (Ibrahim et al., 1994; Holterman et al., 2006, 2011; Agudelo et al., 2011; Gamel et al., 2014, 2017; Kiewnick et al., 2015). However, depending on the intended use of the reference material, other types of material may be used. For example, for interlaboratory comparisons and validation, gDNA extracted from nematode-infested material (including both target and non-target gDNA) may be used depending on the tests which will be carried out.

Nucleic acid reference material should only be generated from biological material which has been identified as described in Section 3.2.2. gDNA should be extracted



* The extent of the characterisation/quality check depends on the storage method and of the intended use of the material. It may include confirmation of the identity of the material and/or characterisation steps (e.g. homogeneity, purity, stability testing)

FIGURE 10 Workflow for the production and storage of nematode reference nucleic acid materials to be included in collections for nematology diagnostics [Colour figure can be viewed at wileyonlinelibrary.com]

after morphological identification of the specimens or isolates.

4.3.1.2 | Preparation and extraction of the material

The next step in the production of nucleic acid reference material is the extraction of gDNA. The selection of the extraction protocol depends on the intended use of the gDNA, the material from which it is extracted and the required quantity. This step is crucial because it can influence the yield and purity of the nucleic acids. Two types of gDNA extraction methods can be used: one solely based on the lysis of the biological material and one based on the lysis of the biological material followed by purification steps. Extraction kits are commercially available for these two types of extraction methods. Lysis buffers containing Proteinase K, β-mercaptoethanol or DL-Dithiothreitol are recommended for gDNA extraction. They effectively degrade proteins of the cuticle and other structures of the nematode, thus releasing free gDNA for subsequent PCR tests, without any further purification or PCR inhibitor removal steps. However, these extraction methods can leave traces of inhibitors that may impair downstream analysis. To get rid of these inhibitors, a purification step (e.g. silica magnetic beads or purification columns) can be used. Any DNA extraction protocol can be used as long as its performance has been demonstrated to be suitable for the intended use (see Table 3; detailed in Braun-Kiewnick & Kiewnick, 2018).

After the extraction of gDNA, the identity of the nucleic acid should be confirmed with a species-specific test (e.g. real-time PCR or sequencing analysis using, for example, Sanger or Illumina sequencing platforms).

4.3.2 | Production of nucleic acid collection composed of WGA materials

Ideally, the reference material in the collection should be indefinitely available. However, the amount of gDNA extracts can be limited. Whole-genome amplification (WGA) allows this limitation to be overcome by generating the copy of an extract from a small amount of gDNA. Therefore, this method may be used to ensure that a constant source of material is available for test validation, routine diagnostics (e.g. first-line controls) and research. Details on WGA technology can be found in Detter et al. (2002) and Nelson et al. (2002).

The starting material to generate WGA reference material is a gDNA extract that matches the requirements detailed in Section 4.3.1 or a previously amplified WGA. Four WGA kits were extensively tested in the EUPHRESCO project Q-AMP 'Development of Validated procedures for whole genome amplification and storage of DNA and RNA'. The Illustra GenomiPhi V2 DNA amplification kit from GE Healthcare was recommended as the best amplification kit overall, when following manufacturers' instructions (Bonants et al., 2011). To guarantee reproducibility and optimal coverage, WGA reaction should be performed in triplicate and subsequently pooled into a single sample. In addition, a different kind of bias can be introduced in the sequences of the WGA products (Sabina & Leamon, 2015).

4.3.3 | Production of nucleic acid collection composed of DNA constructs

DNA constructs can be made available in the form of plasmids or as linear double-stranded DNA fragments.

TABLE 3 Examples of DNA extraction protocols using commercial kits or lysis buffers

Biological material	Extraction protocol	Purification step included	Reference
Single specimen	Lysis buffer	No	Holterman et al. (2006)*
Single specimen	NaOH	No	Holterman et al. (2011)*
Single specimen	Nematode DNA extraction kit for individual nematodes and single cysts (ClearDetections)	No	EPPO (2016) (under revision) - PM 7/129*
Single specimen	GenElute Mammalian DNA Extraction Kit (Sigma-Aldrich)	Yes	Holterman et al. (2011)*
Nematode suspension	Lysis buffer	No	Ibrahim et al. (1994)
Nematode suspension	QIAamp DNA Mini Kit and Lysis Buffer (Qiagen)	Yes	EPPO (2013b) (under revision), PM 7/04 (Appendix 4) Gamel et al. (2014)*
Infested soil	Wizard® Magnetic DNA Purification System for Food (Promega)	Yes	Ollivier and Anthoine (2010)
Infested soil	Soil DNA extraction kit, (Qiagen, formerly Mo-Bio Laboratories Inc., USA)	Yes	EPPO (2009), PM 7/05 (Appendix 2)
Infested potato tuber peelings	Sbeadex maxi plant kit (LGC Genomics)	Yes	EPPO (2016), PM 7/41
Insects	QIAamp DNA mini kit +Protease (Qiagen)	Yes	EPPO (2013) (under revision), PM 7/04

Most of the protocols listed were used for the validation of nematode diagnostic tests (*) or to produce the QBOL collection of reference DNA material.

They can be prepared internally by PCR amplification of target sequences using a gDNA extract obtained from a morphologically identified specimen or isolate, or they can be synthetized by a specialized company. In the latter case, the sequences used for the synthesis of the construct should be reliable specific nematode genomic sequences obtained from databases [e.g. EPPO-Q-bank, Barcode of Life DataSystems (BOLD) or NCBI-Genbank] (Id et al., 2019; Leray et al., 2019) or sequences engineered in a laboratory [e.g. gBlocks, see EPPO, 2021]. For diagnostics in nematology, DNA constructions are mostly used as positive-control templates for molecular tests.

4.3.3.1 | Identification of the material

The sequences constituting the DNA constructs should go through appropriate identification steps. A BLAST should be performed on *in silico* reliable sequences in a database. Afterwards, the sequence of the DNA construct should be confirmed by sequencing.

4.3.3.2 | Preparation and extraction of the material

DNA constructs can be prepared and supplied as lyophilized powder/pellets or diluted in nuclease-free water or Tris-EDTA (TE) buffer. Because pure synthetic DNA constructs lack natural carriers, i.e. the normal cellular debris found in gDNA extracts, they occasionally adhere to the wall of plastic tubes (even to DNA LoBind tubes), which may result in a decrease in measured concentrations. To prevent this binding, synthetic DNA constructs should be diluted or resuspended in molecular-grade water or TE buffer containing 0.1–1.0 mg/mL yeast tRNA.

After appropriate resuspension or dilution, both types of DNA constructs can be further multiplied and purified:

- Linear DNA fragments may be amplified by PCR and further purified using specific PCR clean-up commercial kits (e.g. QIAquick PCR Purification Kit).
- Plasmid constructions may be multiplied in *Escherichia coli* and subsequently extracted and purified using different commercial kits (e.g. Qiagen Plasmid Midi Kit system).

4.3.4 | Characterization of the nucleic acid collection items

The characterization of the nucleic acid items deposited in collections should be performed to ensure they are appropriate for their intended use.

4.3.4.1 | Quantification and purity

The concentrations of nucleic acid materials may vary depending on their category as well as the extraction method and biological material used for their extraction. Aliquots of each batch of nucleic acid should be quantified with accurate methods. In addition, depending on their intended use, the purity, size and integrity of the nucleic acid material may be characterized.

The two techniques used for nucleic acid quantification are UV spectrophotometric quantification and fluorescence-based quantification (Manchester, 1996). Different methods are based on those techniques, which differ in accuracy and are not directly comparable.

Spectrophotometric methods (e.g. nanodrop) quantify the nucleic acid solution and also give an estimation of its purity based on absorbance values. Although these methods are commonly used, they can be unreliable and inaccurate because absorption values are easily affected by other components (e.g. background noise due to different elution/storage buffers could not completely cancelled out).

Fluorescence-based quantification methods (e.g. Qubit, real-time PCR, digital PCR) provide higher specificity and accuracy as they are less affected by other components present in the samples, making them optimal for quantitation of low-level nucleic acid samples.

Real-time PCR is one of the most sensitive methods enabling confirmation of intact DNA and absolute quantification of the DNA template: even picogram quantities of nucleic acid can be measured. Additionally, it can accurately quantify specific nucleic acids, even in the presence of contaminants. Nucleic acid concentration is estimated by comparing the cycle threshold (Ct) value of an item to the Ct value of samples of known concentration. This method is particularly suited for WGA products generated from infested material.

4.3.4.2 | Homogeneity and stability

The assessment of nucleic acid homogeneity should be performed before and after storage to ensure that storage does not affect homogeneity. However, a homogeneity test after storage is not needed when the effect of storage on the homogeneity of a sample is known to be negligible.

Nucleic acids should be sufficiently stable for their intended use. The influence of storage and, when applicable, transport conditions on the stability of the nucleic acid solutions should be evaluated.

Further information regarding the assessment of homogeneity and stability for nucleic acid materials is provided in EPPO PM 7/122(1) (EPPO, 2014).

4.3.5 | Storage

4.3.5.1 | General requirements

Nucleic acid collections are prone to degradation when stored incorrectly. They are especially sensitive to depurination, hydrolytic cleavage, nuclease contamination and oxidation. Therefore, selecting the correct storage devices, conditions (e.g. temperatures, humidity) and

consumables is essential for preserving the integrity, purity, homogeneity and stability of these collections. Regarding consumables, nuclease-free products (i.e. plastic tubes and buffers) should be used. Some plastic materials (e.g. LoBind tubes) limit DNA cleavage and some tube types (e.g. screw-cap tubes) limit evaporation and should preferentially be used to store nucleic acids.

4.3.5.2 | Low temperature storage

Regarding the temperature, the lower the temperature, the higher the nucleic acid stability. For long-term storage in collections, nucleic acid items should be stored at −80°C in Tris-EDTA or in nuclease-free water. Samples may be aliquoted to avoid multiple freeze-thaw cycles and degradation of the reference nucleic acids.

4.3.5.3 | Dry-state storage

Nucleic acid material can be stored using dry-state storage technologies. Dry-state storage can be done at room temperature. An anhydrous atmosphere (low humidity environment) is required for dried DNA storage to prevent DNA hydrolysis (Kansagara et al., 2008).

For instance, Flinders Technology Associates (FTA) was shown to be fully functional for sampling, downstream molecular research and diagnostic testing, long-term storage and maintaining DNA integrity/stability under transport conditions for the plant pest nematode species *D. dipsaci*, *H. schachtii*, and *M. hapla* (Marek et al., 2014; Peng et al., 2017). Other methods may be used for dry-state storage, such as DNA Lateral Flow Device (LFD), GenTegra™ Sample Matrix Tubes and lyophilization.

5 | FEEDBACK ON THIS STANDARD

If you have any feedback concerning this Diagnostic Standard please contact diagnostics@eppo.int.

6 | STANDARD REVISION

An annual review process is in place to identify the need for revision of Diagnostic Standards. Standards 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.

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APPENDIX 1 - PROTOCOL FOR CULTURING PLANT PARASITIC NEMATODE SPECIES ON CARROT DISCS

1. Scope

Some plant parasitic nematodes, such as *Pratylenchus*, *Radopholus*, *Aphelenchoides* and *Ditylenchus*, can be cultured on carrot discs. Several laboratories have successfully maintained isolates by using this protocol for the culturing of species of these nematodes. Carrot discs can be inoculated using two different methods. In this protocol, carrot discs are inoculated by putting them on top of a droplet of sterile water containing nematodes. In other protocols, the carrot discs are first prepared in Petri dishes and inoculated with a drop of nematode suspension later on (O'Bannin & Taylor, 1968; Coyne et al., 2014). This appendix is based on SOPs from CREA and CNR (IT), INIAV (PT), NRC-NPPO (NL) and the University of Évora (PT).

2. Materials

- Laminar flow hood
- Bunsen burner or equivalent
- Stereomicroscope
- Sterile pipette
- Picking needle
- Paring knife
- Wooden cutting board
- Counting dish, cleaned with 70% ethanol
- Sterile Petri dishes Ø 5 cm
- Parafilm
- Carrots with foliage (at least 4, with a minimum diameter of 2 cm)
- Plastic containers with transparent lids
- Tissues
- Weighing paper
- Sterile falcon tube (15 mL) (= plastic centrifuge tube with conical bottom)
- Glass jar (100 mL)
- Mistifier
- Sieve tray with filters for mistifier
- 1% chlorine solution
- Ethanol 70%
- Sterilized tap water
- Streptomycin sulfate solution 0.1%
- 50 mg/L carbenicillin
- 50 mg/L kanamycin
- Miracloth filter
- Small sieve with large mesh aperture (1–2 mm)
- Beaker
- Centrifuge
- Erlenmeyer flasks

3. Procedures

3.1. Preparation

When using an existing carrot disc culture as a nematode source, determine whether the nematode isolate should be disinfected by checking visually whether it is contaminated with bacteria or fungi (see Section 3.3) to reduce the chance of contamination of the new carrot slices. Nematodes retrieved from infested plant material or from soil should always be surface sterilized.

Select carrots that are as fresh as possible (without surface cracks or symptoms of nematode infestations) and with the green foliage still attached. Carrots with a large diameter (max. 4 cm) are most suitable. Remove the foliage and scrub the carrots with water to remove soil and debris, dry them with tissues afterwards. A minimum of 4 carrots should be selected so that slices of 4 different carrots can be used per nematode isolate, to reduce the risk of isolate loss. An extra carrot can be used if needed to ensure adequate number of slices. The carrot discs need to be at least 2 cm in diameter and 0.5 cm thick..

3.2. Extraction of nematodes from existing carrot disc culture

Several options are possible, depending on the facilities in the laboratory. Preferably all steps should be performed under sterile conditions. This is particularly important if you decide not to disinfect the nematodes before inoculating them on new carrot discs.

A. Direct removal by washing

1. Rinse the lid of the Petri dish with sterilized tap water to collect the nematodes from the lid and/or add a few droplets of sterilized tap water onto the previous carrot disc culture.
2. Pour or pipette the suspension with nematodes into a clean glass jar or counting dish.

B. Mistifier

1. Use 1 or more carrot discs for each nematode isolate. Cut the carrot disc(s) into pieces and place them on top of the filter in the sieve tray of the mistifier.
2. Turn the mistifier on for at least one night. The extraction time can be extended if more nematodes are needed.
3. Collect the suspension in a glass jar or counting dish.

C. Centrifuge

1. Cut the carrot disc into small pieces or thin slices and place the carrot fragments on a miracloth filter on a small sieve with large mesh aperture (1–2 mm).
2. Place the sieve in a beaker and fill with sterilized tap water until carrots discs are completely immersed.
3. Let nematodes migrate into the water for 24–48 h.
4. Pipette nematode suspension into 15 mL falcon tubes and centrifuge at 2500 rpm for 2–5 min as many times as necessary to collect all nematodes.

D. Inverted Erlenmeyer flasks system

1. Cut previously inoculated carrot discs (4–6 weeks old) into tiny pieces with a sterilized blade.

2. Place the carrot pieces in an Erlenmeyer flask and add sterilized tap water containing 50 mg/L carbenicillin and 50 mg/L kanamycin until the carrots are submerged, then put a sterilized miracloth around the mouth top and secure with sterilized rubber bands.
3. Invert the Erlenmeyer with carrots inside another Erlenmeyer (bigger mouth top diameter), also with sterilized tap water containing 50 mg/L carbenicillin and 50 mg/L kanamycin, so that the mouth top of the Erlenmeyer with carrots touches the water.
4. Leave inverted Erlenmeyer system at room temperature for 24 h in order for the nematodes to migrate into the water.
5. Pipette the nematode suspension into 15 mL falcon tubes and centrifuge at 2500 rpm for 2–5 min as many times as necessary to collect all the nematodes.

3.3. Surface sterilization of nematodes

Sterilizing (Figure 11) the external cuticle of nematodes is necessary when bacteria or fungi are present in the nematode suspension or on the nematodes themselves. A streptomycin sulfate solution (0.1%) can be used as disinfectant (Speijer & De Waele, 1997). Some higher concentrations (up to twice) of streptomycin sulfate solution can be used if necessary (Coyne et al., 2014).

1. Pour the nematode suspension into a sterile falcon tube (15 mL). Allow the suspension to settle for at least 30 min until a pellet with nematodes is formed at the bottom of the tube (Figure 11A).
2. Use a sterile pipette to discard the water, stopping just above the pellet (Figure 11B).
3. Add 10 mL of 0.1% streptomycin sulfate solution and shake the tube to homogenize the suspension (Figure 11C).
4. Leave the suspension overnight at room temperature.
5. The nematodes should then be washed with sterilized tap water to remove the streptomycin sulfate solution (Figure 11D):
   a. Use a sterile pipette to discard the solution, stopping just above the pellet (Figure 11Da).
   b. Add 1 mL of sterilized tap water and shake to homogenize the suspension (Figure 11Db).
   c. Wait at least 15 min to allow the nematodes to form a pellet at the bottom of the tube.
   d. Use a sterile pipette to discard 1 mL of the solution from the tube (Figure 11Dc).
   e. Check the extracted liquid for the presence of nematodes.
   f. Repeat this washing procedure 3 times.

3.4. Inoculation

Preferably work in a laminar flow hood, but if not possible work as cleanly as possible and open the Petri dishes as little as possible. In either case, inoculation should not be done in an area in which plant material is handled/stored in order to avoid mite contamination.

1. Clean a counting dish and picking needle with 70% ethanol.
2. Pour the nematode suspension into the counting dish.
3. Prepare at least three Petri dishes per isolate.
4. Pipette with a sterile pipette a small droplet of sterilized tap water onto the middle of each Petri dish (Figure 14a).

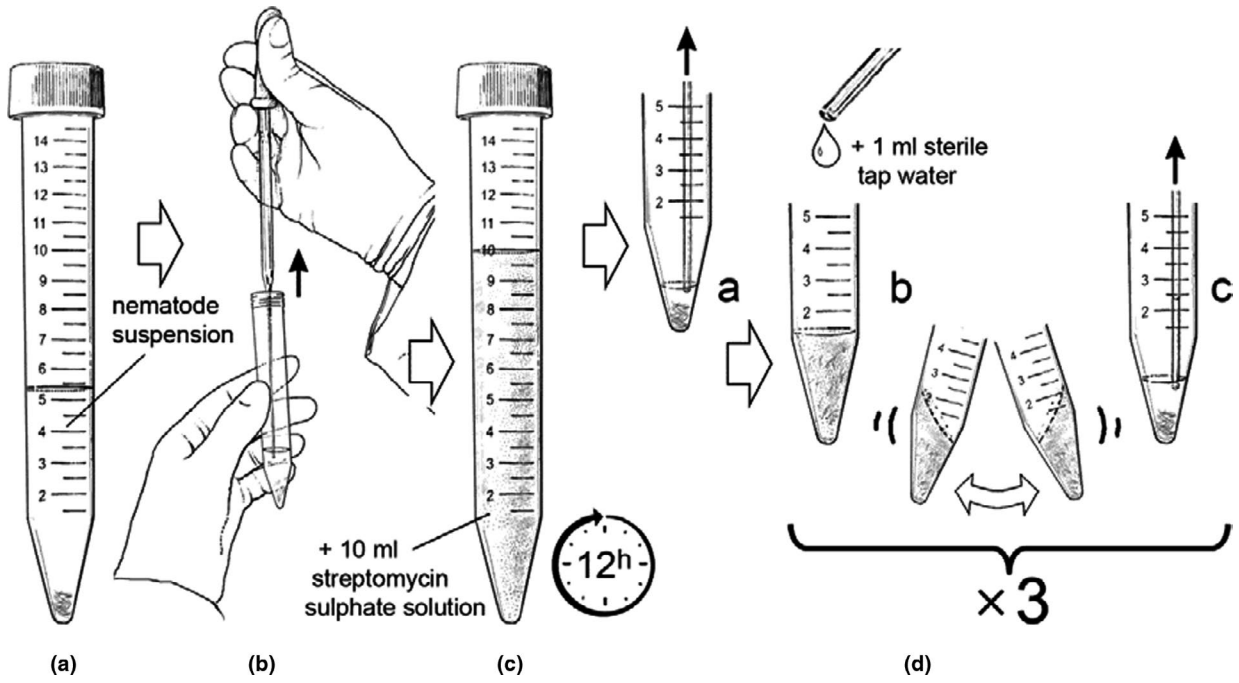

FIGURE 11 Sterilization of nematodes prior to inoculation. See the text for explanation. (Courtesy: A. Troccoli, CNR-IPSP, IT.)

5. Under the stereomicroscope pick or pipette about 20 specimens (a mixture of males, females and juveniles) out of the suspension and place them in the small droplet of sterilized tap water in the Petri dish.
6. Close the lid of the Petri dish directly afterwards and place all repeats per isolate among each other on a tray, same repeat numbers of all isolates per row (Figure 12). Add the carrot discs within 1–2 h.

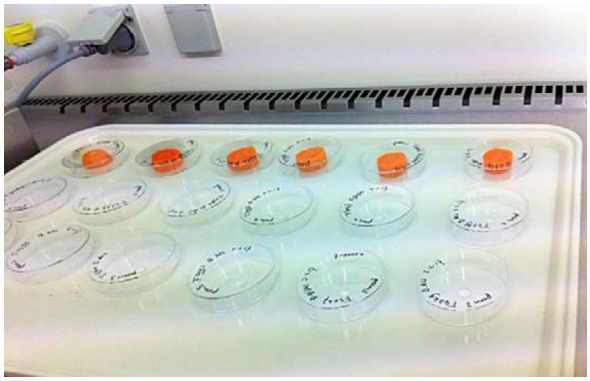

FIGURE 12 Sterile Petri dishes with a droplet of inoculum, with information written on the lid. (Courtesy: NRC-NPPO, NL.)

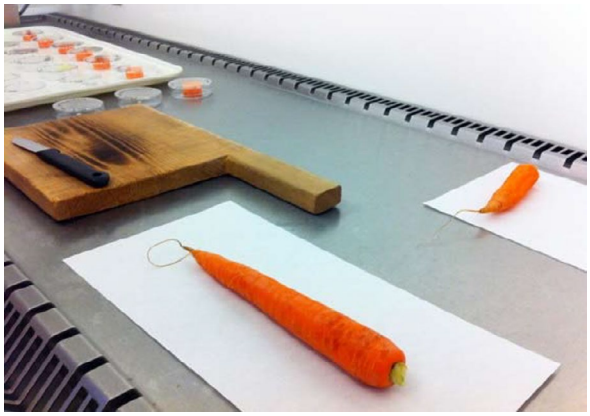

FIGURE 13 Workstation with wooden cutting board and weighing papers with carrots. (Courtesy: NRC-NPPO, NL.)

3.5. Preparing carrot discs

Inoculate at least 3 carrot discs per isolate, derived from different carrots. Use one carrot disc per used carrot as a negative control. For the negative controls, add only a small droplet of sterilized tap water onto the bottom of a sterile petri dish using a sterile pipette.

1. Work in a laminar flow hood under sterile conditions and wear sterile gloves.
2. Flame the surface of a wooden cutting board and a weighing paper to sterilize the surface. Put the paper on top of the cutting board.
3. Flame shortly the surface of two more weighing papers to use one as a clean work spot in the laminar flow hood and the other one to dry the carrot later on.
4. Sterilize the paring knife and the carrot by soaking them in a 1% chlorine solution for 1 min or by spraying them with alcohol (70%) and flaming.
5. Use a surface sterilized weighing paper to dry the carrot and the paring knife.
6. Peel the carrot (thickness: a few millimeters of peel) from the top down, with the growing point of the foliage at the top. Drop the peels on top of a weighing paper to keep the laminar flow hood as clean as possible.
7. Put the peeled carrot on top of the weighing paper (Figure 13) on the cutting board.
8. Cut and remove the top of the carrot with the growth point of the foliage. This part of the carrot will not be used.
9. Cut the carrot into discs, with a thickness between 0.5 and 1 cm, depending on the height of the Petri dishes used. The discs should be 2 cm or more in diameter (Figure 13).
10. Put the carrot disc with the bottom side down on the droplet with nematodes in the Petri dish (Figure 14a). Alternatively, a droplet with the inoculum of nematodes (about 20–40 nem./drop) can be placed directly onto the margin of the carrot disc (not in the centre) (O'Bannin & Taylor, 1968; Coyne et al., 2014) (Figure 14b).
11. Close the Petri dish. Seal the sides with parafilm to prevent contamination and dehydration.
12. Repeat steps 4–11 for the remaining carrots.
13. Put the Petri dishes inoculated with the same nematode isolate in a plastic container (each box with the same isolate) with a transparent lid (Figure 15)

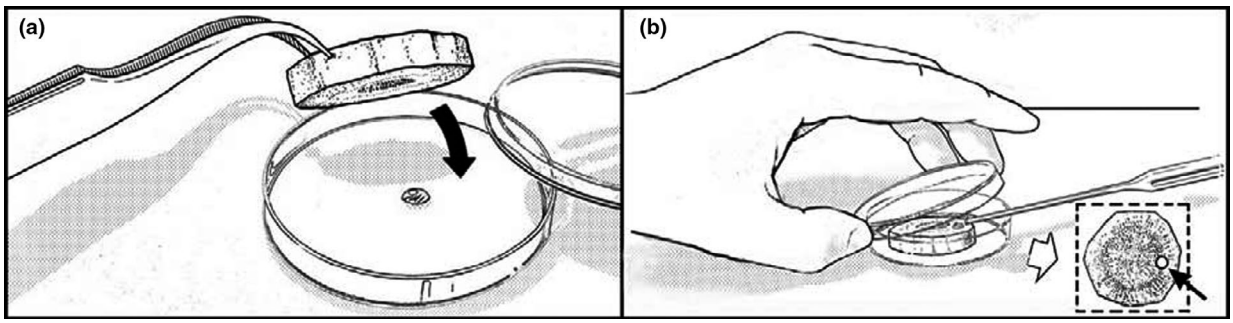

FIGURE 14 (a) Carrot disc put on the droplet containing the nematode inoculum. (b) In the dashed square, the black arrow indicates the inoculum release. (Courtesy: A. Troccoli, CNR-IPSP, IT.)

FIGURE 15 Nematode inoculated carrot disks in Petri dishes in a plastic container with a transparent lid. (Courtesy: NRC-NPPO, NL.)

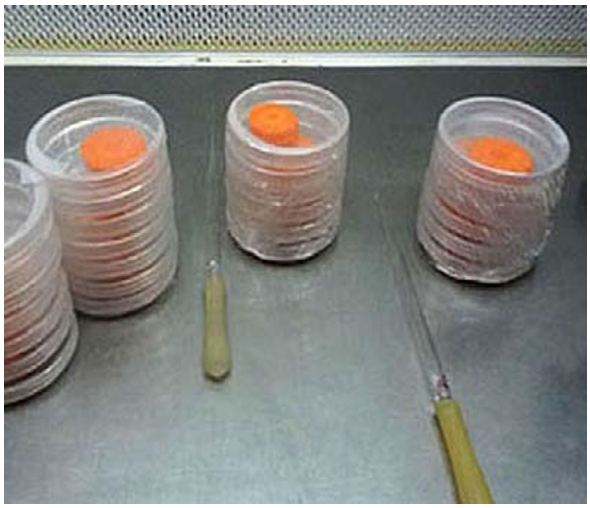

FIGURE 16 Piled up, inoculated carrots in Petri dishes to be put in incubation within a container. (Courtesy: CNR-IPSP, IT.)

or pile them up (Figure 16), with each pile containing the same nematode species, and put them into a larger plastic container.

14. Write on the lid: date of inoculation, isolate number and name of the species (Figure 15).
15. Keep the containers in the dark at ±22°C. Temperature of incubation usually depends on the species: more rapid nematode multiplication occurs at 28°C, although lower temperatures allow the isolate to be maintained for a longer period.

3.6. Maintenance

1. After 2 weeks check for living nematodes in the Petri dishes using a stereomicroscope.
2. The nematodes can be incubated at ±22°C for 6–8 weeks. When the carrot discs start to deteriorate, the nematodes should be transferred to new carrot discs.

APPENDIX 2 - MAINTENANCE OF *BURSAPHELENCHUS* SPP. AND OTHER APHELENCHINA ON PETRI DISHES

1. Scope

Most species of the genus *Bursaphelenchus* and other Aphelenchina have predominantly mycophagous behaviour. As such, isolates can be maintained and multiplied on a mycelial mat, usually of a non-sporulating strain of *Botrytis cinerea* (Mamiya & Shoji, 2009; Shinya et al., 2012). This appendix is based on SOPs from University of Évora (PT), INIAV (PT), CREA (IT) and Latvia.

Isolates kept for a long period, if meant to be used in inoculation trials, need to be revitalized through an inoculation in wood (e.g. autoclaved pine branches) followed by extraction, since a depletion of their virulence may happen after extended subculturing on a mycelial mat (Kiyohara, 1976).

2. Materials

- Autoclave
- Stereomicroscope
- Bunsen burner or equivalent
- Centrifuge
- Counting dish, cleaned with 70% ethanol
- Erlenmeyer flasks or glass tubes with stoppers
- Ethanol 70%
- Glass jar (100 mL)
- Laminar flow hood
- Parafilm
- Picking needle
- Plastic containers with transparent lids
- Sieve 38 µm
- Sterile falcon tube (15 mL) (= plastic centrifuge tube with conical bottom)
- Sterile Petri dishes Ø 9 and/or 5 cm
- Sterile pipette
- Sterilized tap water
- Streptomycin sulfate solution 0.1%
- Tissues (paper towel/cheese cloth fabric)
- Potato dextrose agar (PDA) or malt extract agar (MEA)
- Glycerol

3. Procedures

3.1. Preparation of *Botrytis cinerea* plates

1. PDA is a general purpose basal medium for cultivating fungi. Suspend 39 g of dehydrated media (supplied by commercial suppliers) in 1000 mL sterilized tap water. Glycerol can be added to this substrate (30 mL) to increase the length of survival of nematodes at low temperatures. Heat to boiling to dissolve the medium completely. Alternatively, MEA may also be used (Takemoto, 2008).
2. Sterilize by autoclaving at 121°C for 20 min. Mix well before dispensing to 9 cm Petri dishes.
3. A layer of ≈5 mm of liquid substrate should be poured into each Petri dish.

4. When the substrate has solidified, each plate is inoculated with a mycelial plug cut from the margin of a growing mother culture of *B. cinerea* and placed in the centre. Plates are incubated at ≈25°C for 7–10 days in an inverted position (agar side up) with increased humidity, until the fungus has covered half of the dish.

3.2. Extraction of nematodes from previous *Botrytis cinerea* culture

This step is only performed when one opts to inoculate with a nematode suspension (see Section 3.4.A). If possible, the steps should be performed under sterile conditions. It is particularly important if you decide not to disinfect the nematodes before inoculating them.

A. Direct removal by washing

1. Rinse the lid of the Petri dish with sterilized tap water to collect the nematodes from the lid and/or add a few droplets of water onto the previous *B. cinerea* culture.

2. Pour or pipette the suspension with nematodes in a clean glass jar or counting dish.*B. Baermann funnel (modified)*

1. Remove the content of the Petri dish (fungus + nematodes) and wrap it in one layer of paper towel/cheese cloth fabric.
2. The tray containing the wrapped sample is then filled with sterilized tap water until the entire sample is submerged in water.
3. 18–24 h later, extract the nematodes (38 µm sieve) and pour the suspension into a clean jar or counting dish.

3.3. Surface sterilization of nematodes

See Section 3.3 of Appendix 1.

3.4. *Bursaphelenchus* isolate inoculation

Preferably work in a laminar flow hood, but if not possible work as cleanly as possible and open the Petri dishes as little as possible.

For inoculation, slightly different options are possible, depending on the original nematode isolate.

A. Pure nematode suspension

A drop of the nematode suspension (or the handpicked nematodes, n = 30–50) is placed in the center of the Petri dish with the actively growing mycelium with a sterile pipette.

B. Plates with Bursaphelenchus sp. culture.

From Petri dishes with *Bursaphelenchus* sp. in the mycelial mat, a piece (about 5 × 5 mm) is cut and transferred on new Petri dishes with *B. cinerea* mycelium (see Section 3.1).

C. Flasks with Bursaphelenchus sp. growing on barley.

From tubes containing the nematode isolate growing on barley colonized by *B. cinerea* (see Appendix 3), 2–3 grains are picked and placed on Petri dishes inoculated with the fungus (see Section 3.1). In all cases, the cover of the inoculated plates has to be sprayed with sterilized tap water if not enough humidity was generated and the dish is closed with parafilm to prevent contaminations and dehydration. The lid should be marked with the date of inoculation, isolate number and name of the species.

To allow the nematode isolate to develop, Petri dishes are incubated at 25 ± 1°C in the dark until a high number of nematodes are present/visible in the drops on the lid (Figure 17).

Note: In labs with recurrent attack of mites, incubation should be at 20°C, with the plates floating on water with detergent in or placed on sticky foil to avoid infestation by mites. After the incubation individual plates are sealed in plastic zip bags and stored at low temperature (from 6 to 9°C±1°C).

3.5. Maintenance

After 1–2 weeks check for living nematodes in the Petri dishes using a stereomicroscope.

The plates/cultures can be incubated for about 2–3 weeks. When the mycelium is no longer visible, the nematodes should be inoculated on new *B. cinerea* plates.

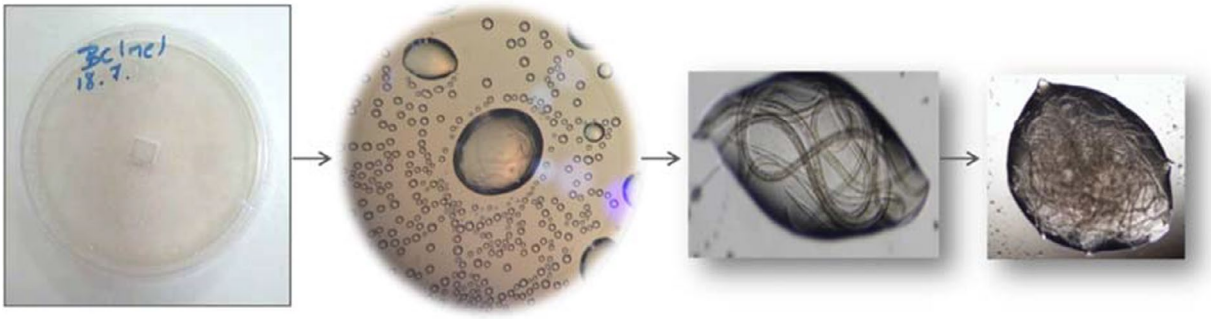

FIGURE 17 Plate of *Botrytis cinerea* for inoculation with *Bursaphelenchus xylophilus*. Nematodes feed on the fungi and can also be found in the drops at the lids of the inoculated plates (courtesy of INIAV, Nematology Lab, PT)

Alternatively, propagated nematode cultures can be stored at ≈9 ± 1°C in the dark for 2–3 months but should be inspected monthly to control the viability of the stored isolate. Whenever a high mortality rate is observed, the multiplication process is restarted on fresh fungus.

APPENDIX 3 - MAINTENANCE OF *BURSAPHELENCHUS* SPP. ON BARLEY SEEDS

1. Scope

For mass inoculum production, species of the genus *Bursaphelenchus* can be maintained and multiplied on a non-sporulating strain of *Botrytis cinerea* growing on barley seeds using this protocol (Mamiya & Shoji, 2009). This protocol is based on SOPs from the University of Évora (PT) and INIAV (PT).

2. Materials

- Autoclave
- Barley seeds
- Bunsen burner
- Centrifuge
- Counting dish, cleaned with 70% ethanol
- Culture of *B. cinerea*
- Erlenmeyer flasks or glass tubes with stoppers
- Ethanol 70%
- Glass jar (100 mL)
- Laminar flow hood
- Parafilm
- Sieve mesh size 38 µm
- Stereomicroscope
- Sterile falcon tube (15 mL) (= plastic centrifuge tube with conical bottom)
- Sterile Petri dishes Ø 9 and/or 5 cm
- Sterile pipette
- Sterilized tap water
- Streptomycin sulfate solution 0.1%
- Tissues (lab paper tissues)

3. Procedures

3.1. Preparation of barley seeds substrate

1. 50 mL glass tubes are half-filled with organic barley seeds (≈15 g), 15 mL of sterilized tap water and tightly covered; alternatively, Erlenmeyer flasks can be used.
2. Tubes are autoclaved at 121°C for 20 min twice and allowed to cool down.
3. In the laminar flow hood, a mycelial plug cut from the margin of a growing mother culture of *B. cinerea* is placed on the grains "white side" downward; tubes are incubated at ≈25°C for 7–10 days. During this period a white colour mycelium should develop around the grains.

3.2. *Bursaphelenchus* isolate inoculation

Preferably work in a laminar flow hood, but if not possible work as cleanly as possible and open the Petri dishes as little as possible.

For inoculation, slightly different options are possible, depending on the original nematodes isolate.

A. Pure nematode suspension

The nematode suspension is placed with a sterile pipette over the mycelium covering the barley grains.

B. Plates with Bursaphelenchus sp. culture

From Petri dishes with *Bursaphelenchus* sp. growing in the mycelial mat, a piece (about 5 × 5 mm) is cut and transferred to the tubes with barley covered by *B. cinerea* mycelium (see Figure 18).

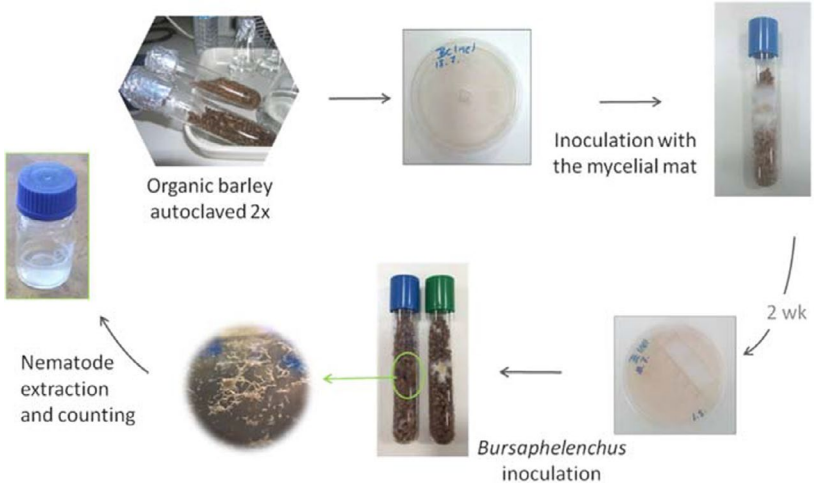

FIGURE 18 Multiplication of *Bursaphelenchus xylophilus* on barley seeds previously inoculated with a non-sporulating strain of *Botrytis cinerea* (after 1–2 weeks). (Courtesy: INIAV, Nematology Lab, PT.)

C. Flasks with Bursaphelenchus sp. growing on barley

From old tubes or flasks containing the viable nematode isolate, 2-3 grains are picked and placed on new tubes with barley seeds colonised by *B. cinerea*.Within about 1 week, after incubation at 25 ± 1°C in the dark, the nematodes will have multiplied and are visible as 'ropes' of nematodes on the tubes wall. Check for living nematodes using a stereomicroscope.

3.3. Surface sterilization of nematodes

See Section 3.3 of Appendix 1.

3.4. Extraction of nematodes from barley substrate

If possible, the steps should be performed under sterile conditions.

A. Direct removal by washing

1. Rinse the tube with sterilized tap water to collect the nematodes on tube walls.
2. Pour or pipette the suspension with nematodes into a clean glass jar or counting dish.

B. Baermann funnel (modified)

1. Remove the content of the tube (seeds + fungus + nematodes) and wrap it in one layer of a paper towel/cheese cloth fabric.
2. The tray containing the wrapped sample is then filled with sterilized tap water until the entire sample is submerged in water.
3. After 18–24 h, extract the nematodes (38 µm sieve) and pour the suspension into a clean jar or counting dish.

3.5. Maintenance

The nematodes can be incubated for about 2–3 weeks. When the mycelium is no longer visible, the nematodes should be inoculated onto new *B. cinerea* plates or transferred to new tubes or Erlenmeyer flasks with barley.

Propagated nematode cultures can be stored at ≈10°C in the dark for 2–3 months but the tubes should be inspected monthly to control the viability of the stored isolate. When a high mortality rate or the appearance of a 'slimy liquid' is observed inside the tube, the multiplication process is restarted on fresh fungus/barley seed + fungus.

APPENDIX 4 - MAINTENANCE OF *DITYLENCHUS DIPSACI* ON ONION

1. Scope

This protocol from ANSES (FR) can be used for maintaining *D. dipsaci* isolates.

The main advantages of this protocol compared to the Faba beans protocol (Appendix 5) is that the preparation and storage of the material can be done in the laboratory, while the Faba beans protocol requires a greenhouse.

2. Materials

- Onion bulbs
- Support for placing onion bulbs
- Scalpel blades
- Glass pipette
- Adhesive tape
- Knife
- Sieve mesh size of 250 µm
- Paper towel or cotton-wool milk filter or equivalent
- Dishes, container or basins in plastic or stainless steel
- Sieve mesh size of 20 µm for nematode suspension recuperation
- Beaker
- Stereomicroscope

3. Procedures

3.1. Preparation of onion bulbs

Onion bulbs (no specific variety is required) to be used for *Ditylenchus dipsaci* propagation must be healthy. Onion bulbs are placed on a rack or support to keep them still. A small excision of 1 cm^2 and a few mm deep is made in the first scale leaves in the upper part of the onion (Figure 19).

3.2. Preparation of the nematode isolate to inoculate

Collect the isolate to be inoculated by extracting the nematodes using Oostenbrink dish (Figure 20a) as described in the EPPO PM 7/119 standard (EPPO, 2013a). Make sure that the nematode suspension is clean, concentrate the nematodes and inoculate the onions bulbs. Before inoculation, the nematode isolate is verified using a stereomicroscope. Counting the nematodes is not necessary before inoculation, but they should be at a high concentration (at least 100 individuals/100 µL).

3.3. Inoculation

1. Inoculate onion bulbs with approximately 100 µL of a concentrate nematode suspension in the small exision made in the first scale leaves (Figure 19a).
2. Seal the inoculation spot with the first excised piece of the scale leaves and a piece of adhesive tape (Figure 19b).

3.4. Culturing on onion bulbs

1. The inoculated onion bulbs are stored for 4–6 weeks at 18/20°C.
2. After the multiplication period, the bulbs are cut into pieces. For each bulb, an infection analysis is carried out on part of the pieces by extracting the nematodes for up to 24 h using the Oostenbrink dish (Figure 20a) [method described in EPPO PM 7/119 (EPPO, 2013a)]. Presence of the nematodes in

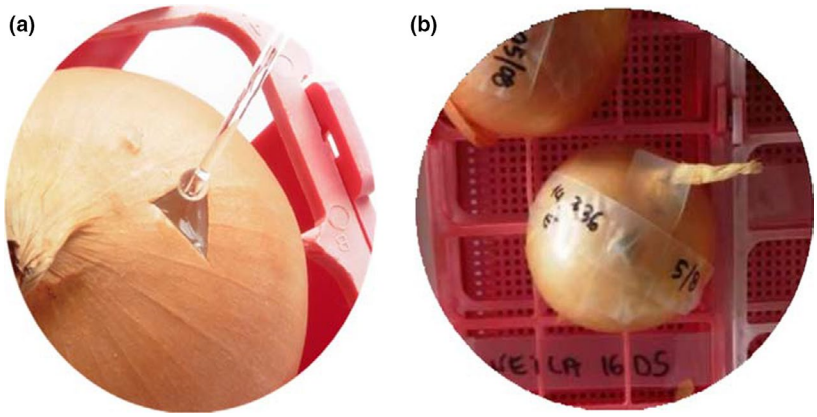

FIGURE 19 Inoculation of onion bulbs with *Ditylenchus dipsaci* isolates. (a) The onion bulb is deposited in a rack and a small excision is made in the first scale leaves in which the nematode is inoculated. (b) The inoculated spot is sealed with the excised piece of the onion scale leaves and a piece of adhesive tape. (Courtesy: ANSES, LSV, Nematology Unit, FR.)

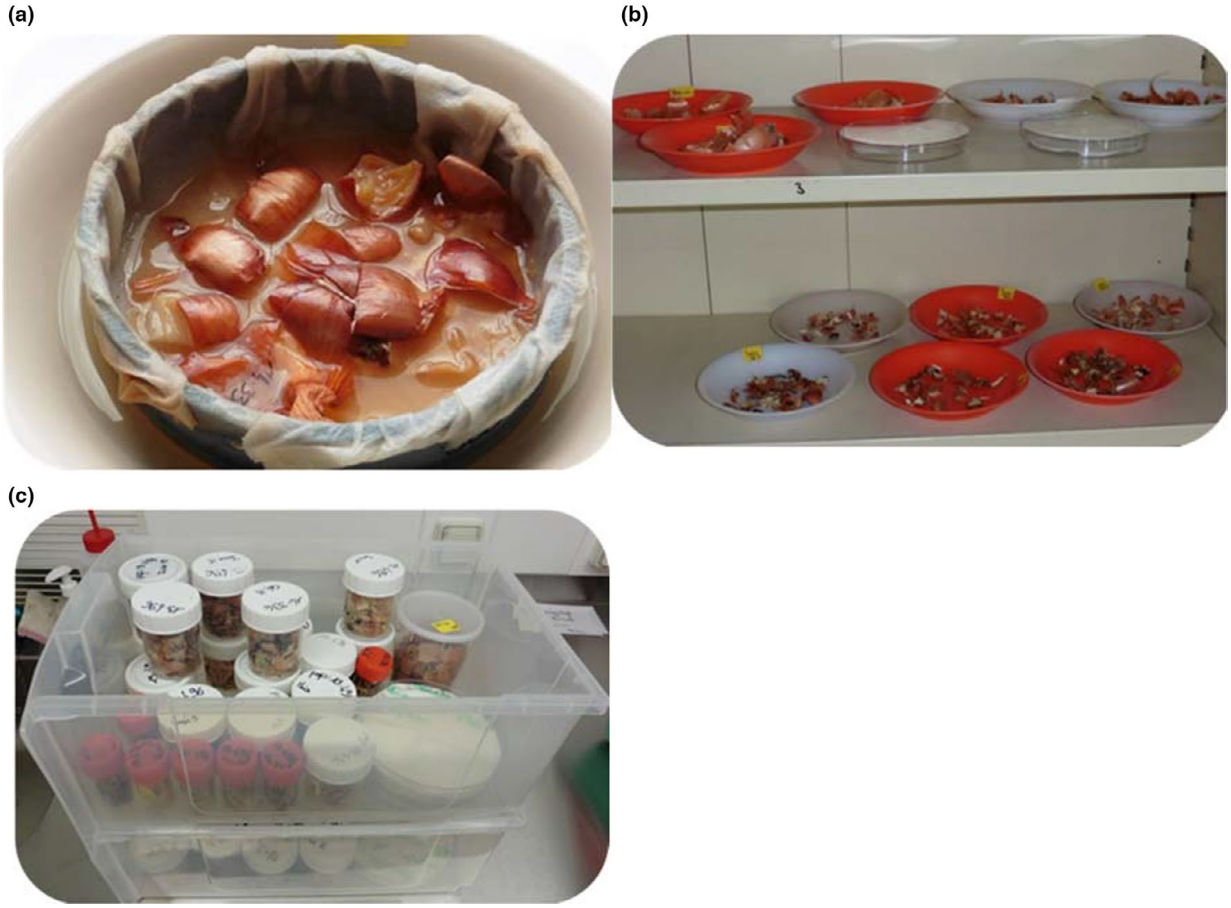

FIGURE 20 Infestation analysis and storage of *Ditylenchus dipsaci* isolates. (a) Oostenbrink dishes for extracting nematodes from onion bulbs. (b) Air-drying infested onion pieces. (c) Storing the dried infested onion pieces in pots. (Courtesy: ANSES, LSV, Nematology Unit, FR.)

the acquired suspension is determined under a stereomicroscope. The rest of the pieces are stored and air-dried at room temperature for ≈1 week (Figure 20b).

3. Only the infested onion bulbs that have multiplied *Ditylenchus* spp. are kept.
4. Dried bulb pieces are stored in closed bottles at room temperature (Figure 20c). The traceability of all these operations enables the monitoring of the isolates in the collection.
5. Determine when the onion bulbs need to be replaced. Include this in the maintenance schedule.

3.5. Transferring isolate to new onion bulb

Onions from the collection are replaced every 4–6 weeks unless the bulb is rotting. The process is repeated to maintain the nematode isolate.

3.6. Storage

Dried bulb pieces are stored in closed jars at room temperature and under dry conditions (Figure 20c).

APPENDIX 5 - MAINTENANCE OF *DITYLENCHUS DIPSACI* AND *D. GIGAS* ON FABA BEANS (*VICIA FABA*)

1. Scope

This appendix describes the maintenance of *Ditylenchus dipsaci* isolates extracted from flower bulbs, or *Ditylenchus gigas*, on Faba bean in the greenhouse. Dutch laboratories have experienced that maintenance of *D. dipsaci* on carrot discs is not suitable (Vreeburg et al., 2014). In addition, this method using Faba beans is less time-consuming than the carrot disc technique, does not produce bad smell and has a low chance of contamination with bacteria or fungi.

2. Materials

- Pots, 5 L terracotta
- Potting soil
- Pottery shards
- Seeds of Faba beans
- Screens
- Dishes Ø 20 cm
- Plastic plant stick (± 150 cm)
- Pipette (80 µL)
- Preparation needle
- Permanent marker
- Paper bag (±20 × 30 cm)

3. Procedures

3.1. Sowing of the faba beans

Place a terracotta pot shard on the bottom of three pots and fill the pots with potting soil to 5 cm below the rim. Sow one Faba bean in the middle of the pot. Place each pot on a clean dish with an inner diameter of 20 cm in the greenhouse at 15–20°C and RH of 70%. Place the three pots preferably in a triangular formation so the sticks later on can lean on each other (pyramid-shape). The temperature should not exceed 25°C. Place a plastic screen between the pots with plants which will be inoculated with different isolates. Make sure that the soil stays moist so that the seeds can germinate and the plants can grow. After approximately 1 month the plants are large enough for inoculation.

3.2. Inoculation of the Faba beans

Prepare a solution with a nematode concentration of 200–300 *Ditylenchus* specimens per 80 µL of tap water in a small glass jar or centrifuge tube. Check the purity of the isolate at least once every 2 years. Use a preparation needle to make a small hole in a leaf axilla, roughly 10–15 cm above the soil. Mark the place with a permanent marker. Pipette 80 µL of the suspension in the hole (Figure 21). Repeat this for all three plants.

FIGURE 21 Droplet on leaf axilla. (Courtesy: NRC-NPPO, NL.)

3.3. Taking care of the plants

Water the plants daily. Tie the plants to a plant stick when they are starting to droop. Prune the plants to a manageable size. Wear gloves. Use a knife to cut the top of the plant when it becomes too long.

3.4. Harvest of the plants

After 3 months the above-ground part of the plant is cut off. The marked stem with symptoms (Figure 22) is cut into small parts and put into a paper bag that should be labelled with the isolate number and inoculation date.

3.5. Extraction of nematodes

To extract the *Ditylenchus*, cut the (dried) stem pieces into ≈2 cm pieces, cross-cut once and use cotton-wool filter or Bearmann funnel/mistifier extraction as described in the EPPO PM7/119 Standard. It is only possible to extract all life stages in fresh harvested material. From dried material only J4 juveniles will be extracted. The reproduction rate is variable, on average between 50–100, occasionally up to 500.

3.6. Storage

A paper bag containing *Ditylenchus*-infested stem parts can be stored at room temperature for at least 1 year.

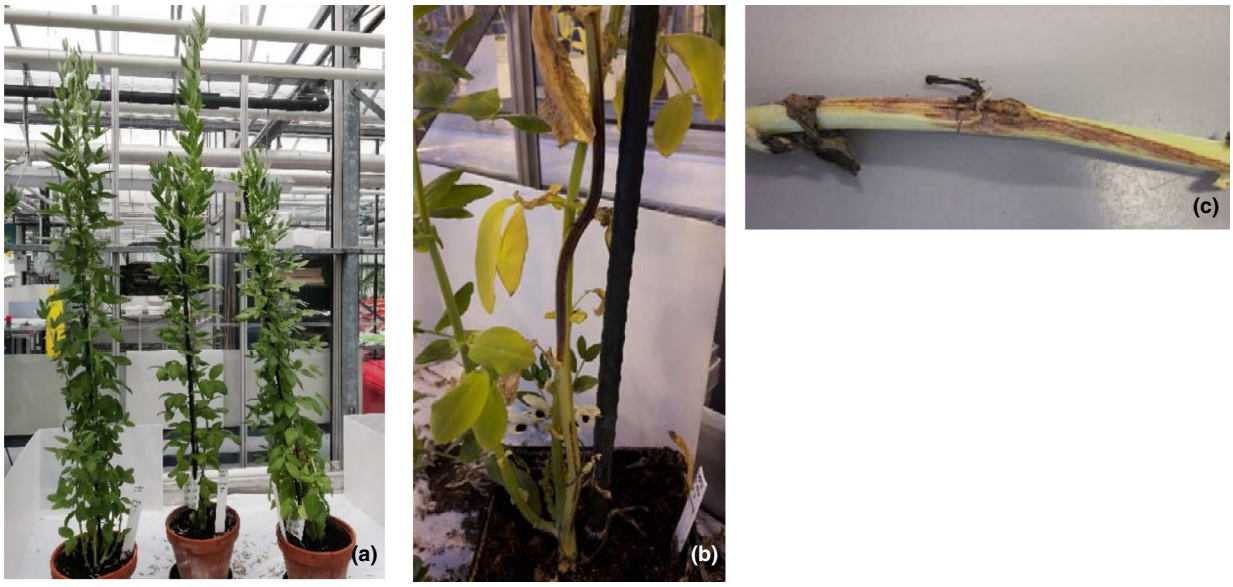

FIGURE 22 (a) Three Faba plants inoculated with one population between shields in the greenhouse. (b) Symptoms of *D. dipsaci* on *Vicia faba*: reddish-brown lesions on the stem, increasing up- and downwards from the inoculated axilla. (c) Bursted stem in lesion. (Courtesy: NRC-NPPO, NL.)

APPENDIX 6 - MAINTENANCE AND MULTIPLICATION OF CYST NEMATODES (HETERODERINAE SPECIES)

1. Scope

This protocol from NRC-NPPO (NL) can be used to maintain and multiply Heteroderinae species; when large amounts of cysts are necessary, several pots can be used per cyst isolate. The number of generations per year and the viable reproduction varies between cyst nematode species. Most temperate species will complete 1–2 generations, corresponding to the natural life cycle, while tropical species can reach multiple generations, both depending on the environmental conditions and their host plant (Moens et al., 2018).

2. Materials

- Potting soil mixture: 69% silver sand + 12% kaolin powder (clay powder) + 17% small hydro grains (2–4 mm) + 1.4% Osmocote Exact Standard [15N-9P-11K + 2MgO (magnesium oxide) + TE (trace elements), 12–14 months active] mixed for 6 min in a cement mill
- Terracota pots, 5 L
- Pottery shards
- Materials for making suspensions for inoculation
- Needles for inoculation
- Sterile syringe

3. Procedures

3.1. Pre-germination of the host plant

Sow the host plant seeds in potting soil and replant them when the root system is large enough or sow the host plant directly in the terracota pot with soil mixture. Be aware that for good development sufficient new young roots should be present so always use very young plants, preferably plants of 2–3 cm in size.

3.2. Preparation of the isolate

1. Ensure the identity and viability of the isolate every time an isolate is inoculated for maintenance or is issued. Identification can be performed by morphological and/or molecular analysis.
2. Assess the isolate to be inoculated by crushing 10–20 cysts and determine the contents. When the eggs and juveniles are healthy, the viability should be at least 50% for *Globodera* spp. and 30% for *Heterodera* spp. and not contaminated with fungi. The total required number of cysts, based on the average cyst content, to achieve an inoculum density of 5 living eggs and juveniles per gram of soil can be calculated.

3.3. Preparation of the pots and inoculation

1. Isolates are preferably set up in two pots to reduce the risk of loss. Place a filter or pottery shard on the bottom and fill the pot with the soil mixture to 5 cm below the rim Place the pots on a clean saucer with an inner distance of 20 cm in the greenhouse.
2. Inoculate the two pots with a suspension resulting in an initial density of 5 live eggs or juveniles per gram of soil. If the suspension contains less than 20,000 live eggs and juveniles inoculate one pot.
3. Stir the inoculum randomly, not making circles. Use a sterile syringe with hollow needle to inject the suspension nearby the root system of the plant. Make sure the needle does not become blocked with soil; if necessary clean it with a wire. Dispense the prepared inoculum per pot via at least five separate injections.

4. Place the labelled pots in the greenhouse at 18°C (day)/13°C (night) for temperate isolates. The temperature must not exceed 25°C. For the tropical isolates set the temperature to 22°C (day)/18°C (night). Here the temperature must not exceed 30°C. RH = 70%.

3.4. Taking care of the plants

1. As the soil mixture already contains a slow release fertilizer, no extra fertilizing is necessary.
2. Watering the pots every day depends on the circumstances. For host plant or propagation tests the moisture percentage should be controlled by weighing and the (weight) moisture percentage must not fall below 10% and not exceed 15% (20% depending on the size of the crop). A measuring cup should be used to ensure a suitable amount of water is added.

3.5. Sampling

If material is needed before the end of the culture, depending on the life cycle of the species, samples of the isolates can be taken (with a scoop) from the soil/roots 12–20 weeks after inoculation.

3.6. End of the culture

The culturing ends when the host plant dies or for non-perennial plants at the latest 1 year after inoculation. Stop the water supply and remove above-ground plant parts. The pot may remain in the greenhouse for up to 3 days so that the soil can dry, but it should not become too dry. Pack the pot contents in a large plastic bag, write the isolate number and species name on the bag, and store this at 4°C until cyst extraction.

3.7. Leave plant and isolate in the existing pot

Depending on the life cycle of the species and the host plant, some cyst cultures do not have to be replaced and can stay for several years in the greenhouse in the same pot. This is the case for species such as *Cactodera cacti* and *Heterodera fici*.

3.8. Extraction and cleaning

Cyst extraction should be performed according to EPPO Standard PM 7/119 (1) *Nematode extraction* and the cysts separated from organic debris, e.g. with ethanol (after Seinhorst, 1974) (EPPO, 2013a).

Pick out the cysts and determine the number of cysts.

If there are <2000 cysts, determine the viability (EPPO, 2017) and when the content is viable enough (see Section 3.2) inoculate it directly on a host plant, as described above in the inoculation paragraph.

If there are more than 2000 cysts and isolate is suitable for storage at 4°C, determine the viability of the cysts by determining the content of at least 100 cysts.

- If the viability is less than 60%: Immediately inoculate the isolate again on a host plant in the greenhouse.
- If the viability is higher than 60%: Store the cysts in a glass jar closed with an oxygen-permeable lid (Figure 23). Depending on the species cysts can be stored for a few months up to several years. Species such as potato cyst nematodes can be stored after extraction for at least 4 years at 4°C, others such as most *Heterodera* spp. are not suitable for long-term storage.

The reproduction rate is variable per species and depends on many factors such as host plant, temperature, root growth and duration of the maintenance on the host plant.

3.9. Ensuring the purity

Check the purity of the isolate at least once every 4 years by:

- identification of cysts
- host plant (bio) testing
- molecular analysis.

APPENDIX 7 - MAINTENANCE AND MULTIPLICATION OF POTATO CYST NEMATODES (*G. ROSTOCHIENSIS* AND *G. PALLIDA*)

1. Scope

This appendix from AGES (AT) can be used for the maintenance and multiplication of the cyst nematodes *G. rostochiensis* and *G. pallida*; for multiplication a larger number of pots needs to be inoculated.

The described procedure is based on the resistance testing procedure from the EUPHRESCO project on *Globodera*: ring testing of methods for identification

FIGURE 23 Dry cyst storage in glass jars, with holes in the lid, in boxes in the coldroom. (Courtesy: NRC-NPPO, NL.)

and resistance testing for potato cyst nematodes (Viaene et al., 2010).

2. Materials

- Potting soil mixture:
  - 60% heat sterilized soil + 40% sand
  - Alternatively: 70% sand (0.3–1 mm) + 16% clay granulate (e.g. Seramis) + 10% sterilized soil + 4% peat
- Pre-germinated susceptible seed potato tubers
- Labelled pots 250–2000 mL (depending on number of cysts available)
- Nematode inoculum
- Strong paper liner
- Glass rod
- Plant stick
- Tap water
- Fertilizer NPK
- Filter paper
- Microscope
- Labelled glass tubes

3. Procedure

3.1. Pre-germination of the host plant

Store susceptible seed potato tubers in a dark room for 6 weeks at room temperature. After sprout development, store the potatoes for 10 days in daylight.

3.2. Preparation of the isolate

1. Ensure the identity of the isolate every time a isolate is inoculated for maintenance or is issued.
2. If more than 20 cysts are available, determine cyst content by crushing and counting the content of a small batch of cysts (after Seinhorst & Ouden, 1966). Calculate the required number of cysts based on the average cyst content to achieve an inoculum density of 5 living eggs and larvae/mL soil.
3. If only a few cysts (1–8) are available, inoculate them directly.

3.3. Preparation of the pots and inoculation

1. Put a strong paper liner into the pots to prevent roots from growing out (Figure 24).
2. Fill the pots 2/3 full with potting soil. The total amount of soil depends on the availability of cysts (250–1500 mL/pot).
3. Stir the prepared cysts into the soil with a glass rod. Use one tuber per pot. Remove all but one sprout from the tuber before planting it. Cover the tuber with the remaining third of soil.
4. Place the pots in the greenhouse at 17.5–19.5°C with a long-day light regime: 16 h (day)/8 h (night).

3.4. Taking care of the plants

1. Measure the temperature in the pots once a day (should not exceed 25°C).
2. Water and fertilize the plants manually during the whole culturing period (this needs experienced staff).
3. Remove extra sprouts emerging from tubers, only one stem is allowed to grow (Figure 25).
4. Tie the plants to a stick to prevent them from falling down.

FIGURE 24 Paper liner (Courtesy: AGES, AT)

FIGURE 25 One stem potato plant. (Courtesy: AGES, AT.)

3.5. End of the culture

1. 8–10 weeks after inoculation cysts appear on the roots. After approximately 12 weeks the potato plants wilt and die (depending on the conditions, root systems should be checked for brown cysts).
2. Stop the water supply and remove the above ground parts of the plants.
3. The pots should remain in the greenhouse for another 2 weeks so that the soil in the pots dries completely (Figure 26).
4. After this, store the pots in a climate chamber at 8°C until cyst extraction.

3.6. Extraction and cleaning

1. Cyst extraction according to EPPO Standard PM 7/119 (1) *Nematode extraction* and the cysts separated from organic debris, e.g. with ethanol (after Seinhorst, 1974) (EPPO, 2013a).
2. Roll the cysts on a filter paper and count them under the microscope (Figure 27).
3. Store the cysts dry in labelled glass jars with holes in the lid (Figure 28) at 4°C in a laboratory refrigerator with a constant temperature regime.

FIGURE 26 Completely dry soil. (Courtesy: AGES, AT.)

3.7. Ensuring the purity

- Bioassay described in the EPPO Standard PM 7/40 (4) *Globodera rostochiensis and Globodera pallida* Appendix 1 (EPPO, 2017)
- Identification of cysts
- Molecular analysis

3.8. Ensuring the viability

Testing the viability of eggs and juveniles can be done by different methods described in the EPPO Standard PM 7/40 (4) *Globodera rostochiensis and Globodera pallida*, Section 4.4 (EPPO, 2017).

3.9. Ensuring virulence

The virulence of isolates can be tested on a set of potato cultivars. An EPPO Standard PM 3/68 *Testing of potato varieties to assess resistance to Globodera rostochiensis and Globodera pallida* was adopted in 2006 (EPPO, 2006).

FIGURE 28 Glass jars. (Courtesy: AGES, AT.)

FIGURE 27 Cysts are counted under the microscope. (Courtesy: AGES, AT.)

APPENDIX 8 - MAINTENANCE AND MULTIPLICATION OF ROOT-KNOT NEMATODES (*MELOIDOGYNE* SPP.)

1. Scope

Root-knot nematodes can be maintained and/or propagated on host plants in the greenhouse as well as outside, depending on the species (temperate or tropical). Host plant choice depends on the species, the purpose and the situation. Plants such as fig trees (for *M. incognita*) and papaya (for *M. javanica*) or barnyard grass (*Echinochloa crus-galli*) (for *M. graminicola*) can be used for maintenance of tropical species, which involves a lower workload due to reduced need for transfer of the isolate to new plants. However, there is a limited range of species that can multiply on some perennials.

This appendix focusses on the maintenance and/or multiplication of *Meloidogyne* isolates on tomato in the greenhouse, as done at the NRC-NPPO (NL); for multiplication several pots can be inoculated. Tomato is a commonly used host plant for propagation, most *Meloidogyne* species multiply well on this plant. Where possible other host plants may be used.

2. Materials

- Pots 5 L, preferably terracotta
- Soil mixture: silver and quartz sand
- Cement mixer
- Osmocote Exact Standard ((15N-9P-11K) + 2MgO (magnesium oxide) + TE (trace elements), 12–14 months active)
- Pottery shards
- Host plants, tomato seeds cv. "Dometica" or other non-infested host plant
- Glass jar
- Closed container for transport of suspension and/or infested plant material
- Screens
- Dishes Ø 20 cm
- Plastic plant sticks (± 150 cm)
- Labels
- Pruning shears
- Pipette (1–5 mL)
- Permanent marker
- Plastic bag
- Paper (at least 40 × 60 cm)

3. Procedures

3.1. Preparation

3.1.1. Sowing and planting of the host plants

Sow seeds in potting soil (optionally mixed with 25% silver sand) and replant when the root system is large enough or sow directly in the pot with soil mixture (Figure 29). Be aware that for good nematode development sufficient new young roots should be present, so always use young plants. If using or buying plants that have already been grown, check the root system in advance for the presence of plant parasitic nematodes.

For tomato, preferably use a cultivar as `Dometica` with a tolerance to wilting diseases such as *Fusarium*. Use young plants with at least 6 leaves. This developmental stage is reached on average 5 weeks after sowing. Plant 1 or 2 tomato plants in the middle of the pot. Water the plant and allow the plant to recover for at least 4 days before inoculation.

3.1.2. Soil mixture and preparation of pots

Prepare the soil mixture in a cement mixer (Figure 30). Mix 2 bags silver sand and 1 bag quarts sand, 25 kg each, into the cement mixer. Add 360 g of Osmocote. Mix for at least 6 min. If stored dry, the soil has a long shelf life.

Isolates are preferably used to inoculate in two pots to reduce the risk of loss. New or reference isolates are used to inoculate in three pots. Place a filter or pottery shard on the bottom and fill the pot with the soil mixture to 5 cm below the rim. Place the pots on a clean dish with an inner diameter of 20 cm in the greenhouse.

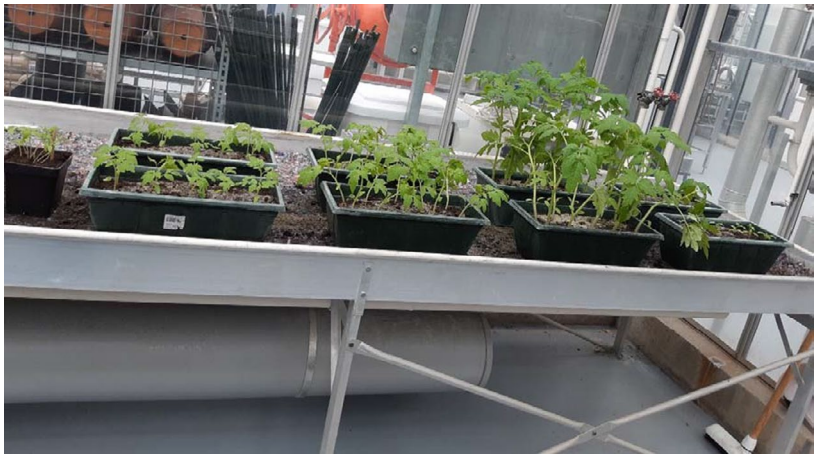

FIGURE 29 Every week 20 tomato seeds are sowed and 1-week-old seedlings are then transplanted in a 50% potting soil/50% silver sand mixture for nematode-free host plants to be used for the maintenance of the nematode isolate. (Courtesy: NRC-NPPO, NL.)

FIGURE 30 Cement mixer used for mixing artificial sandy soil for rearing *Meloidogyne* isolates. (Courtesy: NRC-NPPO, NL.)

3.1.3. Inoculum

Isolates can originate from a diagnostic sample, self-performed sampling, another collection or from the existing collection in the greenhouse.

Extract the nematodes from the infested soil or roots according to EPPO Diagnostic Protocol PM 7/119. Prepare per isolate, per pot, by using a stereomicroscope, a solution containing around 1000 living and healthy *Meloidogyne* specimens in tap water in a small glass jar. If the density of the extract is lower, a minimum of 250 specimens is recommended per pot. Place this jar in a closed container for safe transport to the greenhouse (Figure 31).

3.2. Inoculation and maintenance in the greenhouse

3.2.1. Inoculation of the plants (Figure 32)

Place each glass jar with suspension in front of a prepared pot in the greenhouse (Figure 32). Use the plant label to make 3–4 holes in the soil around the plant in such a way that you can see young roots. Swirl the jar with the suspension such that the nematodes are floating and divide by pouring the suspension over these holes. Rinse the jar afterwards with a small amount of tap water and pour this into the same holes. Use the label to cover the holes with soil. Place the label in the pot with the written part towards you.

3.2.2. Taking care of the plants

Water the plants regularly, depending on the circumstances. Tie the (tomato) plants to a plant stick. Remove all flowers and fruits, unless biological control is applied, using predatory mites, for which some flowers are needed on the plant. Prune the plants to a manageable size.

FIGURE 31 Biosafety transport box with nematode suspensions in glass jars. (Courtesy: NRC-NPPO, NL.)

FIGURE 32 Inoculated tomato plants with barriers between each population. (Courtesy: NRC-NPPO, NL.)

For temperate isolates, set the temperature to 20°C day/15°C night, not exceeding 25°C and for tropical isolates to 22°C day/18°C night; for these isolates the temperature must not exceed 30°C. For both set an RH of about 60–70%.

3.2.3. Sampling

If material (with all stages present) is needed before the end of the culture, a sample from the soil/roots can be taken not earlier then 10 weeks after inoculation (12 weeks for temperate species).

3.2.4. End of the culture

If not perennials, plants are replaced annually or when the plant condition is becoming poor. For perennial

FIGURE 33 Adjustable height table for harvesting *Meloidogyne* infested (tomato) roots. (Courtesy: NRC-NPPO, NL.) [Colour figure can be viewed at wileyonlinelibrary.com]

plants, ensure that they are not becoming too big for the pot they are grown in. Stop watering the plant at least 4 days before ending the culture. Wear a lab coat and disposable gloves. Cut, still in the compartment, the foliage of the plant and transfer the pot with dish (to avoid soil falling from the bottom of the pot) to a table covered with a large piece of paper (at least 40 × 60 cm) (Figure 33). Turn over the pot on the paper and collect the root system in a plastic bag which can be sealed and copy the information from the label onto the bag. The foliage of the plant and the soil should be treated as biohazard waste.

Some host plants such as Gramineae can be split. One part can be replanted and the root system of the other part can be checked for the presence of nematodes in the laboratory.

3.3. Obtaining and storage of isolates

3.3.1. Extraction of nematodes

For extracting *Meloidogyne* sp. from the infested (tomato) root material, cut the roots into small parts of 1 cm and use a suitable method such as the centrifuge method for plant material, cotton-wool filter extraction or processing samples by mistifier according to the EPPO Diagnostic Protocol PM 7/119 (EPPO, 2013a). If it is preferable to collect females, for instance for (isozyme) identification, extract them manually or by using enzymatic maceration and mechanical maceration, followed by centrifuging. The reproduction rate is variable and depends on many factors, such as host plant, temperature, root growth and duration of the maintenance on the host plant, on average between 50 and 200, which can be up to 500 for long-living host plants.

3.3.2. Storage

Infested roots can be stored at 4°C for a couple of weeks, preferably in tissue and/or with some dry soil. Suspensions with *Meloidogyne* stored at 4°C, if not contaminated with bacteria or fungi, will remain in good condition for a couple of months; this is enhanced by adding fresh water after a few weeks.

APPENDIX 9 - MAINTENANCE OF *MELOIDOGYNE* AND POTATO CYST NEMATODES ON POTATO IN CLOSED CONTAINERS

1. Scope

This appendix, from ILVO, can be used for the maintenance and multiplication of *Meloidogyne* and potato cyst nematodes on potato tubers. This protocol is based on Foot (1977) and ILVO experiments.

2. Materials

- Seed potato tuber
- NaOCl solution (e.g. household bleach)
- Sand
- Tap water (can be sterilized if contamination is expected)
- Closed container (0.5 L plastic pot, 10 cm diameter, with lid) (Figure 34)

3. Procedure for potato cyst nematodes

3.1. Potato tuber preparation

Use seed potato tubers as these are healthier than regular ware potato tubers. When not enough tubers are available or tubers are too big, a tuber piece containing one sprout can be used instead of a whole tuber. The piece with the eye is removed carefully from the tuber with a cork borer and left to dry before planting in the sand.

1. Disinfect a potato tuber (a known susceptible variety) in a solution containing 5% NaOCl solution for 4 min. Alternatively, tubers can be soaked for 30 min in 1% chlorine (Foot, 1977).
2. Rinse well with tap water.

FIGURE 34 Closed container with cysts visible on potato roots. (Courtesy: ILVO, BE.)

3. Dry tubers spread out at room temperature, with the eyes exposed to daylight, so they can develop shoots (can take days to a few weeks).

3.2. Preparation of the pots and inoculation

1. Prepare nematode inoculum by soaking cysts in root exudate (potato or tomato) for 1 to several days. Inoculum from cysts soaked in exudate for 10 days yielded more cysts than from cysts soaked in tap water (Nicole Viaene, pers. comm.).
2. Put 200 g of dried clean sand and 30 mL of tap water in a closed container. Use river sand (fine sand sold at construction stores). Avoid sea sand as it can contain salts, although it could be rinsed to remove the salt. The sand can be autoclaved (see Foot, 1977) or dried in an oven (100°C, 16 h). Simply drying in the open air also works, provided the sand is not contaminated (check origin).
3. Place the germinating potato tuber in the container with developing roots and shoots (buds) in the sand (do not cover the whole tuber).
4. Crush the cysts and collect the eggs and juveniles released from the cysts in a beaker. Add about 1000 eggs and juveniles of *Globodera* spp. to the sand (at time of planting) in no more than 5 mL of tap water. One can also inoculate with cysts if the exact inoculum amount does not matter.
5. Roots can be left to grow, but not for too long before inoculation: maximum until they reach the outside of canister (Foot, 1977). Juveniles prefer growing roots to enter at the tip. Inoculating germinating tubers immediately after placing them in the sand yielded more cysts than inoculating them when roots were 1 cm or more (own tests).

3.3. Storage and extraction

1. Close the container tightly (good fitting lid).
2. Store the closed containers in the dark at 20°C for about 14 weeks or until new cysts have formed and turned dark brown.
3. Collect the cysts after drying roots and sand. The majority of the cysts can be picked from the roots. Cysts can also be harvested by an appropriate method (see EPPO PM 7/119).

4. Procedure for *Meloidogyne*

4.1. Potato tuber preparation

Similar to Section 3.1 of this appendix.

4.2. Preparation of the pots and inoculation

1. Put 200 g of dried clean sand and 30 mL of tap water in a closed container. Place the germinating potato tuber in the container with developing roots and shoots (buds) in the sand (do not cover the whole tuber).
2. Inoculation can be performed using egg masses, eggs or second-stage juveniles at time of planting or during the first 2 weeks (about 1000 eggs/J2 per container). Add no more than 5 mL of tap water to avoid rotting of the tuber.

4.3. Storage and extraction

1. Close the container tightly (closely fitting lid).
2. Store the closed containers in the dark at about 20°C. After 8–14 weeks, newly formed egg masses are visible on the roots stuck to the wall of the container. The time needed to develop egg masses varies between species. It is not so easy to observe egg masses from the outside of the container as it is for brown cysts: egg masses can be confused with a grain of sand.
3. Take out the tuber with its roots and wash carefully in beaker with tap water to remove the sand, but not the egg masses. Roots can be further processed to obtain juveniles or eggs (see EPPO PM 7/119).

APPENDIX 10 - FIXING OF LIVE NEMATODE SPECIMENS

1. Heat fixation

One of the best methods is adapted from Seinhorst (1966a), which kills and fixes nematodes in one process. The specimens are collected in a very small drop of water (Seinhorst, 1973) in a glass staining block or similar deep concave vessel. A fixative, such as triethanolamine formalin (TAF) or Formal-acetic fixative 4:1 (FA 4:1) (refer to Table 4 for alternative fixatives) is heated to about 55–70°C and 3–4 mL is quickly added to cover the nematodes (Figure 35). The fixative can also be heated in a small tube stood in boiling water and then poured or pipetted onto the nematodes. If a pipette is used it should have a wide aperture to allow a quick flow and should be heated with the fixative. The method fixes glands and gonads appropriately, and nuclei are often clearly visible. Netscher and Seinhorst (1969) reported improved results when propionic acid was used in place of acetic acid in the killing solution, followed by fixation in 4% formaldehyde. Netscher (1970) poured hot fixative onto nematodes that had been concentrated on a vacuum filter.

FIGURE 35 Excess 70°C TAF added to nematodes suspended in a small drop of water. Nematodes are placed in a small amount of water, to allow rapid mixing with the fixative. TAF heated to 70°C is added in excess to the water droplet. The combination of killing and fixing the nematodes in one step results in reduced shrinking of nematodes. (Courtesy: NRC-NPPO, NL.)

Alternatively, a few specimens can be killed by transferring them to a drop of water on a plain or cavity glass slide, which is then heated on a hot plate at 65°C for several seconds until the nematode habitus is characteristic of heat relaxation. The slide should be removed from the heat as soon as nematodes are killed as overheating will damage the specimens. The specimens are immediately transferred to cold fixative or fixed on the slide by adding cold 'double-strength' fixative (see Table 4).

A method for handling samples comprising many specimens is to heat the nematodes en masse in a tube of water before fixation. The volume of suspension is decreased by centrifuging the solution at ca. 1500 rev/min for 1–2 min; the nematodes can also settle slowly under gravity. The supernatant is decanted so that nematodes remain in a few millilitres of water. The tube is then agitated to distribute the nematodes freely in suspension before being placed into a beaker of hot water at 65°C. Nematodes are typically killed within 2–5 min, but a thermometer can be placed within the tube to ensure specimens are not damaged. An equal volume of cold 'double-strength' fixative is immediately added.

Golden (in Hooper, 1970) placed live nematodes into 2–3 mL of water in a watch glass stored in an oven at 43°C. After ca. 12 min, the watch glass is filled with fixative at 43°C (from a bottle that has been kept in the oven) before being removed from the oven and stored at room temperature. After 16–24 h the specimens can be processed into glycerol. Various workers have recommended different times and temperatures for killing nematodes in solution using an oven; Zuckerman (1960) reported 60°C for 5 min, Kline (1976) up to 4 min at 70°C.

2. Other methods

Vapour-phase perfusion can kill nematodes without heat (Maggenti & Viglierchio, 1965). Nematodes in 0.5 mL of water in a small, shallow, open vessel are placed on a grid over 3 mL of a killing and fixing solution, then kept in the bottom of a wide-mouth 60 mL jar with a screw cap. The jar is sealed and left for 24 h, during which time the fixative molecules leave the solution as gas, enter the water in the nematode container and diffuse into the nematodes. Three parts of formalin (40% formaldehyde) to one part of either water, 100% propionic acid or 37% HCl are recommended for the killing and fixing solution.

Iodine has also been used as a killing agent for ciliated protozoa and other small organisms, with Staniland (1950) stating that nematodes were rapidly killed using an iodine solution of 1 part in 2000. This method uses a 0.1% Lago's solution (I_2 0.1 g, KI 0.2 g, distilled water 100 mL); an equal volume of this solution is added to a suspension of nematodes. Hasbrouck (1959) made temporary mounts in a mixture of 0.001% iodine and 0.5% concentrated picric acid in FAA (see below). Killing and fixation were rapid, and sclerotized and glandular structures clearly differentiated.

TABLE 4 Selected nematode fixatives

TAF (Courtney, Polley & Miller, 1955)	Formalin (37.4% formaldehyde) 7.6 mL, triethanolamine 2 mL, distilled water 90.4 mL For 'double strength', use formalin 7.6 mL, triethanolamine 2 mL, distilled water 45.2 mL
The appearance of nematodes after fixation in TAF is remarkably lifelike. The solution remains stable for a long period of time. The triethanolamine neutralizes any free formic acid and, being hygroscopic, prevents specimens from drying even if the fixative evaporates. However, Hooper et al. (1983) noted cuticle degeneration of some nematodes stored in TAF for 20 years and so it is not recommended as a long-term fixative. Specimens processed to glycerol after a year or two in TAF remain satisfactory.	
FA 4:1 or FA 4:10 (FP4:1)	Formalin 10 mL, glacial acetic acid (propionic acid) 1 mL/10 mL, distilled water up to 100 mL
Nematodes are rarely distorted in these fixatives, but specimens can potentially discolour and the posterior half of tylenchid stylets become transparent after a number of days, especially in FA 4:10. Seinhorst (1954) observed that the cuticle of *Paratrichodorus pachydermus* swelled in FA 4:10. Although the above can be used for long-term fixation, Netscher and Seinhorst (1969) found that 4% formaldehyde was better following killing with FA or FP 4:1.	
Formalin-glycerol fixative (Golden in Hooper, 1970)	3% formaldehyde solution + 2% glycerol: formalin 8 mL, glycerol 2 mL, distilled water 90 mL
Nematodes can be infused directly from this fixative to glycerol by slow evaporation. This method also has the advantage that the specimens will not dry if vials evaporate. De Grisse (1969) recommends a similar fixative of FG 4:1 (formalin 10 mL, glycerol 1 mL, distilled water 89 mL)	
Ditlevsen's or FAA	95% ethanol 20 mL, formalin 6 mL, glacial acetic acid 1 mL, distilled water 40 mL
As this contains ethanol a certain amount of shrinkage nearly always occurs, although this quality could, on occasion, be useful particularly in enhancing structures such as incisures and annulations.	
5–10% formalin	Dilute solutions of 2–4% formaldehyde
Dilute solutions may be used for fixing dead nematodes, but the specimens may appear dark and granular. The addition of a small amount of $CaCO_3$ (Baker, 1945) neutralizes free formic acid and may prevent granulation. Muscles often show clearly in specimens killed and fixed by adding hot formalin (Hooper, 1986). Netscher and Seinhorst (1969) recommend 4% formaldehyde for fixing nematodes killed by formalin-propionic acid 4:1 (FP4:1). Boag (1982) found that very weak solutions of formaldehyde (0.078%) or TAF (1/128) preserved nematodes for 4 months. Although the long-term effect is not known, it does mean that fixed extracts can be examined in the weaker solutions without risk of deterioration even if they are returned to stronger solutions of storage. Use of such weak solutions means that operators are subjected to reduced risk from formaldehyde fumes.	
Cold fixation (Stynes & Bird, 1981**)**	Phosphate buffer, 4% paraformaldehyde
Good fixation of *Anguina agrostis* has been reported by cooling nematodes alive at 5°C in phosphate buffer and then adding 4% paraformaldehyde (freshly made from powder) in phosphate buffer at 5°C. Specimens mounted in the buffered paraformaldehyde exhibited morphometrics closest to live nematodes, whereas those subsequently processed to glycerol by various methods exhibited shrinkage of some structures.	
Restoration (Franco & Evans, 1978)	Aqueous $KMnO_4$
The appearance of cuticular structures, stylet tip and knobs, and some internal structures of heteroderid juveniles fixed in 4% formaldehyde were restored using potassium permanganate. A drop (ca. 35 µL) of aqueous $KMnO_4$ is added to 4 mL of nematode suspension and incubated overnight at room temperature. Specimens are mounted for examination in the $KMnO_4$-treated fixative.	

Hoff and Mai (1964) killed and preserved specimens by injecting 5 mL of a suspension of nematodes in water into 20 mL of liquid nitrogen, which was then maintained at −35°C. When specimens are required, the block of ice containing them is allowed to thaw at room temperature and specimens can then be fixed and processed. Specimens can be maintained for several months at −35°C.

APPENDIX 11 - PREPARATION OF FIXED NEMATODES FOR SLIDE MOUNTING OR STORAGE

Processing fixed nematodes into glycerol can be carried out using slow or rapid methods, with differing advantages for each technique. Following Seinhorst's (1959) glycerol-ethanol method offers very good results and is a relatively quick technique. Slow methods, which can take several days or even weeks to process, will result in specimens in excellent condition (Garber et al., 1982). Malcevschi and Zullini (1973) also report satisfactory results by rapidly heating fixed specimens in 0.4 mL of 20% glycerol in a well slide at 50–60°C until they are infused with anhydrous glycerol. Ryss (2017) has recently published a simple express technique for fixing and processing nematodes into glycerol. The specialized stains described in Table 5 can be incorporated into the preparation method prior to slide mounting in glycerol. The rapid glycerol method after Seinhorst (1959) (see Section 1.1) and the slow glycerol method (see Section 2) are recommended but other methods may be used and are also briefly described (see Sections 1.2–1.5).

TABLE 5 Specialized stains for nematode specimens

Acetic orcein	Glacial acetic acid 55%, distilled water 45%, orcein powder 1% (this solution is unstable and should be prepared immediately before use) Carnoy's solution: glacial acetic acid 10%, absolute ethanol 60%, chloroform 30%, ferric chloride 1 g (optional)
Hirschmann (1962) made temporary mounts of nematodes to study the reproductive systems. Nematodes were killed by gentle heat before being fixed in Carnoy's solution. Specimens were stained in 1% acetic orcein for about 1 h and mount in 45% acetic acid. Yuen (1966) used this method for staining general somatic nuclei and the reproductive system. Hirschmann (1959) used a modification of Mulvey's (1955) propionic orcein method to stain the cephalic framework. Hirschmann and Triantaphyllou (1968) also stained specimens in 1% acetic orcein without previous fixation and examined them in dilute stain. Triantaphyllou (1975, 1979) gives details for staining chromosomes and Triantaphyllou (1981) treated fresh material with 0.5% colcemid, before staining, to improve separation of chromosomes.	
Nile blue B and toluidine blue	
Rodriguez-Kabana and King (1977) used toluidine blue for the differential staining of internal organs of nematodes. Fixed specimens are stained in 0.5% (w/v) toluidine blue in 0.05 M phosphate buffer (pH 4.6) for 7 h at 60°C, then differentiated in 0.01 M phosphate buffer (pH 4.6) for 5 h at 50–60°C. Kostyuk (1978) placed live nematodes in toluidine blue, 5 parts of 1% solution to 1 part glacial acetic acid, for 25 h at 37°C. Similar effects were achieved by using a 0.2% solution of Nile blue B with 0.1% acetic acid and 10% formalin for 24 h at 37°C.	
Gold chloride	Aqueous solution of 0.1% $AuCl_3$
Hasbrouck (1959) described a staining method for differentiating genital tracts, pharyngeal glands and the nerve ring. Fixed nematodes are washed in distilled water, then placed in an aqueous solution of 0.1% $AuCl_3$ and 0.1% $HgCl_2$ until they are light yellow. The specimens are washed for 10 min in distilled water, then placed in 1% formic acid and exposed to photoflood light, breaking down the $AuCl_3$ to colloidal gold, which stains the organs bright red. Nematodes can then be processed to glycerol and mounted permanently. Yadav and Chawla (1980) used $AuCl_3$ to stain phasmids in hoplolaimid nematodes.	
Silver nitrate	
Croll and Maggenti (1968) stained the peripheral nervous system of *Thoracostoma californicum*. Live nematodes were placed in a hypertonic solution of 10% $NaNO_3$ for 5 min, which shrinks them by exosmosis, then in 0.5% $AgNO_3$ for 15 s and finally in distilled water. Endosmosis occurs in the hypotonic $AgNO_3$, which is flushed into the tissues. This treatment kills the specimens, but after illumination colloidal silver is deposited in the peripheral nervous system, which remains visible in specimens subsequently processed to glycerol. This method is successful only on live specimens. Malakhov and Spiridonov (1982) stained hypodermal structures by soaking live nematodes in 0.1% $AgNO_3$ until paralysis before rinsing in distilled water and transferring specimens to photographic developer. Nematodes are rinsed again in distilled water before being fixed in 4% formaldehyde. The process could be stopped earlier by using the photographic fixative sodium thiosulphate. Bedding (1968) used 0.5% $AgNO_3$ to deposit metallic silver on specimens so that surface structures, such as body annulations and lateral incisures, are more easily seen. Rodrigues-Kabana and King (1976) reported improved staining with $AgNO_3$ (0.2 N for 3–4 min) when fixed specimens were pre-treated with 0.01 N iodine solution for 1–10 min or for 1 min with bromine solution (1 mL Br_2 in 390 mL w/v KBr)	
Vital staining	
It is difficult to use vital stains on nematodes because their cuticle is almost impermeable to most and few plant-parasitic forms ingest liquid in vitro. Doncaster and Clark (1964) showed that two species of rhabditid nematode ingested methyl red and neutral red pH indicator dyes with their food in vitro. The dyes were absorbed in different concentrations by different regions of the intestinal wall. Smith (1965) found that *Panagrellus redivivus* would sometimes ingest the redox indicator, 1-naphthol−2-sodium sulphonate-indole; the excretory system subsequently stained red. Günther (1973) obtained some reaction with a few dyes out of many tested	
Acid fuchsin	Acid fuchsin 0.5 g, distilled water 300 mL, 10% HCl 25 mL Add the water to the acid fuchsin, followed by the acid. The solution may be kept in a clear glass bottle.
Most commonly used to stain endo-parasitic nematodes in root material before de-staining plant tissue.	

1. Rapid glycerol methods

1.1. Method after Seinhorst (1959)

The glycerol-ethanol processing method after Seinhorst (1959) is routinely employed at the NRC-NPPO, Wageningen (NL) and numerous nematology laborarories.

1. After 14 days (after fixation) in 4% formalin solution in a saturated environment, transfer the nematodes with a needle to a fixation dish with a solution of 96% ethanol (20 mL), glycerol (1 mL) and distilled water (79 mL) (S1 solution) (Figure 36).
2. Place the dish in a desiccator with a small volume of ethanol 96% and place in an incubator at 40°C.
3. After a period of 16–24 h, remove solution S1 under the dissecting microscope; be careful not to damage or pick up the nematode specimens.
4. Add a solution of glycerol (7 mL) and 96% ethanol (93 mL) (S2 solution).
5. Cover the dish with a suitable coverslip and place it in a Petri dish on a filter paper moistened with glycerol (Figure 37) in an incubator (40°C).
6. After 2–3 h, add a few drops of dehydrated glycerol and store overnight in the incubator (40°C).
7. Remove the coverslip and place the fixation dish in a desiccator with silica gel for at least 2 days to remove the last traces of water.

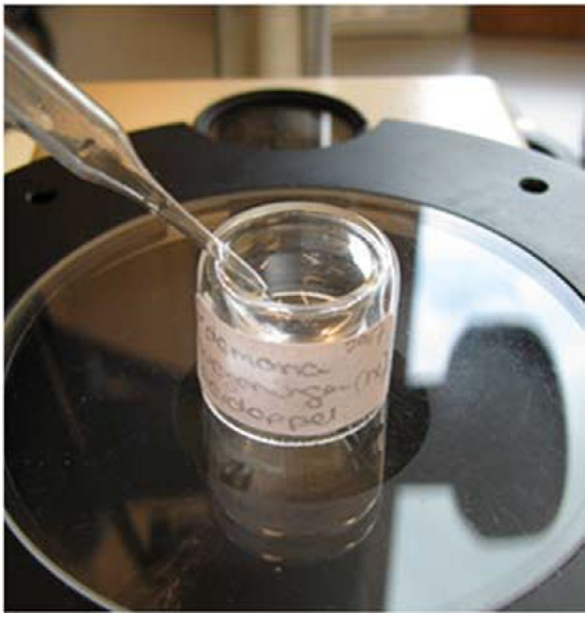

FIGURE 36 Fixed specimens added to glycerol solution. (Courtesy: NRC-NPPO, NL.)

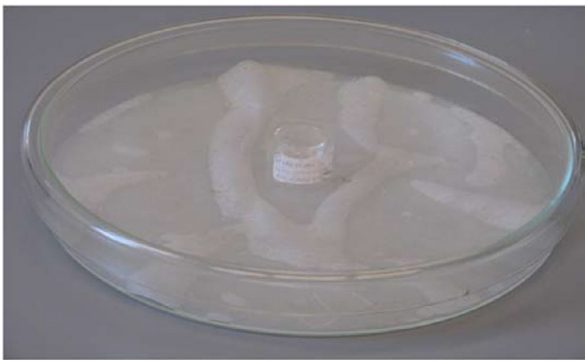

FIGURE 37 Specimens in solution on a filter paper infused with glycerol. (Courtesy: NRC-NPPO, NL.)

1.2. Method of Tarjan (1973)

Tarjan (1973) placed fixed specimens into hot (72°C) lactophenol for 1–3 min and subsequently into glycerol at 72°C. Siddiqi (1964) put fixed nematodes into a drop of hot lactophenol on a cavity slide and continued heating until the solution thickened before transferring into a warm mixture of 75% glycerol and 25% lactophenol for 5 min, and finally into glycerol.

1.3. Method of De Grisse and Choi (1971)

De Grisse and Choi (1971) transferred nematodes into a small drop of water in a cavity block and placed this in a vacuum desiccator containing an excess of ethyl alcohol. A dish of $CaSO_4$ was also included and supported on a mesh above the block. Air was evacuated from the desiccator until the ethyl alcohol boiled under the reduced pressure. The desiccator then remained under reduced pressure for 4 h before air was allowed to enter via a drying tube of $CaSO_4$. The cavity block containing the nematodes, now in ethyl alcohol, was topped up with Seinhorst solution II, which was then allowed to evaporate.

1.4. Method of Maggenti and Viglierchio (1965)

Maggenti and Viglierchio (1965) recommended dehydrating fixed specimens by processing to absolute ethanol in 5% increments or in automatic solvent exchanger (Viglierchio & Maggentti, 1965). The specimens are then transferred to a 5% solution of glycerol in absolute ethanol in a watch glass placed inside a Petri dish, the alcohol being allowed to evaporate overnight.

1.5. Method of Ryss (2017)

Ryss's rapid method processes a mass nematode suspension from fixative to glycerol using a modification of Seinhorsts hot fixation, with 4% formalin or TAF. For each nematode suspension, a single 1.5 mL Eppendorf tube is filled with fixative and 'floated' in a water bath at 100°C for 2–4 min. The nematode suspension to be fixed is placed in a 1.5 mL Eppendorf tube and stored vertically for 10 min or centrifuged at 3000 rpm for 30 s to allow the nematode to settle in the bottom of the tube. Once the nematode suspension has settled, using a pipette the top 2/3 of the supernatant is removed from the nematode suspension and replaced with hot fixative. Should the nematode mass exceed 1/3 of the level of the Eppendorf tube, it is recommended to retain water at double the height of the nematode suspension. The Eppendorf tube with the nematode suspension is sealed and floated in a water bath for 1 h at 90°C before being stored for a minimum of 48 h at room temperature prior to processing to glycerol. Fixed samples can be kept for an indefinite period before further processing.

To process the fixed nematodes to glycerol without damage, a stratified column of glycerol–water solutions containing different ratios of glycerol to water is required. To create the stratified column on a microscope slide, it is necessary to prevent the drops of liquid from spreading on the glass surface. The use of a hydrophobic beeswax–paraffin wax [histology mixture of beeswax and paraffin (1:5) with a 65°C melting point] ring attached to a glass microscope slide or similar alternative is recommended to contain the drops of liquid. Two drops of anhydrous glycerol are placed on the bottom of the wax ring and spread to form an even layer. Three drops of distilled water are placed on top of the glycerol, protecting the nematodes from the concentrated glycerol. Excess fixative is removed by washing the nematodes in distilled water three times before approximately 170 mL of the nematode solution is transferred into each wax ring. As a result, a high lens of liquid is formed within the hydrophobic wax ring, stratified in the increasing density of glycerol from top to bottom. Nematodes are

contained in the upper water layer, whilst the thin layer of glycerol is at the base. This lens of liquid is retained overnight to evaporate in a dust-free environment to allow the nematodes to be slowly infused with glycerol. The nematode glycerol solution may now be stored or processed directly onto permanent slides following a suitable method.

2. Slow glycerol method

After fixation, transfer nematodes to a 1.5% dilute glycerol solution in a glass staining block or small Syracuse watch glass (following the use of Ditlevsen's fixative, the inclusion of 7.5% ethanol to the solution is recommended). A trace amount of copper sulphate or picric acid can be added to prevent growth of mould; picric acid has also been reported to prevent clearing and fading of the basal part of tylenchid stylets (Hooper, 1970), but it does cause discolouration of the nematode cuticle over time. Transfer the glass staining block to a partially closed Petri dish and then to an oven set at 40°C or a desiccator. The diluted glycerol will evaporate very slowly; with most nematode species, evaporation to pure glycerol takes about 4 weeks but the period varies. Some species can be processed more rapidly, others can distort or collapse if the process is not very gradual.

APPENDIX 12 - PROCEDURES FOR RESTORING SLIDE-MOUNTED TYPE SPECIMENS

The following techniques are utilized at Fera Science (UK) during slide curation.

1. Restoring glass slides without a wax support

1. Take a clean glass slide and carefully remove the coverslips from the aluminium Cobb slide. In some cases, the coverslips may have adhered to the aluminium and will need to be carefully cut away using a scalpel.
2. Place the coverslip seal side up in the centre of the glass slide. Secure the coverslips to the glass slide using masking tape (Figure 38) and label the glass slide.
3. Working in a fume cabinet, place the 100 mL beaker with approximately 20 mL of anhydrous glycerol on a heating block at 65°C.
4. Place a drop of distilled water on the coverslip and gently clean the coverslip with lens tissue.
5. Using the microscope, asses the orientation and position of the nematode on the slide and the location of the remaining glycerol (Figure 39). Cut and remove seal in a position closest to the nematode but some distance from the remaining glycerol. Ensure that the nematode habitus curves away from the area to be cut away so that air bubbles will not become trapped around the nematode when the glycerol enters.

FIGURE 38 Coverslips on a Cobb slide removed and secured to a glass slide. (Courtesy: Fera Science Ltd, GB.)

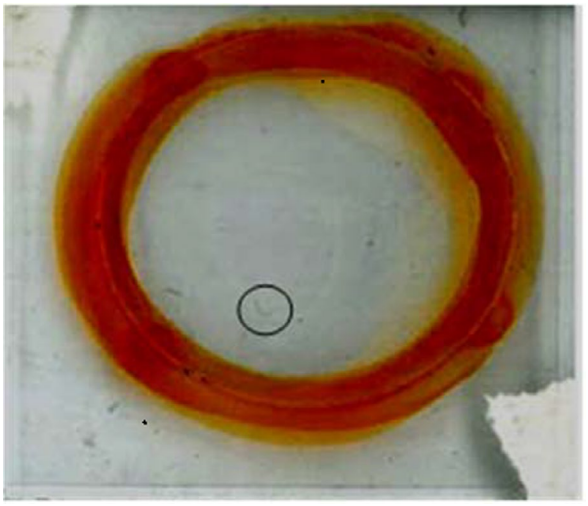

FIGURE 39 Recommendation of where to break the slide seal in relation to the position of the nematode and remaining glycerol. (Courtesy: Fera Science Ltd, GB.)

6. Using a scalpel cut away a section of the seal, taking care not to break the coverslip (Figures 40 and 41).
7. Place a drop of distilled water on the lens tissue and gently clean away any debris.
8. Using a pressurized air canister, spray short bursts to remove any remaining debris. Take the beaker of glycerol from the heating block, dip a needle into the glycerol and transfer a drop of approximately 25 µL of glycerol to the cutaway area of the seal (Figure 42).
9. Clean the needle and return the beaker of glycerol to the heating block.
10. Place the slide on the heating block for approximately 30 s. Return the slide to the microscope and observe the movement of the glycerol into the slide (Figure 43).
11. Repeat the addition of glycerol, if necessary, until the slide is fully re-infused. If the glycerol ceases to move then insert a hypodermic needle gently under the edge of the coverslip and return to the heating block for 30 s (Figures 44 and 45).
12. If no further glycerol moves under the coverslip, or a large air bubble is trapped at the opposite side to the broken seal, then remove a small section of the seal from this opposite side of the coverslip.

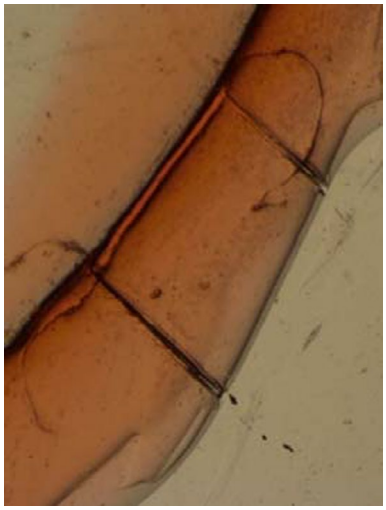

FIGURE 40 Incisions into the slide sealant. (Courtesy: Fera Science Ltd, GB.)

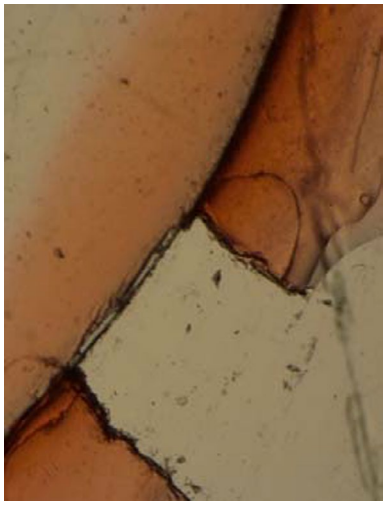

FIGURE 41 Removed section of sealant. (Courtesy: Fera Science Ltd, GB.) [Colour figure can be viewed at wileyonlinelibrary.com]

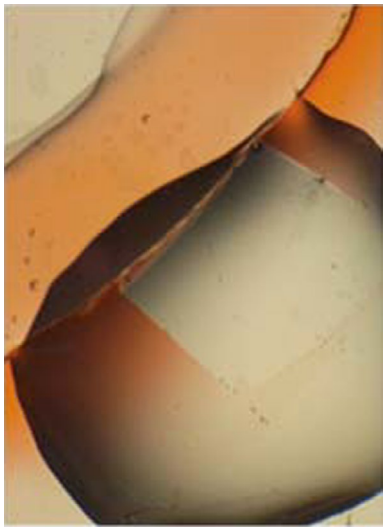

FIGURE 42 Glycerol placed onto the break in the seal. (Courtesy: Fera Science Ltd, GB.)

13. Gently apply pressure to the coverslip to push any bubbles towards the hole in the seal.
14. Once the slide is fully re-infused with glycerol, gently apply pressure to the coverslip to move any bubbles away from the nematodes. This also releases the nematodes if they have adhered to the slide or coverslip (they will move freely once released).
15. If there are too many air pockets that cannot be moved away from the nematodes (Figure 46), it may be necessary to remount the specimens.
16. Place a drop of distilled water onto the lens tissue and gently clean away any glycerol from the slide.
17. Place the slide on a slide-ringing stand, and re-seal it. Allow the seal to fully dry before repeating to give a robust double seal.

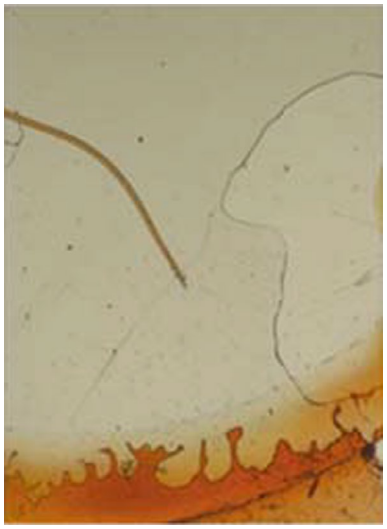

FIGURE 43 Glycerol infusing into the slide and specimen. (Courtesy: Fera Science Ltd, GB.)

FIGURE 44 Coverslip lifted with a hypodermic needle to encourage infusion of glycerol. (Courtesy: Fera Science Ltd, GB.)

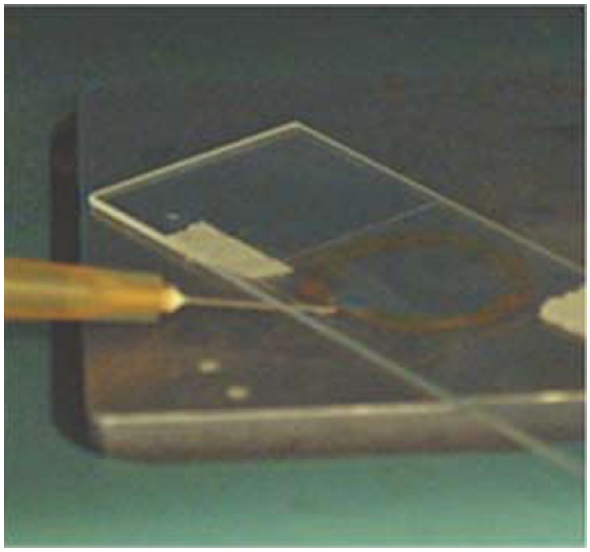

FIGURE 45 Slide with needle heated to 65°C to encourage further infusion of glycerol. (Courtesy: Fera Science Ltd, GB.)

FIGURE 46 Air pockets may obscure the view of important morphological features. (Courtesy: Fera Science Ltd, GB.)

18. Once dry, examine using a compound microscope to ensure the slide is now restored and return the slide to the collection.

2. Restoring glass slides with a wax support

1. Working in a fume cabinet, place a 100 mL beaker with approximately 20 mL of anhydrous glycerol on a heating block at 65°C.
2. Place a drop of distilled water on the coverslip and gently clean the coverslip with lens tissue.
3. Using the microscope, asses the orientation and position of the nematode on the slide. Cut away the seal in a position furthest from the nematode where there is the least amount of wax (Figure 47).
4. Place (a chisel-shaped) scalpel on the coverslip approximately 2 mm inside the wax. Insert fine forceps under the coverslip and grip it firmly.

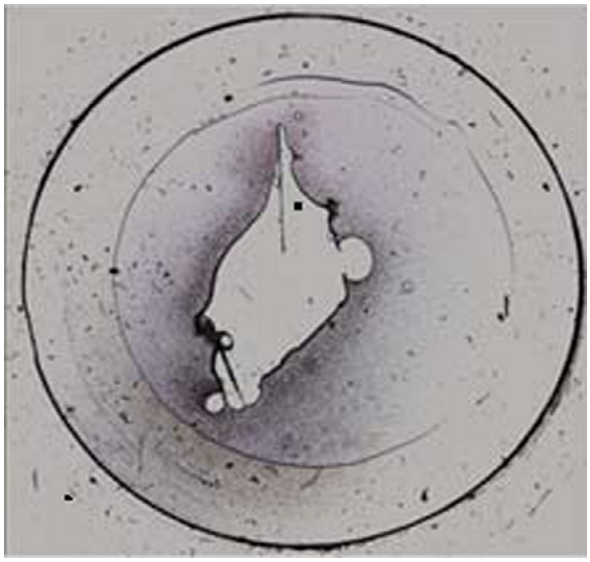

FIGURE 47 Wax-supported coverslip, with the position of the nematode specimen and te suggested section to remove indicated. (Courtesy: Fera Science Ltd, GB.)

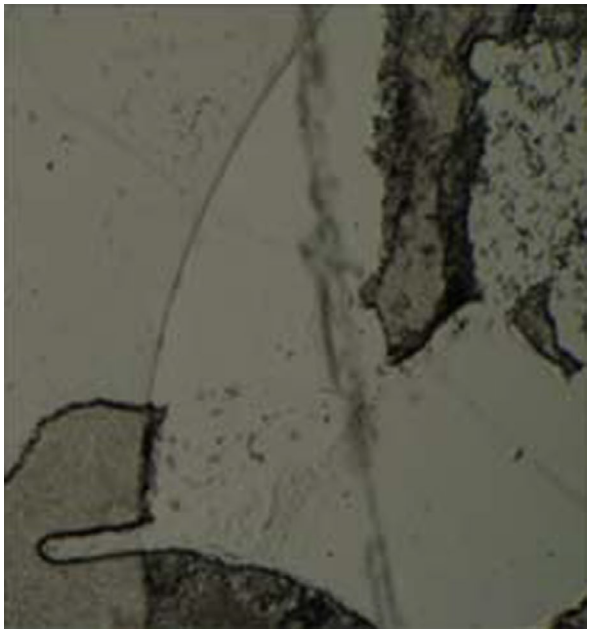

FIGURE 48 Section of coverslip and wax removed from slide. (Courtesy: Fera Science Ltd, GB.)

5. Taking great care, simultaneously apply light downward pressure on the coverslip and upward pressure with the tweezers to break a small piece of the coverslip (Figure 48).
6. This should have exposed the void inside the wax. If not, repeat step 3 until this has been achieved.
7. Take the beaker of glycerol from the heating block, dip a needle into the glycerol and transfer a drop of approximately 25 μL of glycerol to the void inside the wax (Figure 49).

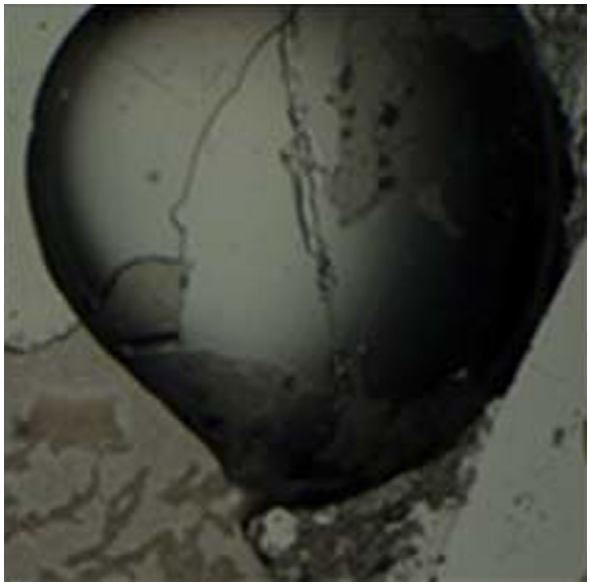

FIGURE 49 Glycerol placed onto break in the seal. (Courtesy: Fera Science Ltd, GB.)

1. Observe glycerol infusing under the coverslip. Do not heat the slide as the wax will melt and the specimen may be lost.
2. If the glycerol ceases to move then insert a hypodermic needle gently under the edge of the coverslip. Add more glycerol if necessary (refer to Figures 44 and 45).
3. Once the slide is fully re-infused with glycerol, gently apply pressure to the coverslip to release the nematodes if they have adhered to the slide or coverslip (they will move freely once released).
4. Remount the nematodes on new slides following the procedures outlined in the section 'Slide mounting nematodes'.

APPENDIX 13 - PRESERVATION OF SYMPTOMATIC PLANT MATERIAL

The following preservation method has been developed for use at the NRC-NPPO, Wageningen (NL).

1. Clean the plant parts carefully with water to ensure that the material remains intact. Dry with tissue and select an appropriately sized portion of the plant for preservation.
2. Half-fill the glass vial with a 6% formalin solution in demineralized water. Transfer the plant material to the vial.
3. Fill the vial with the formalin solution, ensuring the plant material is submerged.
4. If the colours of the plant material need to be maintained, add 0.2% copper sulphate to the formalin solution. After 1 year this is replaced by 6% formalin solution without copper sulphate.
5. Close the vial with the lid/stopper and leave in a suitable chemical storage container for about 6 months.
6. After the fixation period, pour off the formalin solution leaving the plant material in the vial.
7. Rinse the vial and plant material with 6% formalin solution if sand or loose plant parts are present.
8. If necessary, fix the plant material to a glass plate and place the plant material in a clean vial half-full of 6% formalin solution. Foam can be used to affix the glass plate to the vial.
9. Fill the vial to just below the bottom of the stopper with 6% formalin solution. Remove any bubbles of air in the liquid with a wooden stick. Lubricate the milled part of the stopper with a thin layer of Vaseline.
10. Place the stopper in the vial and check that the formalin solution is not touching the stopper. If the stopper is touching the formalin, remove the excess formalin solution with a pipette.
11. Turn the stopper a quarter to ensure the Vaseline is well distributed between the stopper and the vial.
12. Place plastic film over the stopper. Fix this firmly to the bottle and hold in place with an elastic band.
13. Finally tightly seal the plastic with wire. Fix the knot with a drop of adhesive and put a label on the vial.
14. Store the vials in a ventilated, dark cabinet at room temperature.