European Union Reference Laboratory

for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL–*B. xylophilus*-Extraction (BXE)_Version 01

February/ 2023

Protocol for the extraction of *Bursaphelenchus xylophilus* from wood

Foreword

These methods are recommended by:

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Contents

1. Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934; Nickle, 1970), is the only plant-parasitic nematode (PPN) listed among the 20 priority quarantine organisms in the European Union (Commission Delegated Regulation (EU) 2019/1702), and is also classified as an EU quarantine organism (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2021/2285 amending Implementing Regulation (EU) 2019/2072 as regards the listing of pests). The totality of EU countries must conduct official controls at EU borders and perform annual surveys for this nematode species. If detected, eradication measures should be taken to avoid their spread within the EU territory (Commission Implementing Decision (EU) 2012/535 and Regulation (EU) 2016/2031). This PPN causes the pine wilt disease (PWD) (Figure 1A), which in general only affects *Pinus* spp., known as the most susceptible host; however, it can also be found in other trees species of *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* (Evans *et al*., 1996). After *B. xylophilus* was exported from North America through infected timber to Japan at the beginning of the 20th century, the nematode spread further to other Asian countries (China and Korea). It was detected for the first time in Europe, in Portugal, in 1999, and 2008 in Spain (Mota *et al*. 1999; Abelleira *et al*. 2011; Fonseca *et al*. 2012). Check the EPPO Global Database (EPPO, 2022) for more updated information on geographical distribution.

As common among Aphelenchid species, the *B. xylophilus* feeds on both fungal (mycophagous) and plant cells, and is transmitted to dead or dying trees during oviposition by insect vectors from the genus *Monochamus* (Coleoptera: Cerambycidae) or to healthy trees during maturation feeding by the vector beetle (Mota & Vieira. 2008). The life cycle of *B. xylophilus* involves two forms: the propagative form (J1 to J4 and adult stages), which is present under suitable conditions, and the dispersal form (dauer juveniles J_{III} and J_{IV}) that is induced under unsuitable conditions, such as desiccation, food shortage, or environmental deterioration due to overpopulation. The dispersal form is intimately related to the vector beetle (Futai 2013). Thus, *B. xylophilus* can be found and detected in adult insects of *Monochamus* spp. (Figure 1B). When *B. xylophilus* is transmitted during oviposition, the nematodes remain relatively close to the introduction site. However, when transmission occurs through the young shoots and when the tree succumbs to PWD, the nematodes are distributed throughout the whole tree, destroying wood tissues such as epithelial cells, parenchyma cells of axial and radial resin canals, cambium and phloem. *Bursaphelenchus xylophilus* can also be found in roots, even when the above-ground part of the tree is already dead, dried out or felled. Symptoms may first appear on one or a few branches but often develop quickly throughout the crown, and trees may die only 1 or 2 months after symptoms appear. Symptoms start with needle discolouration, progressing rapidly from a greyish green to yellow and brown. *Bursaphelenchus xylophilus* feeds on fungi in the wood, including the bluestain fungi that are transmitted by engravers and other bark beetles.

Next to their occurrence in vector insects (*Monochamus* spp.), *B. xylophilus* can be present in several coniferous products: wood, wood products and packaging, wood chips/shavings, plants for planting, branches, isolated bark, but not in needles, cones or seeds. Therefore, samples of imported wood and standing conifer trees should be taken and verified for the presence of the nematode.

Figure 1 – Symptoms of Pine wild disease on *Pinus* caused by *Bursaphelenchus xylophilus* (A), and adult insect of *Monochamus* spp., vector of *B. xylophilus* (B). Pictures from INRAE-Biogeco and INRAE Orléans, France.

2. Terms, abbreviations and definitions

- Container: bowl, beaker, vial, tube, pot, jar, device, etc
- D: Detected
- ND: Not Detected
- EU: European Union
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- J1: first second-stage juveniles
- J2: second-stage juveniles
- J3: third-stage juveniles
- J4: fourth-stage juveniles
- J_{III}: Dauer juvenile (dispersal third-stage)
- J_{IV}: Dauer juvenile (dispersal fourth-stage)
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes
- PWN: pine wood nematode
- PWD: pine wood disease

3. Purpose and scope

The EURL recommended protocols are based on literature reviews and IPPC and EPPO standards[1], when available. These recommended methods, including operational procedures, were performed with the scientific experience and technical expertise of the EURL team. These methods have been adapted, optimised and further validated by the EURL laboratory.

The purpose of this EURL recommended protocol[2] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostic analyses by providing them with details on analytical operating procedures for the detection and identification of *Bursaphelenchus xylophilus*.

This document describes a EURL diagnostic protocol for the extraction of *B. xylophilus* from coniferous wood and bark (in the form of chips or small pieces of wood). The proposed extraction method also extracts other nematodes present in these matrices, including species from the *Bursaphelenchus* group. After nematode extraction, the protocols EURL-BXI_Version 01, 2022 and EURL-BX-qPCR_version 01, 2022, can be applied to the extracted nematodes.

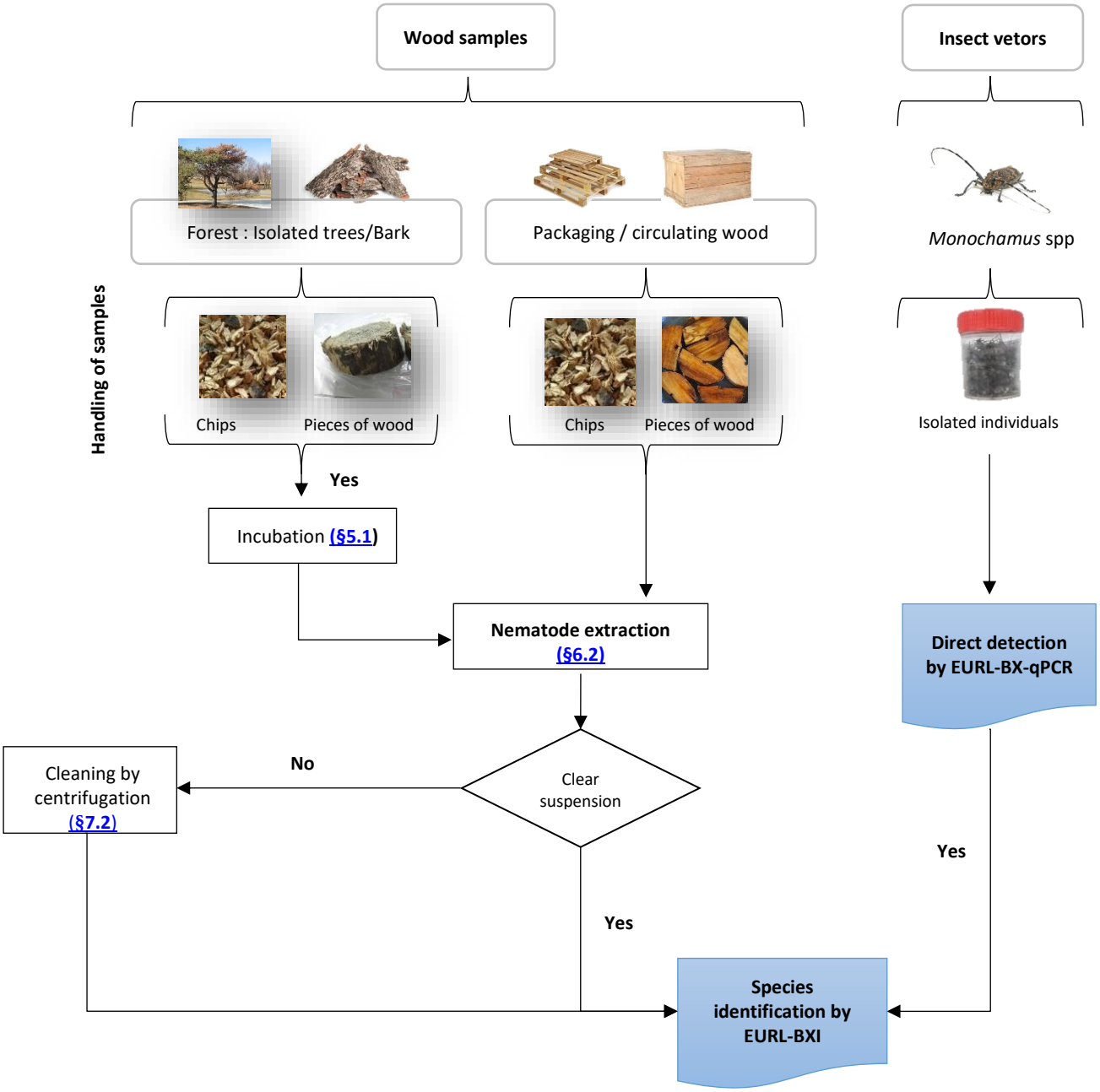
The material linked to this diagnostic protocol, such as slide presentations, technical videos & media, can be found on the EURL Plant Parasitic Nematodes website.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations. All actions taken in accordance with this method must be performed by employees who have attended relevant training.

[1] For more information on *B. xylophilus* biology, detection, and identification, view the standards EPPO PM 7/4 (4) and IPPC ISPM27 (DP10).

[2] The use of chemical brands or equipment in this recommended protocol does not imply the exclusion of others, which may also be appropriate.

4. Schematic procedure of the methods



5. Handling of samples

5.1 Wood

1) The size and volume of (sub) samples to be taken for *B. xylophilus* testing must allow optimal migration with standard laboratory equipment and limit the risks of dissemination linked to the vector.
2) Use all or part of the raw sample (e.g. 400 mL if possible) of wood chips, discs or bark. Pieces of approximately 1cm and not larger than 3cm x 3cm x 5cm should be used. Apply a cutting method that does not generate heat to avoid killing the nematode if present in the sample. These can be pruning scissors, a small saw, or even a drill that is not applied for too long to avoid heating.
   a. If taking a sampling of the raw sample, homogenise thoroughly before sampling.
   b. The 3cm x 3cm x 5cm width mentioned above is based on the size of the *Monochamus* larval chamber. This maximum is requested to avoid the risk of dissemination via an insect emerging from the samples.
3) Nematodes may occur in very low numbers in a sample and thus leading to difficult detection. Therefore a preliminary incubation of wood samples is highly recommended to allow nematode multiplication before extraction, increasing detection probability. In case of incubation, samples are slightly moistened (in the case of dry wood), put in a sealed plastic bag and incubated at approximately 25 °C for a minimum period of 15 days from the date of arrival. For more details on incubation and when applicable, check the updated EPPO PM 7/4.
4) The wood extract obtained after extraction can be stored at positive cold while waiting for their analysis.
5) During analysis, the remaining samples must be kept in the same conditions as mentioned in point 3) until the end of the analysis.

5.2 Insects

1) Depending on the containment level of the laboratory, it is necessary to be very vigilant that the insects are dead before opening the sample. The risk of *B. xylophilus* dissemination is very high for this type of sample. If extraction does not require nematode mobility, insects can be killed by exposing them to -20°C.
2) For more information regarding the extraction of *B. xylophilus* from insect vector, please check the the updated EPPO PM 7/4.

6. Extraction from wood material

Live nematodes are extracted from infected wood samples using the Baermann funnel[3] or the Oostenbrink dish. This protocol details the extraction procedure using the Oostenbrink dish. Briefly, small pieces of wood or wood shavings are immersed in water and placed on an Oostenbrink dish, where the live nematodes migrate from the wood into the water for a set time. The suspension is collected and further analysed for the presence or absence of nematodes. The detailed procedure is described below.

6.1 Material and consumables

6.1.1 Equipment and small materials

- Sieve greater than or equal to 40 µm for migration (or any perforated support such as kitchen strainer)
- Sieve of 20 µm for suspension recuperation (or possibility to use a sieve of 25 µm)
- Dishes, containers or basins
- Small container (for nematode suspension recovery)

6.1.2 Consumables

- Milk filters (e.g. cotton-wool milk filter), wiping paper or any similar paper (1 or 2 layers)
- Beakers, bowls and containers
- Knife or pair of scissors, spoon or spatula
- Kaolin (quantity to be adjusted, according to the centrifuge container diameter (e.g. approximately 10 g for a container of ~ 8.6 cm of diameter)
- $MgSO_4$ Solution (for preparation, **see Appendix §7.1**)

6.2 Extraction procedure

1) Place the pieces of wood on wiping paper or milk filter held by the perforated support (Figure 1).
2) Place the support in a dish and fill the dish with water so that the support's contents (e.i. wood) are in permanent contact with the water (add more if absorbed) (Figure 1).
3) Live nematodes migrate through the paper/filter and sink to the bottom of the dish.
4) Allow nematodes to migrate for 24 to 48 hours at room temperature.
5) Pour the nematode suspension contained in the dish onto a 20 µm sieve.
6) Collect nematodes on top of the sieve into a beaker using a water bottle.
   a. **If the suspension is clear**: the extraction is finished, and nematodes can be observed for detection and identification (morphology and/or molecular analysis).
   b. **If the suspension is dirty**: clean it via centrifugation (**see Appendix §7.2**).

[3] The Baermann funnel technique and Oostenbrink dish are described in the EPPO (2013). PM 7/119 (1): Nematode extraction. EPPO Bulletin. 43 (3), 471-495.

Figure 1 –Extraction of *B. xylophilus* from bark using the Oostenbrink dish. Picture ANSES-LSV, France.

7) The obtained suspension is kepp at room temperature if nematode detection or identification is carried ou on the day of extraction, otherwise, store at 4°C.
8) After nematode extraction, the protocols EURL-BXI_Version 01, 2022 and EURL-BX-qPCR_version 01, 2022, can be applied to the extracted nematodes.

If you have any question about this protocol, please, send an e-mail to eurl.nematode@anses.fr.

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These recommendations were prepared by the EURL consortium composed of ANSES - Plant Health Laboratory - Nematology Unit and ILVO - Plant Unit - Nematology in the frame of EURL activities.

7. Appendices

7.1 Preparation of a solution of MgSO4 with a 1.18 density

1) Sulphate Dilution.

For example, to prepare about 20 L of a solution with a density close to 1.18:

- Weigh about 7.2 kg of $MgSO_4$.
- Add 12L of water (hot water is better for dissolution),
- Mix until $MgSO_4$ is fully dissolved,

2) Cooling: Allow to cool to room temperature (e.g. overnight).

3) Adjustment of Density, if needed.

- Measure the density of the solution with the density meter,
- Adjust the density to 1.18 by adding small volumes of water (at room temperature) or $MgSO_4$ (stir the mixture after each addition prior to measuring the density again and repeat if necessary).

4) Filtration, Conditioning and Storage

- Filter through a sieve of a maximum 40 µm and store the solution in a container at room temperature.
- Keep the solution away from light to prevent algae growth.

Note: *Adjust the quantities if other products than* $MgSO_4$ are used. *The quantities of product to be added, depending on the desired density, are shown in the following table:*

Required quantities in grams of each product to be diluted in water, adjusting the quantity to obtain a final solution of 1 litre at the indicated densities (based on SOUTHEY, 1986)

Desired Density (20°C)	**1,15**	**1,18**	**1,22**
Sugar	401	484	588
$MgSO_4$ (pure)	166	200	245
$MgSO_4$ $7H_2O$	339	409	503
$ZnSO_4$ (pure)	156	187	229
$ZnSO_4$ $7H_2O$	279	335	410

Check the density with a density meter before the use of the final product.

7.2 Cleaning of the nematode suspension via centrifugation

1) Nematode suspension are transferred to a centrifuge container with a wash-bottle jet of $MgSO_4$ (density of 1.18).
2) Put a spoonful of kaolin in the centrifuge container (quantity to be adjusted, see **§7.1.2**).
3) Fill the container to about three-quarters of its volume with water.
4) Homogenise the suspension.
5) Centrifuge the suspension for 2 min at 900 g.

Note: *Centrifugation*[4] *speeds and times are approximate*

[4] EPPO (2013). PM 7/119 (1) describes that centrifugation speed and time are not critical and can vary from **2 to 5 min** and from **700 to 2900 g.**

6) Empty the supernatant through a pre-moistened 20 µm sieve. Rinse immediately and abundantly to remove any remaining $MgSO_4$ and collect the contents of the 20 µm sieve into a collecting container using a wash bottle with water.
7) The obtained suspension can be kept at room temperature if nematode detection is carried out the same day; otherwise stored at 4°C.
8) The nematode suspension can be further used for nematode detection by morphometric and/or by molecular analysis.

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