European Union Reference Laboratory
for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL–Globo-Viability (GV)_Version 01

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Protocol for determining the viability of eggs and juveniles of *Globodera rostochiensis* and *G. pallida*

Foreword

These methods are recommended by:

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1 Introduction

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber, 1923) and *Globodera pallida* (Stone, 1973) are plant-parasitic nematodes that cause significant losses in potato (*Solanum tuberosum*). Given the damage caused by these nematodes, they are regulated by a majority of countries in the world (EPPO 2017) and classified as quarantine organisms in the European Union (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2019/2072) in order to avoid their spread within the EU territory and the introduction of new populations.

The application of this protocol is part of the monitoring to assess the spread of these pests in Europe according to European Council Directive 2007/33/EC of 11 June 2007 (under revision).

The EURL diagnostic protocols are based on literature reviews and IPPC and EPPO standards, when applicable. This diagnostic protocol, including operational methods, was chosen as the basis, and with the scientific experience and technical expertise of the EURL team, some of these methods have been adapted, optimised and further validated by the EURL laboratory.

The purpose of this EURL recommended protocol[1] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostics analyses by providing them with details on analytical operating procedures for identification of *G. rostochiensis* and *G. pallida*.

Warning and safety precautions: The user of this method 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] The use of chemical brands or equipment on this recommended method does not imply the exclusion of others, which may also be appropriate.

2 Terms, abbreviations and definitions

- Attractant: Chemical substance present in the root exudate of host plants
- Cysts: the sclerotised cuticle of the deceased Heteroderinae, particularly *Globodera-, Heterodera-* and *Punctodera* females, usually filled with eggs and juveniles
- EPPO: European and Mediterranean Plant Protection Organization
- Hatching test: Analyse the viability of the eggs by observing the hatching of juveniles from the eggs/cysts following exposure to root exudates of host plant
- IPPC: International Plant Protection Convention
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematode(s)
- Swollen stages: sedentary immobile nematode stages inside the root, e.g. third-stage juveniles (J3), fourth-stage juveniles (J4) and females of *Globodera* spp.
- Vermiform: mobile, worm-shaped, e.g. the second-stage juveniles (J2) and males of *Globodera* spp.

3 Purpose and scope

The purpose of this document is to describe a EURL diagnostic protocol for testing the viability of eggs and juveniles of *Globodera rostochiensis* and *G. pallida.* In this protocol, the viability determination can be performed by visual assessment and a hatching test. The described protocol allows a qualitative analysis and the detection of viable juveniles in cysts of *Globodera pallida* and *G. rostochiensis*.

4 Material and consumables

4.1 Equipment

- Stereoscopic microscope with episcopic and diascopic illumination (magnification to a minimum of 50X)

4.2 Consumables and small materials

- 150 µm sieves
- Counting dish
- Fishing tool, small metal tip, paintbrush or other appropriate tool or instrument suitable for handling cysts and vermiform nematodes
- Pipette
- Container (Petri dishes, glass vials, wash glass disc, etc)
- 96 multiwell plate
- microscope slides and coverslips
- Tissue grinders-homogenisers
- Small scalpel or a syringe needle
- Root exudate (production is described in appendix **§7**)

5 Determining viability with a hatching test

An easy way to determine if a *Globodera* cyst contains viable juveniles is to induce their hatching. Indeed, if juveniles hatch and come out from the cyst, this means they are alive. In nature, juveniles within cysts hatch in the presence of their host plant. This presence is detected via the perception of the root exudates of the host plant as they contain chemical attractants acting as hatching factors. Hatching tests performed on Globodera sp. are based on this host-parasite relationship and consist of exposing the cyst to root exudates of the host plant (potato or tomato in the case of *G. pallida* or *G. rostochiensis*).

- ***Note:*** When sampling is performed during the autumn (after potato harvest, for example), samples can include newly formed cysts that may be dormant. In that situation, it is preferable to place cysts at +4°C for at least 2-3 weeks to break the dormancy.

5.1 Setting up the test

1) Place a 150 µm sieve on a small watch glass dish filled with 4 to 5 ml of root exudate (see 7.1 for production information). One sieve is prepared for each sample.
    - ***Note:*** *The test can also be set up in 96 multiwell plate with 200 µl of exudate. Each well corresponding to a sample.*
2) For each sample, place the cysts in each sieve, or if using a well plate, 20 to a maximum of 30 cysts per sample, separately in each well. Seal the test with the lid to prevent spillage and evaporation.
3) Positive control: Aside prepare a batch of approximately 20 cysts (i.e. cysts containing viable juveniles) for each target species to ensure the validity of the test.
4) Incubate the test at room temperature in dark conditions to prevent exudate degradation and algae growth until they are checked for hatching.

5.2 Examination of the hatching test

5) Check the watch glass (or multiwall plate) every 1-2 weeks for the presence of hatched juveniles in the sample. At each examination time, old exudate is replaced by fresh exudate.
    - Note: Also, make sure there are hatched juveniles for positive controls. If it is not the case, the viability test is not considered valid
6) As soon as one juvenile hatches, **the test is positive.**
7) When no hatching occurs after three checks (i.e. up to 45 days), the cysts are crushed, and the viability of juveniles is assessed by visual examination (**see §6**).
8) **When viable juveniles are detected, the test result is positive**; otherwise, **it is negative**.
9) After the analysis, the small equipment (sieves, glass vials, Petri dishes, plates etc.) are disinfected with boiling water or bleach.

6 Determination of viability by visual assessment

1) Soak the cysts for a period of at least 12 hours in a Petri dish or any other container filled with tap water and sealed to avoid evaporation (the cysts can be soaked for more than 12 hours (up to 1 week), but they should be kept cold (4 – 6 °C) to reduce fungal growth.
    - ***Note:*** *Most hydrated cysts sink to the bottom of the container.*
2) Transfer the cysts with an appropriate tool to a drop of water on a glass slide or a tissue grinders-homogenisers and crush the cysts. Observe the crushing and the release of the eggs and juveniles from the cysts through the stereoscopic microscope.
3) Check that no eggs and juveniles are left in the cysts. If necessary, remove the contents from the cysts with a syringe needle (or other appropriate tools) or crush several times until all the content is released from the cysts.

4) Rinse the crusher and slide glass, collect and transfer the suspension to a counting dish.
    - ***Note:*** *If many eggs and juveniles are present in the content, dilute the suspension to facilitate observation.*
5) Allow the suspension to settle (all eggs and juveniles at the bottom of the dish) for approximately 10 min.
6) Check for the viability of the eggs and juveniles through the stereoscopic microscope at a magnification of 25x to 50x. The decision of whether the eggs and juveniles are alive or dead is made based on their appearance (see Table 1 and Fig. 1A to L).
    - ***Note:*** *After crushing the eggs and juveniles, one can soak them for at least 12 hours in water to allow the rehydration of dry eggs and larvae (old cysts). It eases the visual examination as some juveniles become mobile after a few hours in tap water.*

Table 1. Characteristics of live and dead eggs and juveniles of *G. rostochiensis* and *G. pallida*. These characteristics are also detailed in the EPPO PM 7/40 (4), 2017.

Live eggs (Fig 1. A, B and C)	**Dead eggs (Fig 1. G, H and I)**
a Whole egg intact	**a** Damaged, broken and empty
b Egg with a smooth shell	**b** no smooth shell
c Clear, transparent egg with distinct contents or with a black line in the middle of the egg	**c** Grained, grey/black content, no structure
d The curved juvenile lies pressed against the egg wall	**d** Shrivelled, disintegrated juvenile in the egg
e Sometimes, the clear pharynx and stylet are visible	**e** No clear pharynx and stylet visible
Live juveniles (Fig 1. D, E and F)	**Dead juveniles (Fig 1. J, K and L)**
a Juvenile has a clear pharynx, stylet visible	**a** No clear pharynx, complete or partially grey/black structure
b Juvenile with smooth cuticle	**b** Cuticle shrivelled or no longer intact
c Intestine has a grey, contiguous grainy content	**c** Intestine transparent, body with transparent spots or completely transparent
d Clear oblique separation between pharynx and intestine	**d** No clear separation between pharynx and intestine
	e Juvenile is kinked or in a semicircle
Included in the counts:	**Not included in the count**
Heads	Tails, empty shells

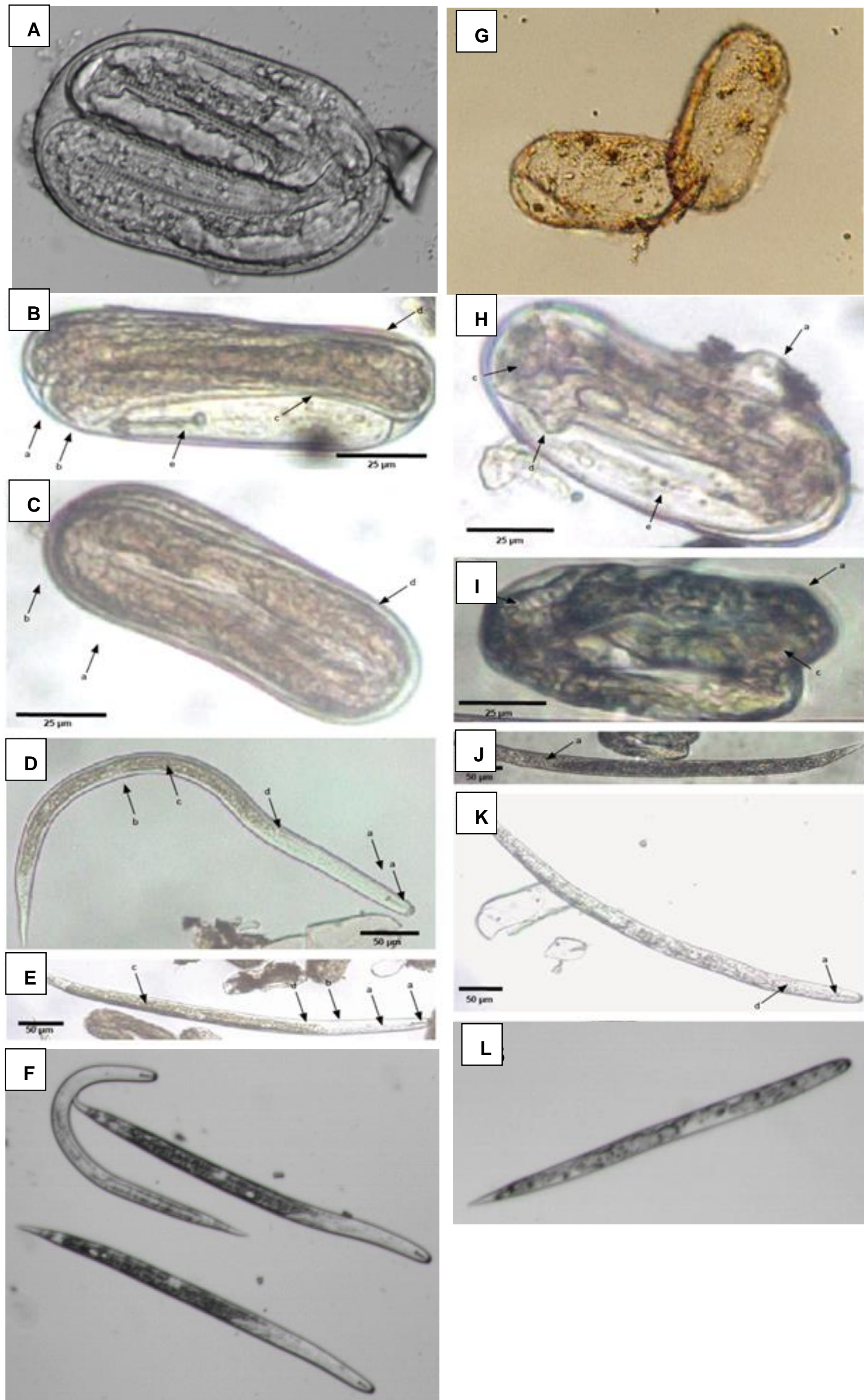

Figure 1. (A to L) Characteristics of live and dead eggs and juveniles of *G. rostochiensis* and *G. pallida* (Picture adapted from EPPO PM 7/40 (4), 2017, Picture A,F,G and L from IVO, Belgium).

7 Appendix

Root exudate production for hatching tests

1) Use a potato variety susceptible to *Globodera pallida* and *G. rostochiensis* (e.g. Desiree).
2) Germinate potato tubers on top of containers with tap water so that the roots can develop for 3 to 4 weeks.
3) Provide 200-500 ml of water per tuber.
4) Place the tubers at room temperature and protect them from light to avoid fungal growth.
5) The root exudate (water containing the attractant emitted by the potato roots) is passed through a 40 µm sieve and then packaged (tubes or plastic pots) and frozen.
6) The root exudate can be stored at -20°C for several years. After thawing, it can be stored between 4 and 8 °C for 3 weeks.

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

Acknowledgements
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8. References

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