International Rules for Seed Testing 2023

Validated Seed Health Testing Methods

7-031: Filtration method for detection of *Ditylenchus dipsaci* in *Medicago sativa* (alfalfa); *D. dipsaci* and *D. gigas* in *Vicia faba* (faba bean) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2022, Cairo, Egypt

Effective from 1 January 2023

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-031: Filtration method for detection of *Ditylenchus dipsaci* in *Medicago sativa* (alfalfa); *D. dipsaci* and *D. gigas* in *Vicia faba* (faba bean) seed

Host: *Medicago sativa* L. and *Vicia faba* L.
Pathogen(s): *Ditylenchus dipsaci* Kuhn, 1857*; Ditylenchus gigas* n. sp.

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Revision history

Version 1.0, 2018-01-01: New method
Version 1.1, 2021-01-01: Sample size and Methods revised
Version 1.2, 2023-01-01: Materials, Methods and Critical control points revised

Background

Ditylenchus dipsaci is known as the stem and bulb nematode. It occurs in most temperate areas of the world, including the Mediterranean region (Volvas *et al*., 2011). About 30 different biological races have been identified in many different hosts. Although they are morphologically similar, they are host specific (Esquibet *et al*., 2003). In 2011, what had previously been identified as 'giant race' (Sturhan and Brzeski, 1991; Kerkoud *et al*., 2007) was genetically characterised and became known as *D. gigas* (Volvas *et al*., 2011). EU phytosanitary rules and regulations require that all seed lots for national and international trade must be tested for the presence of *D. dipsaci* and *D. gigas* on alfalfa. One of the aims of the EU TESTA project (https://secure.fera.defra.gov.uk/testa/) was to harmonise within Europe an efficient detection method for *Ditylenchus* sp. on alfalfa and faba bean. In a pretest, a GEVES sieving method was compared to a NIAB decantation method (GEVES, DGAL MOA 13 and NIAB 013 STNEM beans v5 and described in the EPPO protocol PM 7/87 [1] *Ditylenchus destructor* and *Ditylenchus dipsaci*, Bulletin 38, 2008). The GEVES method was chosen for the validation test and two different PCR protocols (Kerkoud, 2007; Wood, 2014) were used to validate results (GEVES, ISHI-Veg/TESTA WP5/ISTA 2015).

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions. It is assumed that this procedure is being carried out in a Nematology laboratory by persons familiar with the principles of Good Laboratory Practice, Good Nematology Practice. This test protocol involves the heating of nematodes on microscope slides, as part of the examination process. Measures to reduce the risk of shattering the glass are outlined at that stage in the procedure.

D. dipsaci is a quarantine pest in some areas of the world on alfalfa seeds (EPPO A2 list no. 174, EU Annexe designation: II/Council Directive 2000/29/CE Chapter II a.4) and should be handled according to each country's regulations (CCP).

Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. If possible, use an alternative chemical, e.g. Gel Red™ (Biotium). Use ethidium bromide according to safety instructions. It is recommended to work with solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an 'ethidium bromide' designated area in order to keep ethidium bromide contamination to a minimum.
- Also read and take into account safety precautions included in any commercial kit used.

Ultraviolet light

Ultraviolet (UV) light must not be used without appropriate precautions. Ensure that UV protective eyewear is utilised when working with ethidium bromide.

Treated seed

This method has been shown to be suitable for the detection of *Ditylenchus* spp. on treated seed using physical treatment in the TESTA project. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample (total number of seeds tested) and subsample size to be tested depend on the desired tolerance standard (maximum acceptable number of seeds infested) and detection limit (theoretical minimum number of nematodes per sample which can be detected). An example of a recommended minimum sample size for faba bean with a detection limit of 1.5 nematodes per 100 g in the seed lot and a zero tolerance, is 900 g, using ISTA sampling methodology calculation adapted to sieving method (Macarthur *et al*., TESTA Deliverable 2.4). The minimum sample size should be 100 g of seeds for alfalfa and 300 g for faba bean (TESTA WP2-Sampling), and the maximum subsample size should be 100 g of seeds for alfalfa and 300 g for faba bean. The whole sample is tested.

Materials

Reference material: known nematode of *D. dipsaci* and *D. gigas* or standardised reference material

Containers: plastic basin with a capacity of 1000 ml maximum

Sieves: 250 µm sieve cover with soft filter paper or equivalent nematode-permeable container (i.e. non-woven plant growth bag) and 20 µm mesh sieve

Plate: 9 cm diameter glass Petri dish

Rinse: pipette with tap water

Observation: stereo-microscope and microscope, slide and coverslide

Harvest of nematodes: hair, eyelash, hair brush device

Ultra pure water and chemicals: for PCR preparation

Sterile microtubes: 1.5 ml; 0.2 ml

Microliter pipettes: e.g. Gilson, Finn, with sterile filtered tips (1–1000 µl)

DNA extraction: Two kits were used during validation experiments – Macherey Nagel NucleoSpin Tissue, protocol 'animal tissue' and Qiagen blood and tissue kit. An equivalent kit may be used if validated by the laboratory applying the method.

Conventional thermocycler

Electrophoresis equipment: 1.5–2 % agarose gels

DNA visualising system: BET or analogue reagent, UV imaging apparatus

PCR primers (Kerkoud *et al*., 2007 based on two forward primers (DdpS1 and DdpS2) and one reverse (rDNA2)):
Forward: DdpS1 (5'-TGGCTGCGTTGAAGAGAACT-3') is specific to *D. dipsaci*, while DdpS2 (5'-CGATCAACCAAAACACTAGGAATT-3') anneal to both *D. dipsaci* and *D. gigas*;
Reverse: rDNA2 (5'-TTTCACTCGCCGTTACTAAGG-3') rDNA2 can be used with rDNA1 5'-TTGATTACGTCCCTGCCCTTT-3' (Vrain *et al*., 1992) as a nematode amplification control.

Methods

1. Sieving method

1.1 Place a 250 µm mesh sieve or equivalent nematode-permeable container (i.e. non-woven plant growth bag) in a plastic basin.

1.2 Line the sieve with soft filter paper (wipes) and humidify it.

1.3 Pour the seeds into the lined sieve.

1.4 Add water (approx 1 l depending of the size of the plastic container): seeds need to be completely covered by water during soaking. If seeds are not completely covered, it is possible to add water. Allow nematodes to migrate during a minimum 24 h at room temperature (Fig 1a, b).

1.5 After migration, the sieve containing the seeds and the filter paper is removed and the water containing nematodes is filtered through a 20 µm mesh sieve (Fig 1c, d).

1.6 Rinse the 250 µm sieve or equivalent nematode-permeable container (i.e. non-woven plant growth bag) and the basin with tap water through the 20 µm mesh sieve in order to collect all nematodes.

1.7 Transfer everything collected from the 20 µm mesh sieve to a glass Petri dish. Add the minimum quantity of water needed to create a suspension to ensure all nematodes are recuperated (CCP) (Fig 1e, f).

1.8 Examination under binocular magnifier and microscope for identification of the *Ditylenchus* genus. The identification of *D. dipsaci* and *D. gigas* is based on morphological characters observed under stereo-microscope at low magnification: aspect of the body (long, thin); swimming style of the nematode

(undulating); shape of head (round to slightly flattened and not swollen compared to the body); shape of the tail (pointed conidial and short); head and tail slightly transparent and middle of the body is more dense. Moving nematodes are considered alive and motionless and degraded nematodes are considered dead. Identification of suspect nematodes can be done either by morphological criteria (2) or PCR (3).

2. Morphological confirmation
Examination with binocular magnifier and microscope for identification of *D. dipsaci* or *D. gigas*. Suspect nematodes are harvested and placed individually between slide and coverslides for identification. Examination of the specific morphological characters is done at high magnification (×100) and nematodes need to be immobile. To ensure immobility, nematodes are heated (to approximately 60 °C for about 10 to 30 s). The following observations are made under a microscope: the size of the stylet (10–12 µm); body size (1000–1300 µm for *Dd* and 1373–1950 µm for *Dg*); number of lateral lines (4) and the shape of tail (should be conical and pointed). Morphological characters are described in PM 7/87 (EPPO 2008) and Volvas *et al*. (2011) (Fig 2a, b). All of the above characters must be present on alfalfa seeds to identify nematodes as *D. dipsaci*. On faba bean seeds, nematodes with all the above criteria can be identified as *Ditylenchus* sp. However, as morphological criteria cannot be differentiated between the two species, except at the adult stage, a PCR is required.

Morphological characters for *Ditylenchus dipsaci* and *D. gigas* identification

	According to PM 7/87 (1) Bulletin EPPO bulletin 38, 2008		According to Volvas *et al.*, 2011	
Characteristics	*D. dipsaci* female	*D. dipsaci* male	*D. gigas* female	*D. gigas* male
Body length (µm)	1000–1300	1000–1300	1780 (1561–1932)	1557 (1373–1716)
Stylet length (µm)	10–12	10–12	12 (11.5–13)	11.6 (11–12.5)
Body width (µm)	36–40	37–41	48.9 (43.0–56.4)	56.7 (34.3–63.0)
Number of lateral lines	4	4	4	4
Vulva position (%)	82 (79–82)	–	81.5 (80–83)	–
Vulva–anus distance	1¾–2¼ tail length	–	228 µm (208–266)	–
Pharengial length (µm)	6.5–7.1	6.5–7.3	8.5 (7.3–9.3)	8.3 (6.7–10.7)
Tail length (µm)	14–18	11–15	20 (16.8–27.6)	17.9 (15.7–20.0)

3. Polymerase chain reaction (PCR) confirmation

3.1 Each suspect nematode is placed in a microtube with 180 µl of kit extraction buffer for further extraction. Add a negative control (a nematode different than *D. dipsaci*), a positive control (*D. dipsaci*) and a negative process control (extraction buffer).

3.2 DNA extraction is performed using a commercial kit according to the instructions provided.

3.3 Use Kerkoud primers and nematode amplification control primers in a separate PCR reaction with the same DNA extract. Using water as a negative PCR control.

3.4 The mix and program for Kerkoud PCR is described in the tables below.

3.5 For the nematode amplification control, the mix is described in a table below (*Example of PCR mix and program for nematode amplification control*); same program as the Kerkoud primers.

3.6 Fractionate 10 µl of the PCR products containing a loading buffer on an agarose gel of 1.5 %, with 100 bp ladder (migration conditions 180 V for 45 min) for example. Stain with ethidium bromide in a bath and rinse in water.

3.7 An electrophoresis profile showing both a 517 bp (DdpS2 / rDNA2) and a 707 bp band (DdpS2 / rDNA2) identifies *D. dipsaci*. If only the 707 bp band is observed, the tested individual is identified as *D. gigas* (Fig 3).

3.8 The profile for the nematode amplification control can give one or several bands around 700–1400 bp (Use the 1 kb ladder for the electrophoresis run). Some bands can appear in the extraction control (Fig 4).

Example of program for Kerkoud protocol

Number of cycles	Temperature	Duration
	94 °C	1 min
40	94 °C	30 s
	55 °C	30 s
	72 °C	45 s
	72 °C	5 min
2H15	10 °C	

Decision table

Nematode amplification control	DNA amplified by Kerkoud PCR	Follow-up
positive / negative	707 bp	*D. gigas* identification
positive / negative	517 bp and 707 bp	*D. dipsaci* identification
positive	negative	No *D. dipsaci* or *D. gigas* identification
negative	negative	Not valid, test must be repeated

General methods

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the scientific name of the pathogen at the genus or species level, and the presence of mobile (alive), motionless, or motionless and degraded (dead) nematodes observed.

Quality assurance

Critical control points (CCP)

D. dipsaci is a quarantine pest on alfalfa seed in some areas in the world, so national regulations must be respected. The 250 µm sieve or equivalent nematode-permeable container (i.e. non-woven plant growth bag) and 20 µm sieve used in the filtration protocol must be rinsed thoroughly to ensure all nematodes present are collected. To avoid cross contamination, sieves, basins and Petri dishes must be carefully cleaned, rinsed with hot water, disinfected using sodium hypochlorite and then put through a dishwasher cycle after each use.

Media and solutions

Example of PCR mix for nematode amplification control

Compound	Concentration	Final concentration	Volume in 20 µl
Sterile ultra pure water			9.24
Buffer	10×	1	2
$MgCl_2$	50 mM	1.5	0.6
dNTP	2 mM	0.2	2
rDNA2	10 µM	0.5	1
rDNA1	10 µM	0.5	1
Platinum Taq	5 U/µl	0.04	0.16
DNA matrix			4

Example of PCR mix for Kerkoud protocol

Compound	Concentration	Final concentration	Volume in 20 µl
Sterile ultra pure water			6.2
Buffer	10×	1	2
$MgCl_2$	50 mM	1.5	0.6
dNTP	2 mM	0.2	2
DdpS1	10 µM	0.5	1
DdpS2	10 µM	1	2
rDNA2	10 µM	1	2
Platinum Taq	5 U/µl	0.05	0.2
DNA matrix			4

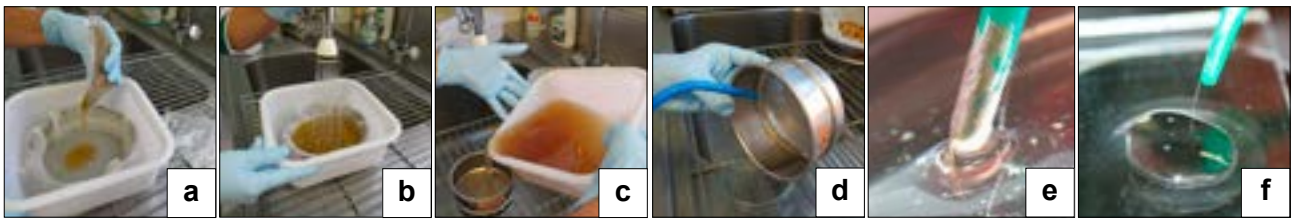

Figure 1. a–f Nematode extraction by filtration method.

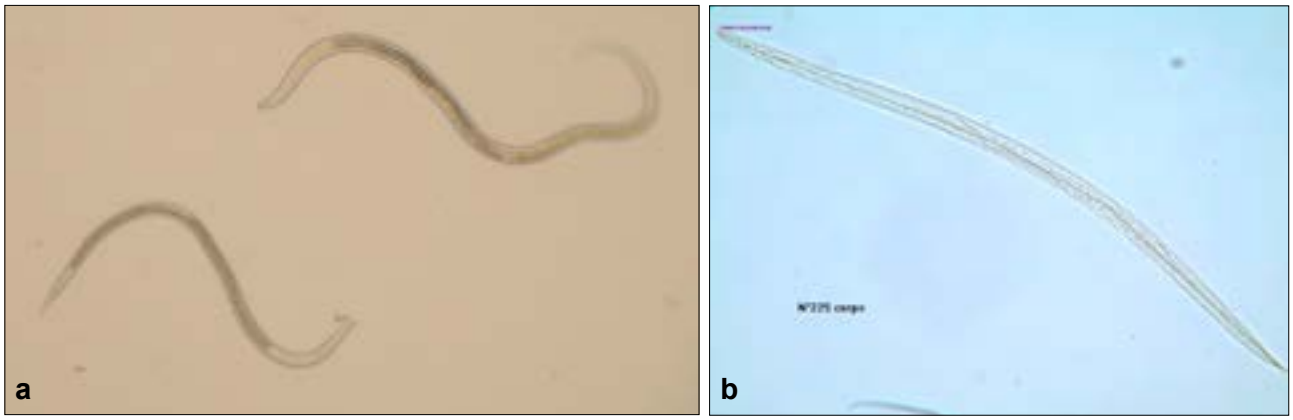

Figure 2. a *Ditylenchus dipsaci* extracted from alfalfa. **b** *Ditylenchus gigas*.

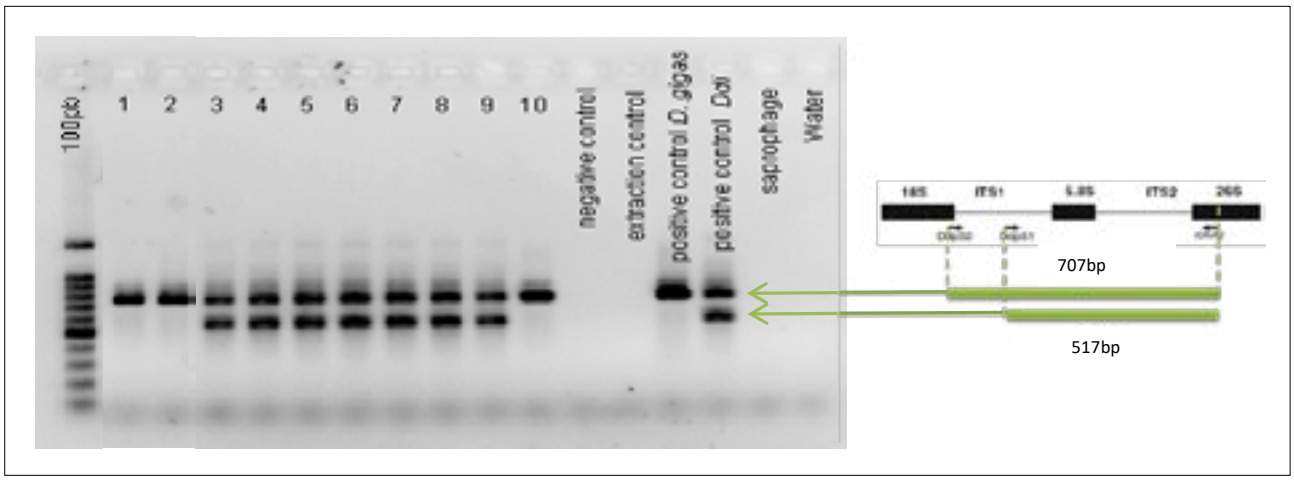

Figure 3. Aspect of electrophoresis gel after amplification of specific primers DdpS1 and DdpS2.

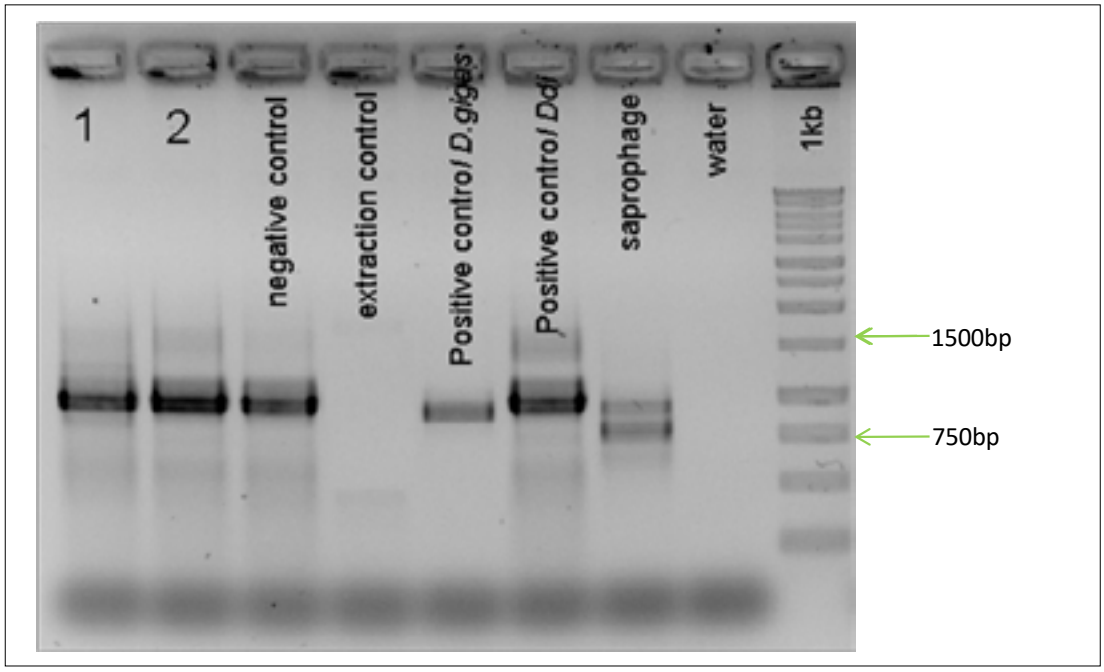

Figure 4. Aspect of electrophoresis gel after amplification of control amplification primers rDNA1 and rDNA2.

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