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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/85 (2)

Diagnostics
Diagnostic

PM 7/85 (2) *Plasmopara halstedii*

Specific scope

This Standard describes a diagnostic protocol for *Plasmopara halstedii*.[1]

Specific approval and amendment

Approved in 2008–09. Revised in 2014–06

Introduction

The oomycete *Plasmopara halstedii*, which causes downy mildew on cultivated sunflower, is known on every continent of the world. It is considered as a major disease in all sunflower producing countries in Europe (Sackton, 1981). In addition, fungicide tolerant isolates as well as new pathogenic races have appeared and are spreading, and representing a threat to sunflower crops (Roeckel-Drevet *et al.*, 2003).

The significant hosts are the wild and cultivated species of *Helianthus*, including sunflower (Leppik, 1966; Novotel'nova, 1966), but over 100 host species from a wide range of genera in the family *Asteraceae* have been reported. Wild *Asteraceae* hosts (e.g. species of the genera *Helianthus*, *Artemisia*, *Xanthium*, etc.) may also occur widely in the corresponding areas but their potential as reservoirs of the pest is not yet confirmed (Viranyi, 1984; Walcz *et al.*, 2000).

This pathogen is mainly soil-borne and seed-borne (Basavarju *et al.*, 2004) but may also be wind-borne.

In the EPPO region the pathogen is present wherever sunflowers are grown. Further information on the biology and epidemiology of the pest can be found in the EPPO data sheet on *P. halstedii* (EPPO/CABI, 1997). Details on its geographical distribution can be found in the EPPO Plant Quarantine Retrieval System (PQR) (2007).

Identity

Name: *Plasmopara halstedii* (Farlow) Berlese & de Toni

Synonyms: *Plasmopara helianthi* Novotel' nova
Taxonomic position: Oomycetes; Peronosporales;
EPPO code: PLASHA
Phytosanitary categorization: EU Annex designation:II/A2

Detection

Plasmopara halstedii may induce symptoms of various kinds depending on the age of tissue, level of inoculum, cultivar reaction and environmental conditions (moisture and temperature). Symptoms result from systemic or local infections; those being caused by systemic infections being more typical.

Systemically infected sunflower plants may have some degree of stunting and the leaves show pale green or chlorotic mottling which spreads along the main veins and over the lamella (Fig. 1). Young leaves of severely affected plants often become entirely chlorotic, curl downward, and become rigid and thick (leaf chlorosis is illustrated in Fig. 2). Under moist conditions, a white downy growth composed of sporangiophores and sporangia of the oomycete develop on the lower leaf surface and observations have shown that their extent strictly corresponds to the chlorotic areas on the upper leaf surface (Fig. 3). Due to internode shortening, an infected sunflower may have a cabbage-like appearance.

Heads of infected sunflower plants have a reduced size and face upwards (Fig. 1), bearing no or a limited number of seeds, the viability of which is poor. The economical loss can be considerable.

The root system of infected sunflower is underdeveloped, with significant reductions in secondary root formation and with a dark brown appearance on their surface.

[1]Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Fig. 1 Field symptoms of *P. halstedii* on sunflower plant. Leaf chlorosis, stunting and upturned head (Photo: Hedvig Komjati).

Fig. 2 Leaf chlorosis on sunflower cotyledon leaves – plants 5–6 days older than those from Fig. 4 (Photo: Hedvig Komjati).

Other, less common symptoms associated with systemic infection include damping-off of seedlings, pith discoloration of the stem and/or capitulum, disturbance of inflorescence, twisted leaves and basal gall (Sackton, 1981). Furthermore, systemic downy mildew infection may be localized to lower stem tissues (cotyledon or hypocotyl infections (Ljubich & Gulya, 1988; Viranyi & Gulya, 1996)). In moist conditions, such infections are characterised by white sporulation on the cotyledon leaves and the stems of seeds at cotyledon stage (Figs 4 and 5).

Local leaf infections causing angular leaf spotting may also be observed (small, angular, pale green spots, delimited by the veins). Systemic infection (pathogen growing through the petioles into the stem) may subsequently occur when environmental conditions are favourable and the infection pressure is high (Ratai-Vida, 1996).

Before fructification, *P. halstedii* may be confused with herbicide phytotoxicity. After fructification, it may be confused with *Albugo tragopogonis* but in this case typical large chlorotic blister-like pustules form on the upper side of leaves. Such deformation of the foliage does not occur with *P. halstedii.*

Identification

This oomycete is an obligatory biotrophic plant pathogen and morphological identification is possible only *in vivo*, on the host plant. Consequently, morphological identification from seed and soil samples requires a bioassay to be conducted. PCR tests have also been developed for identification from seed and may be adapted for identification of *in planta* grown structures (sporangiophores bearing sporangia). An ELISA test is described in the literature (Bouterige *et al.*, 2000), but there is little experience in the EPPO region with using this method. Therefore ELISA is not recommended.

Detection from seed is difficult because seed infection in lots is highly variable and sometimes very low due to blending/dilution of seeds with various infection levels. The bioassay tests described in this section and the PCR tests give an indication of the number of seeds that can be tested in a single test. The total number of seeds to be tested in order to detect the pest at different infection levels in a lot needs to be determined statistically (useful guidance is given in table 1 and 2 of ISPM 31 *Methodologies for sampling of consignments* (IPPC, 2008)).

Bioassay

Detection from seed

Detection from seed is based on the observation of signs of sporulation on germinated seedlings at cotyledon leaf stage. The appearance of any other symptoms such as distortion

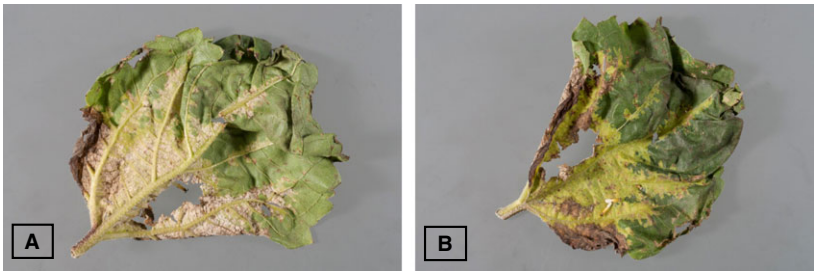

Fig. 3 White downy growth developed on the lower leaf surface (A) and corresponding to the chlorotic areas on the upper leaf surface (B) Photo Courtesy The Food and Environment Research Agency (Fera), Crown Copyright.

Fig. 4 Sporulation of *Plasmopara halstedii* of surface of artificially inoculated sunflower cotyledon leaves (Photo: Hedvig Komjati).

or discoloration of young plantlets is not a specific sign of infection. In addition, ensuring good growth conditions (temperature of 16–17°C and relative humidity of 95 to 100%) is critical for the detection of *P. halstedii.*

The method is presented in Appendix 1.

Detection from soil

This test is recommended for testing soil from fields where infestation is suspected. A soil sample with a standard rate of at least 1 500 mL soil ha^{-1} should be collected from at least 100 cores ha^{-1} preferably in a rectangular grid of not <5 m in width and not more than 20 m in length between sampling points covering the entire field. The whole sample should be used for the test. The detection from soil is based on the ability of the pathogen to infect young sunflower seedlings when the roots are 1–2 cm long and the soil is water saturated. In such conditions motile zoospores migrate through soil-pores to the roots (Gulya *et al.*, 1997). A fast and comparatively simple bioassay method was developed for diagnostic purposes, based on the method of Gulya & Radi (2002). Soil samples from the suspected field should be taken after the vegetative period (late autumn at the earliest) or before sowing during springtime the following year. Samples should be taken from the rhizosphere level (up to 20 cm) as potentially infected plant debris may have been incorporated onto the soil surface. Detection from soil is based on the observation of signs of sporulation in germinated seedlings at cotyledon leaf stage.

The method is presented in Appendix 1.

Fig. 5 Sporulation of the oomycete at soil level on the stem of sunflower seedling in a climatic chamber. (Photo: Aranka Kormany)

Morphology

Morphological identification is possible by microscope.

Mycelium

Mycelium is composed of intercellular, colourless, aseptate hyphae 6–20 µm diameter, often irregularly shaped and swollen, bearing small, rounded, vesicular haustoria, 5–10 µm in diameter, growing in all plant tissues.

Sporangiophores

Sporangiophores are hypophyllous, or occasionally epiphyllous, arborescent, 300–450 (750) µm × 7–14 µm, obconical or pointed at the base, branching in the upper half, with the apex of the branching axis frequently swollen: branches in the form of a whorl of 7–8, produced monopodially at right angles to the main sporangiophore axis, each with 2–5 secondary branches 40–86 µm long, bearing 3–5 tips, 8–15 µm long, diverging at right angles (Figs 6 and 7).

Sporangia

Sporangia are ovoid to ellipsoid; 18–30 µm × 14–20 µm papillate; germinating to give 20 reniform, biflagellate zoospores (Fig. 8).

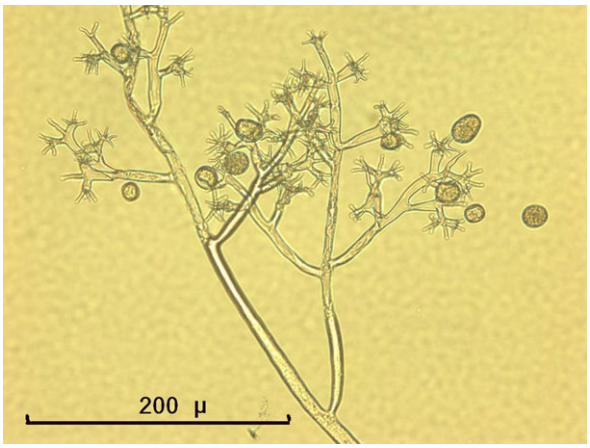

Fig. 6 Sporangiophores of *Plasmopara halstedii* with sporangia (Photo: Aranka Kormany).

Fig. 7 Sporangiophores with sporangia (Photo: Aranka Kormany).

Antheridia
Antheridia are club-shaped; approximately 12 × 30 µm; and formed on distant hyphal branches (diclinous).

Oogonia
Oogonia are spherical; 30–40 µm in diameter, and colourless.

Oospores
Oospores are formed in all the vegetative organs of the host especially in roots and leaves; just under the epidermis; spherical, (15)-23-(30) µm diameter (aplerotic); yellow-brown with a slightly wrinkled wall; 3 µm thick; and germinate to give sporangia. (Hall, 1989).

Molecular methods

P. halstedii-specific primers (PHAL-F/R) have been developed by Ioos *et al.* (2007) for the direct detection of the oomycete in sunflower seed samples by the Polymerase Chain Reaction (PCR).

This method may be adapted for identification of *in planta* grown structures (sporangiophores bearing sporangia).

A duplex real-time PCR has also been developed by Ioos *et al.* (2012). Details of the tests are presented in Appendices 2 and 3.

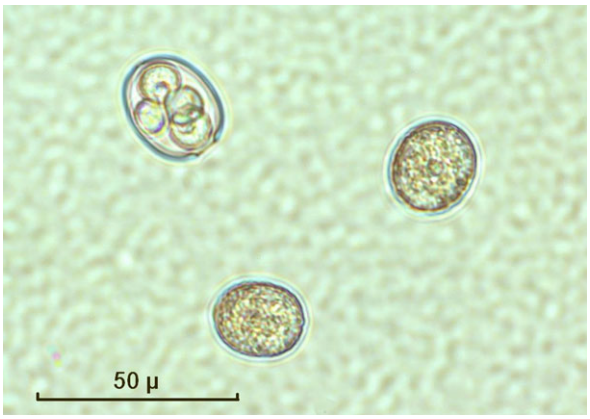

Fig. 8 *Plasmopara halstedii* sporangia and formation of zoospores in a sporangium (Photo: Aranka Kormany).

Reference material

P. halstedii pathotypes designated as 100, 330, 700, 710 and 730 can be obtained from the Szent István University culture collection on request (R. BÁN, Institute of Plant Protection, Szent István University, H-2103 Gödöllő, Hungary. E-mail: Ban.Rita@mkk.szie.hu).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

Further information

Further information can be requested from Ferenc Virányi (Institute of Plant Protection, Szent István University, H-2103 Gödöllő, Hungary, e-mail: Viranyi.Ferenc@mkk.szie.hu). Bacterial suspension harbouring the Internal Amplification control or positive control plasmids may be provided by R. Ioos upon request, providing that the laboratory is authorized to handle and keep genetically modified bacteria.

Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int

Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols 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.

Acknowledgements

This protocol was originally drafted by E. Dormanns-Simon & A. Kormány (Plant Protection and Quarantine Laboratory – Csongrad County Government Office (formerly: Csongrád County Agricultural Office), Directorate of Plant Health and Soil Conservation, Hódmezővásárhely, Hungary). The molecular section of the protocol was prepared by R. Ioos (Plant Health Laboratory, Mycology Unit – ANSES, Domaine de Pixérécourt, Malzéville, France) and Á. Halász (Plant Health and Molecular Biology National Reference Laboratory – National Food Chain Safety Office, Directorate of Plant Health, Soil Conservation and Agri-environment, Budaörsi út 141-145., H-1118 Budapest, Hungary).

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Appendix 1 – Bioassay

Detection from seeds

The test should preferably be performed with three replicates (of 400 seeds). Four hundred seeds are surface sterilized (by 0.5% NaOCl for 10 min, thoroughly rinsed by sterile water), rolled in sterile wet paper and incubated at 20°C until germination occurs.

After germination, seeds are removed from the paper and placed on the surface of containers with water saturated sterile substrate (compost or sand). Pots are placed on a tray. Seeds are covered by a layer of sterile substrate of 1 cm maximum.

Pots are then placed in a climatic chamber at 16–18°C and relative humidity of 95% to 100% and covered by loose plastic bags. Twelve hours of daylight artificial illumination are required from the second day. The substrate should be kept saturated and the temperature should not exceed 18°C.

When the cotyledon leaves appear (about 10 days) the young plants should be observed for signs of sporulation.

An alternative system is to germinate surface-sterilized seeds in Petri dishes under the same conditions as for con-

tainers with substrates (16–17°C and a relative humidity of 95–100%), although maintaining the required relative humidity is much more difficult.

If one of the replicates is positive the pest is considered detected.

Detection from soil

Each sample should be placed in containers on a tray and the soil should be saturated slowly with water until it flows out into the tray. In each container, seeds of susceptible sunflower breeding lines susceptible to all know races of *P. halstedii* infection (e.g. HA-89 or Peredovic), should be planted (the quantity of seed depending on the size of the container). It should be ensured that the seeds used are not contaminated with *P. halstedii*. The seeds are then covered by a 1 cm layer of soil that should also be watered. A minimum of 50 seeds should be sown for each soil sample. The containers should be placed into a climatic chamber at 16–18°C and relative humidity of 95% to 100% and preferably covered by loose plastic bags. During the first 3 days the temperature can be increased to 20°C to accelerate germination but after the germination, the temperature should not exceed 18°C. Twelve hours of daylight artificial illumination is required from the fourth day.

After 2 weeks the cotyledon leaves begin to appear and the young plantlets begin to grow fast. Observation of plants should be conducted about 2 weeks after sowing, at the 2 cotyledon leaf stage, before the plants become too elongated. Plastic bags should be kept on the plants for the entire period.

If the young plants are infected, sporulation of the pathogen should appear on the cotyledon leaves or on the lower part of the stem (at soil level). The number of seedlings showing sporulation should be determined under a dissecting microscope. Other symptoms such as stunting or deformation may also appear (due to possible unfavourable conditions) but the detection of the pathogen can only be positive if sporulation is observed.

A positive control to monitor the suitability of test conditions may be included which can be a spiked subsample of the original soil sample. However, the main limitation for the inclusion of such positive controls is that infested material is not easy to obtain and to maintain.

Note for both Bioassays: in a climatic chamber it is usually difficult to keep low temperature and 100% humidity when the illumination is strong. Low temperature (below 18°C) and constant water saturation is crucial for the germination of the spores. Daylight illumination is necessary because if the young plants do not get enough light they develop long thin stems (become etiolated), and the pathogen will not infect the loose tissues and the elongated cells.

Appendix 2 – Detection of *Plasmopara halstedii* in sunflower seed by conventional PCR

1. General information

1.1 Scope of the test is the detection of *Plasmopara halstedii* in sunflower seed using conventional PCR (Ioos *et al.*, 2007).

1.2 The PCR primers are designed to target the large-subunit (LSU) ribosomal DNA (28S rDNA) sequence in *Plasmopara* spp.

1.3 Amplicon size is 308 bp.

1.4 Oligonucleotides

Target: *Plasmopara halstedii*

Forward primer: PHAL-F: 5′TATCTCTAAGTTGCTTATAC-3′

Reverse primer: PHAL-R: 5′AGCATATACAGCACATACG-3′

1.5 The conventional PCR reactions were carried out on a GenAmp 9700 thermocycler (Applied Biosystems).

1.6 PCR fragments were separated together with a 100 bp DNA ladder (Invitrogen), by a 1 h electrophoresis on a 1% agarose gel at 4 V cm^{-1}.

1.7 Images were recorded with a CCD camera and a GELDOC 2000® system (Biorad).

2. Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 The recommended DNA extraction method is described in Ioos *et al.*, 2007.

2.1.2 For DNA extraction from sunflower seed samples, 35 g of seed (representing c. 500 seed) are sampled. The seeds are directly ground for 1 min in a mixer equipped with a vessel attachment of appropriate volume (e.g. Microtron MB 550, Kinematica, Lucerne, Switzerland, with a 125 mL attachment). Approximately 500 µL of ground powder (90–110 mg) are transferred into a sterile 2 mL microcentrifuge tube with 500 µL of DNeasy® lysis buffer (Qiagen). Four microlitres of the RNase provided by the manufacturer and 10 µL of 10 mg mL^{-1} proteinase K (Sigma-Aldrich, Lyon, France) are also added. Genomic DNA is then extracted following the manufacturer's instructions, except that the lysis buffer incubation step is increased to 20 min.

2.1.3 The DNA samples are kept at −20°C for long-term storage, and at 4°C for routine amplifications.

2.2 Conventional PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	n.a.	10.78	N.A.
Taq DNA polymerase buffer (*Invitrogen*)	10×	2	1×
$MgCl_2$ (*Invitrogen*)	25 mM	1.6	2 mM
dNTPs (*Invitrogen*)	25 mM	0.12	0.15 mM
Bovine Serum Albumin (BSA) (*SIGMA*)	10 mg mL^{-1}	1.6	0.8 µg $µL^{-1}$
Forward primer (*PHAL-F*)	10 µM	0.9	0.45 µM
Reverse primer (*PHAL-R*)	10 µM	0.9	0.45 µM
Taq DNA Polymerase (*Invitrogen*)	5 U $µL^{-1}$	0.1	0.025 U $µL^{-1}$
Subtotal		18	
Template DNA (40–60 ng)		2	
Total		20	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

2.2.2 PCR conditions: The cycling profile for *P. halstedii*-specific PCR includes an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation, annealing and elongation for 20 s at 94°C, 30 s at 58°C and 1 min at 72°C respectively, and a final extension step at 72°C for 7 min.

2.2.3 Any IAC (internal amplification control) can be used. The IAC, developed from *Populus trichocarpa* × *P. deltoides* cv 'Beaupré' genomic DNA is available upon request from R. Ioos (for the address see Acknowledgements section). Amplification of the IAC with primer PHAL-F/R will yield an approximately 800 bp fragment.

3. Essential procedural information

A negative control (no target DNA) should be included in every experiment to check the absence of contamination, as well as a positive control (genomic DNA from a reference strain of *P. halstedii*, or a subcloned *P. halstedii* PHAL-F/R PCR product).

The positive control, but not the negative control, should yield a 308 bp DNA fragment. Subsamples yielding a 308 bp fragment should be considered as infected by *P. halstedii*, whereas subsamples not yielding such amplicons should be considered as negative, provided that the 800 bp IAC amplicon is produced.

DNA extracts that do not yield the IAC amplicon should be diluted and tested again by PCR. If the dilution does not overcome the inhibition effect, the DNA extract should be considered as not suitable for PCR analysis.

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic IAC) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed.

Conventional PCR tests.

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of the 308 bp size.

When these conditions are met:

- A test will be considered positive if amplicons of 308 bp are produced.
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available (from Ioos *et al.*, 2007)

4.1 Analytical sensitivity data: The PHAL-F/R PCR test was able to detect 3 pg of *P. halstedii* DNA in a 20 ng μl^{-1} sunflower seed DNA extract. The detection threshold determined for the conventional PCR test was estimated to be five *P. halstedii*-contaminated seeds/500 seeds but should be carefully interpreted since this limit was inferred from naturally infected seeds in which the overall *P. halstedii* biomass content was not known. Nevertheless, PCR may yield false negative results with samples with a very low infection level, but proved to be more sensitive than the biological test (Ioos *et al.*, 2007).

4.2 Analytical specificity data: This test has proven to be specific for *P. halstedii*, regardless of the race and the geographical origin (22 isolates), and differentiates this species from another species of *Plasmopara (Plasmopara viticola)*, 14 isolates of *Phytophthora* spp., and from 15 isolates of fungi commonly occurring on sunflower seeds (for details see Ioos *et al.*, 2007).

4.3 A test performance study was organized according to EN ISO 16140 in 2007 involving 10 European laboratories from the European Mycological Network (EMN). The collaborative study showed that the accuracy, analytical sensitivity and selectivity were 96.6%, 94.7%, and 100%, respectively, whereas the accordance and concordance (i.e. qualitative repeatability and reproducibility) of the test were estimated to 97.5% and 87.0%, respectively (Ioos & Iancu, 2008). These results showed that this method fulfils the requirements to be considered fit for regulatory purpose.

Appendix 3 – Detection of *Plasmopara halstedii* in sunflower seed by duplex real-time PCR

1. General information

1.1 Scope of the test: detection of *Plasmopara halstedii* in sunflower seed using duplex real-time PCR (Ioos *et al.*, 2012).

1.2 The PCR primers/hydrolysis probes are designed to target the large-subunit (LSU) ribosomal DNA (28S rDNA) sequence in *Plasmopara* spp. A universal primer pair (18S uni-F/-R) and a hydrolysis probe (18S uni-P) target the 18S rDNA region.

1.3 The amplicon sizes are 94 bp for qPHAL-F/-R and 150 bp for 18S uni-F/-R = .

1.4 Oligonucleotides

Target: *Plasmopara halstedii*

Forward primer: qPHAL-F: 5′TTCCAGTGTCTATAATCCGTGGT-3′

Reverse primer: qPHAL-R: 5′GCACATACGCCGAGCGTA-3′

Probe: qPHAL-P: 5′FAM-TCGGCGAGCGTGTGCGTGT-BHQ1-3′

Target: *Helianthus annuus*

Forward primer: 18S uni-F: 5′GCAAGGCTGAAACTTAAAGGAA-3′

Reverse primer: 18S uni-R: 5′CCACCACCCATAGAATCAAGA-3′

Probe: 18S uni-P: 5′JOE-ACGGAAGGGCACCACCAGGAGT-BHQ1-3′

1.5 The real-time PCR reactions were performed with a Rotor-Gene 6500 (Corbett Research, Mortlake, Australia) set with an autogain optimization for each channel, which was performed before the first fluorescence acquisition.

1.6 The C_t value was determined using the Rotor-Gene software, version 1.7.75, setting the threshold line at 0.02.

2. Methods

Nucleic acid extraction and purification

The recommended DNA extraction method is described in Ioos *et al.*, 2012;. The *Helianthus annuus* seed sample (1000 seeds) is ground for 1 min in a mixer mill (e.g. Microtron MB 550, Kinematica, Lucerne, Switzerland). Five hundreds microlitres of the seed powder is collected using a sterile spatula and transferred in a sterile 2-mL microcentrifuge tube. Total DNA extraction is carried out using commercial DNA extraction kits according to the manufacturer's instructions. The use of NucleoSpin Plant II with PL1 lysis buffer (Macherey-Nagel, Düren, Germany) is recommended in Ioos *et al.* (2012). The DNA extract (100 µL) is tested after a 10-fold dilution in 1× Tris-EDTA buffer (Sigma-Aldrich).

The DNA samples are kept at −20°C for long-term storage, and at 4°C for immediate or short-term use.

2.1 Duplex Real-time Polymerase Chain Reaction – duplex real-time PCR

2.1.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	12.17	N.A.
Reaction buffer** (*Eurogentec*)	10×	2.00	1×
$MgCl_2$** (or alternatives) (*Eurogentec*)	50 mM	2.00	5 mM
dNTPs mix** (*Eurogentec*)	5 mM	0.80	0.2 mM
Plasmopara halstedii			
Forward primer (*qPHAL-F*)	30 µM	0.03	0.05 µM
Reverse primer (*qPHAL-R*)	30 µM	0.20	0.3 µM
Probe 1 (*qPHAL-P*)	10 µM	0.10	0.05 µM
Helianthus annuus			
Forward Primer (*18S-uni-F*)	30 µM	0.20	0.3 µM
Reverse Primer (*18S-uni-R*)	30 µM	0.20	0.3 µM
Probe 2 (*18S-uni-P*)	10 µM	0.20	0.1 µM
DNA Polymerase** (*Eurogentec*)	5 U μL^{-1}	0.10	0.025 U μL^{-1}
Subtotal		18.00	
DNA dilution		2 µL of template DNA (0.8–20 ng)	
Total		20 µL	

* Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

** Reagents supplied with the qPCR core kit No Rox (Eurogentec)

2.1.2 PCR conditions: include an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 10 s at 95°C and 45 s at 65°C respectively.
Applied PCR kit: qPCR core kit No ROX (Eurogentec).

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated 18S uni-F/-R/-P, systematically used in duplex with PHAL-F-R-P in order to check the quality of DNA extraction. This 18S uni-F/-R/-P combination targets a conserved region within the 18S rDNA gene from a wide range of plants (Ioos *et al.*, 2009).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed.

Verification of the controls

- The PIC and PAC (as well as IC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if an exponential amplification curve is generated with the DNA extract.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available (from Ioos & Fourriier, 2011; Ioos *et al.*, 2009, 2012)

4.1 Analytical sensitivity data: The detection limit is 456 plasmidic copies of the target DNA per PCR tube. The detection threshold determined for this duplex real-time PCR is less than a single *P. halstedii*-contaminated seed per 1000 seeds.

4.2 Analytical specificity data: no cross reaction was noted: The test did neither cross-react with 1 ng of DNA from any of the fungi commonly isolated from soil or sunflower seed, nor with 1 ng of DNA from any other *Plasmopara* spp., thus confirming the anticipated in silico analytical specificity. (See Ioos *et al.*, 2012.)

4.3

Reproducibility	
Calculated coefficient of variance for a given level of the pest (see PM 7/98)	0.45% for a target concentration of 2.26×10^4 copies of the target DNA*
	0.52% for a target concentration of 2.26×10^3 copies of the target DNA*
	1.98% for a target concentration of 2.26×10^2 copies of the target DNA*
	1.74% for a DNA extract from a naturally infected *H. annuus* seed sample
Repeatability	
Provide the calculated % of agreement for a given level of the pest (see PM 7/98)	2.21% for a target concentration of 2.26×10^4 copies of the target DNA*
	1.52% for a target concentration of 2.26×10^3 copies of the target DNA*
	1.69% for a target concentration of 2.26×10^2 copies of the target DNA*
	4.04% for a DNA extract from a naturally infected *H. annuus* seed sample

*Per PCR tube.

4.4 The robustness of the tool was assessed with target DNA template concentrations close to the limit of detection, by assessing the effects of 10% variation for the DNA template volume, reactional volume and ±2°C hybridization/polymerization. The qualitative results of the test were not affected by volume or temperature variations (see Ioos *et al.*, 2012).