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Diagnostics
Diagnostic

PM 7/28 (2) *Synchytrium endobioticum*

Specific scope

This Standard describes a diagnostic protocol for *Synchytrium endobioticum*.[1]

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

Specific approval and amendment

Approved in 2003-09.
Revision approved in 2017-06.

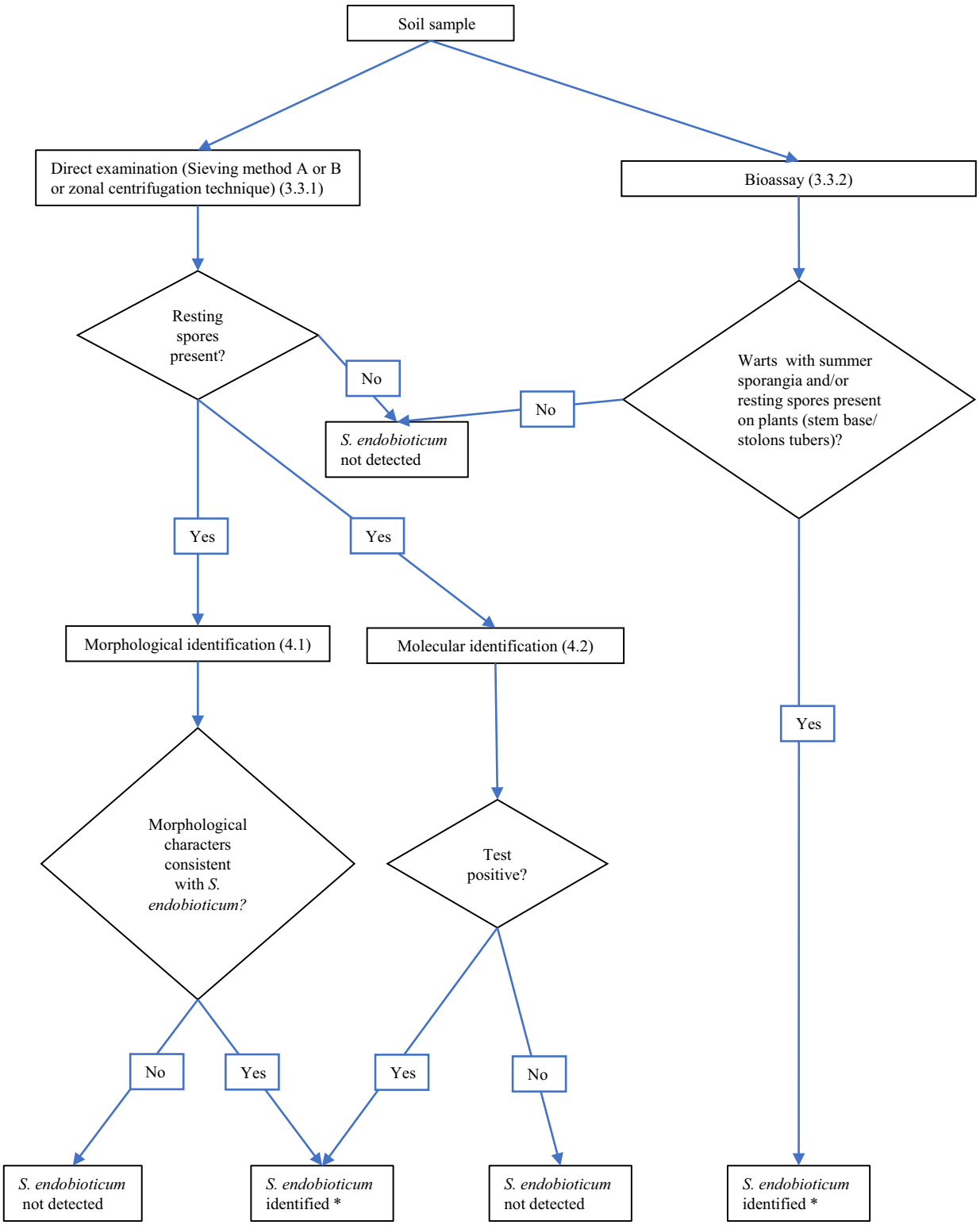
1. Introduction

Synchytrium endobioticum causes potato wart disease (Langerfeld, 1984). At the end of the 19th century the disease spread from its original range in the Andean region of South America to parts of North America and Europe. Eventually, the fungus was found in potato-growing countries all over the world, including Asia, Africa and Oceania. It is a highly destructive disease under conditions favourable to disease development; infected tubers may then be largely converted to warts. Although a number of solanaceous crops can be infected experimentally, potato is the principal host. Favourable conditions for the development of the fungus are cool summers, with an average temperature of 18°C or less, and an annual precipitation of at least 700 mm. However, new outbreaks have been reported from areas in South-Eastern Europe where summer temperatures are higher. The capacity for natural spread of *S. endobioticum* is limited. The fungus may be spread by human assistance on infected potato tubers, in soil, as such or attached to plants, or with potato waste. Due to the limited capacity for natural spread, the disease has been controlled effectively by statutory actions in many countries. Strict phytosanitary control and obligatory cultivation of resistant cultivars have allowed the eradication of the pathogen in some countries. Eradication is very slow because the fungus survives in soil for decades. Because of this, and the destructive nature of the disease, the fungus is considered as an important quarantine pest worldwide.

Synchytrium endobioticum is an obligate parasite which does not produce hyphae but sporangia, which contain motile zoospores. Summer sporangia are thin-walled and short-lived. They are formed in the potato tissue and give rise to new zoospore infections. Resting spores (sometimes also referred to as winter sporangia, winter spores, resting sori or winter sori) are thick-walled and remain viable for extremely long periods of time even in the absence of a host. These can be produced throughout the growing season. They are released from decomposing warts into the soil. Diagnosis of *S. endobioticum* concerns both the plant, on which warts may have formed, and the soil, which may carry resting spores. An EPPO Standard PM 3/59 *Procedure for descheduling of previously infested plots* was initially approved in 2003 (revised version published in this issue of the EPPO Bulletin). The tests that have to be implemented in the framework of this procedure are described in this protocol.

Numerous pathotypes (races) have been described in *S. endobioticum* (Baayen *et al.*, 2006). These are defined by their virulence on differential potato cultivars. Pathotype 1(D1) is now rarely reported infecting potato in Europe because few potato cultivars are susceptible to it, apparently due to the availability of dominant resistance in the host. Other pathotypes are now reported to occur more frequently in Western Europe, and are particularly found in the rainy mountainous areas of Central and Eastern Europe. They also occur outside Europe (e.g. in Newfoundland, Canada and in the Asian part of Turkey). Resistance to these pathotypes is rare, so control is more difficult. Pathotypes other than 1(D1) are distinguished through differential virulence

[1]The use of brand names of chemicals, equipment or commercial kits in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.



* For viability testing and pathotype identification see sections 4.3 and 4.4 respectively.

Fig. 1 (A) Flow diagram for the detection and identification of *Synchytrium endobioticum* in soil. [Colour figure can be viewed at wileyonlinelibrary.com]

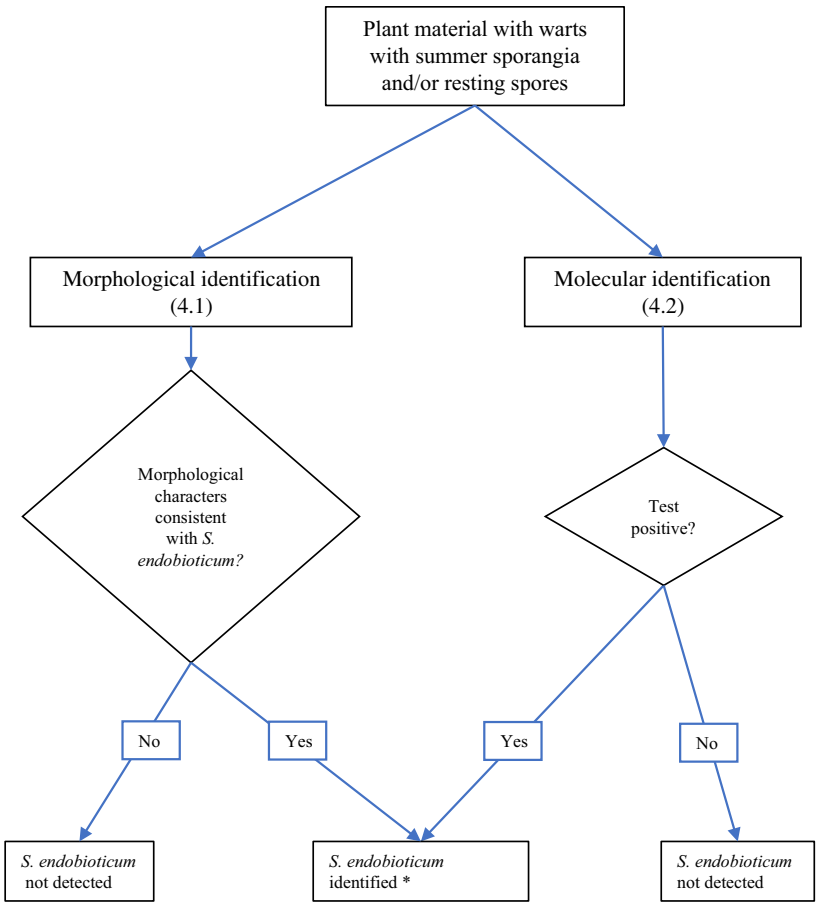


Fig. 1 (B) Flow diagram for the detection and identification of *S. endobioticum* in plant material with warts. [Colour figure can be viewed at wileyonlinelibrary.com]

to specific potato cultivars (Stachewicz, 1980; Langerfeld & Stachewicz, 1993; Langerfeld *et al.*, 1994; Stachewicz *et al.*, 2000), but otherwise seem to be more closely related to each other than to pathotype 1(D1). Internal measures recommended in EPPO countries require that pathotypes are identified. Consequently, this protocol also covers pathotype identification.

The flow diagrams describing the diagnostic procedures for the detection and identification of *S. endobioticum* in soil samples and in plant material showing warts are presented in Fig. 1A and B, respectively.

2. Identity

Name: *Synchytrium endobioticum* (Schilbersky) Percival.
Synonym: *Chrysophlyctis endobiotica* Schilbersky, *Synchytrium solani* Massee.
Taxonomic position: Fungi; Chytridiomycota; *Chytridiales*.
EPPO Code: SYNCEN.
Phytosanitary categorization: EPPO A2 List no. 82; EU Annex designation I/AII.

3. Detection

3.1. Disease symptoms

The typical symptoms of potato wart disease on tubers are the proliferating warts which may vary markedly in form but are primarily spherical to irregular (Fig. 2A, C, D). The infection invariably originates in eye tissue, but may expand to engulf the whole tuber. Warts vary in size from less than pea-sized proliferations to the size of a fist. Above-ground warts are green (Fig. 2A, B, D), but later become black, and subterranean warts are white to brown, becoming black on decay. Early infection of young developing tubers results in their becoming so distorted and spongy as to be scarcely recognizable. In older tubers, the eyes are infected and develop into characteristic, warty, cauliflower-like protuberances (Fig. 2C). These are initially whitish (or green if exposed to light), but gradually darken and eventually rot and disintegrate. The whole tuber may be entirely replaced by the warty proliferation. Similar warts occur on stolons (Fig. 2D). Roots are not known to

Fig. 2 Symptoms of infection of potato (*Solanum tuberosum*) by *Synchytrium endobioticum*. (A) Warts formed on the tubers at the soil surface during the growing season. (B) Warts formed on emerging sprouts, which do not develop into shoots but obtain an irregular, warty cauliflower-like appearance. (C) Warted tubers as observed at harvest. (D) Heavily infected plant showing yellowish warts on subterranean tubers and greenish warts at soil level. (E), (F) Fresh wart tissue under a stereoscope with the remnants of summer sporangia (brown/black dots/circles) after releasing zoospores. (H) Potato wart disease – leaf deformation. (G) Resting spores as seen under the light microscope. Credits: (A), (E), (F) courtesy Fera, York (GB); (C) Biologische Bundesanstalt für Land- und Forstwirtschaft, Kleinmachnow (DE); (B), (D), (G) HLB B.V., Wijster (NL); (H) courtesy Benaki Phytopathological Institute (GR).

be infected. Small greenish warts may form in the position of the aerial buds at the stem bases. Deformation of leaves may also occur (Fig. 2B, H). The disease does not kill the host and, in the case of subterranean symptoms, may not be evident until harvest.

3.2 Possible confusions

Proliferation of eyes ('pseudo-wart') (Fig. 3)

Simultaneous germination of all buds in one eye results in wart-like outgrowths very similar to those caused by potato wart disease. However, these pseudo-warts consist of abundant pointed shoots compacted together and do not start rotting when ripe. No summer sporangia or resting spores are present in the affected tissue. The individual apices of the shoots are somewhat less swollen and more recognizable as buds than are the outgrowths of wart. The cause of this symptom is not clear, but it is thought to be physiological or varietal and to be stimulated by treatments of tubers with various chemicals.

Fig. 3 Pseudo-warts (courtesy ILVO, BE).

Powdery scab

On tubers, enlargement and division of host cells, due to infection by *Spongospora subterranea* f.sp. *subterranea* forces the periderm to rupture, resulting in scab-like outgrowths which, in very wet soil, can develop into hollowed-out areas or very large cankers. Inside fresh lesions, ovoid, irregular, or elongate spore balls (diameter 50–51 µm on average and range 18–100 µm; Falloon *et al.*, 2007) can be observed, consisting of an aggregate of closely associated resting spores (Fig. 4). Powdery scab can, in contrast to *S. endobioticum*, attack roots. Milky white galls, 1–10 mm in diameter or more, develop on roots and stolons (Fig. 5). In the severe canker form of powdery scab the tuber is induced to form knob-like protuberances, which often become totally covered by scab tissue, thus closely resembling wart symptoms.

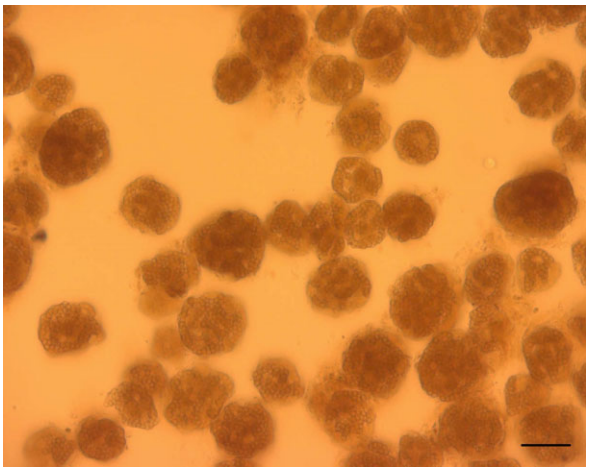


Fig. 4 *Spongospora subterranea* f.sp. *subterranea* spore balls produced in scab-like outgrowths on potato tubers (scale bar 50 µm) (courtesy SASA, GB).

Fig. 5 Galls (black arrow) of powdery scab (*Spongospora subterranea* f.sp. *subterranea*) on roots and stolons of a potato plant (courtesy CLPQ, BG).

3.3. Detection of resting spores in soil

Several methods are available for the detection of resting spores in soil; these are presented below. Soil samples are collected from fields according to EPPO Standard PM 3/59 *Soil tests and descheduling of previously infested plots* (EPPO, 2017). Quantities of soil indicated below correspond to the maximum quantity that can be processed in a single test. Guidance on sampling is given in EPPO Standard PM 3/59. The sample is composed of 60 cores for 0.33 ha and the final total weight usually varies between 20 and 25 kg. 500 g from the bulk sample is used by the laboratory to select sub-samples (200 g) for direct examination. The remaining soil may be used for bioassays.

3.3.1. Direct examination

3.3.1.1. Sieving method A (Pratt, 1976). From the 500 g (see above), two sub-samples of maximum 100 g (depending on the type of soil) are air-dried at room temperature and then suspended in 900 mL of tap water for 24 h, and all soil aggregates thoroughly broken up. The suspension is wet-sieved through an electromagnetic sieve shaker (e.g. Fritsch Analysette 3, A. Christian Ltd, Gateshead, GB) with successive mesh sizes of 500, 250, 125, 71, 40 and 25 µm. The fractions held on the 40 and 25 µm sieves are washed onto filter paper, air dried, and transferred to 50 mL centrifuge tubes. Chloroform (15 mL) is added to each tube, stirred and the tubes are centrifuged at approximately 800*g* for 15 min. The supernatant is filtered through hardened filter paper (e.g. Whatman no. 50). Washing with chloroform is repeated, usually twice more or until no more material can be floated off. The residue collected on the hardened filter paper is resuspended in 1 mL of lactoglycerol (according to Pratt, 1976) or water. The quantity of lactoglycerol/water is slightly increased if necessary for accurate counting, for example for soil with a high content of organic matter, and examined under the microscope for the

presence of resting spores of *S. endobioticum*. Whenever needed these can be counted and the number per gram of soil estimated. Quantification can also be performed with molecular techniques, as described for the zonal centrifuge technique (see 3.3.1.3).

Recovery rates of 80–95.5% have been reported using sieving method A (Pratt, 1976). These recovery rates were determined by adding a known number of resting spores to a soil sample. Repeatability was evaluated on 4 subsamples of infested soil. A variation of 39% from the mean was noted and considered acceptable (Pratt, 1976). The EPPO Panel on Diagnostics in Mycology considers that recovery rates may vary according to different types of soil (e.g. soil with high content of organic matter shows a lower recovery rate).

3.3.1.2. Sieving method B (adapted from van Leeuwen, et al., 2005). Samples are processed using two sieves (upper sieve 75 µm, lower one 25 µm). From the 500 g (see above), two sub-samples of maximum 100 g (depending on the type of soil) are air dried at room temperature and mixed with tap water and poured on the upper sieve. The soil is washed with tap water through the upper sieve until no fine material is left. The material held on the lower sieve is subsequently transferred with water (approximately 40–50 mL) to a beaker until no material is left on the lower sieve. Approximately 2–3 g of kaolin (Roth art. 8361.1 bolus; see Appendix 1) is added to the beaker to facilitate precipitation of organic matter, including resting spores, and shaken on a vortex mixer for 10 s to mix the fraction and kaolin thoroughly. This mix is then transferred into tubes and centrifuged at approximately 1018*g* for 5 min. Subsequently, the supernatant is removed and tubes are filled with saturated calcium chloride solution ($CaCl_2$) (see Appendix 1). This solution with a specific gravity (s.g.) of 1.4 separates particles with a lower and higher s.g. during centrifugation. The tubes are shaken for 10 s on a vortex mixer and then centrifuged at approximately 1018*g* for 5 min. The supernatant is collected in 100 mL glass jars and left to settle overnight. Approximately 2–2.5 mL of supernatant is collected per subsample and the number of (floating) resting spores present per subsample is determined in a counting chamber (Fig. 6) covered with a glass slide (resting spores stick to the lower side of the slide). Whenever required these can be counted and the number per gram of soil estimated. Quantification can also be performed with molecular techniques, as described for the zonal centrifuge technique (see 3.3.1.3). Recovery rates are not yet available.

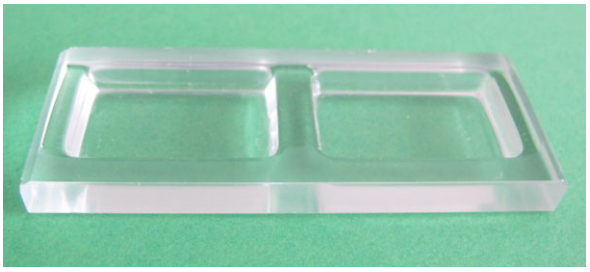


Fig. 6 Counting chamber (courtesy Main Inspectorate of Plant Health and Seed Inspection - Central Laboratory, PL). (Note: the counting chambers shown here are specifically manufactured for the Main Inspectorate of Plant Health and Seed Inspection - Central Laboratory. Nematode counting chambers are also appropriate.) [Colour figure can be viewed at wileyonlinelibrary.com]

3.3.1.3. Zonal centrifuge technique (Wander et al., 2007). The method is suitable for high-throughput analysis.

For this method, automated, sophisticated machinery is needed. This machinery is available from Instrumentenmakerij de Koning v.o.f. (Zierikzee, NL), and is called the Hendrickx centrifuge. Service-based processing of soil samples using this machine is also possible at ILVO, Belgium. The zonal centrifuge technique was originally developed for extraction of free-living nematodes (Hendrickx, 1995). The entire process from sample supply to removal of resting spore suspension is automated, and the method is as follows:

The subsample size is 200 g soil. The machine mixes the soil up to a volume of 1 L using tap water. Then the following ingredients are automatically added (in order) to the centrifuge rotor, which rotates horizontally at 15 000*g*: (1) saturated calcium chloride solution ($CaCl_2$, s.g. 1.4), (2) tap water, (3) 100–500 mL soil suspension, and (4) kaolin suspension (see Appendix 1). The separation liquid has a certain s.g. which separates particles with a lower and higher s.g. during centrifugation. The supernatant of water and $CaCl_2$ solution containing resting spores is collected in a small beaker. The rotor and tubing are automatically cleaned after each sample. Resting spores are counted as described above (see 3.3.1.2). When the objective is to evaluate the number of resting spores and not their viability these are quantified with real-time PCR. For the samples to be tested using real-time PCR the small beakers are kept for 1 h to overnight in a refrigerator, after which they are filtered over 25 mm nylon net filters (e.g. Millipore NY2002500, 20 µm pore size). Each nylon filter is placed in a 2 mL microcentrifuge tube and DNA extraction and real-time PCR are conducted as described in Appendix 4. Resting spores that have been stored in extracts from *S. endobioticum*-free soil should be used to establish a standard curve and translate the Ct value to the number of spore equivalents.

To determine the recovery rate, sandy loam soil samples spiked with known numbers of resting spores (2/20/200/2000 per 100 g) were analysed together with controls (the same number of resting spores spiked in extracts of *Synchytrium*-free soil). The average recovery rate was 50–70%. Three soil samples spiked with 1 spore per 100 g allowed detection of the pathogen in two out of three cases. Recovery was also tested with sandy soil and with loam

soil, with similar results. The method was also performed with a recently naturally infested soil sample (four subsamples processed) and yielded an average of 2912 resting spore equivalents per g of soil, which indicates an exceptionally high level of infestation.

3.3.2. Bioassays

In order to be able to interpret the test, negative controls of soil free from *S. endobioticum* and positive controls of infested soil should be included. In practice, the concentration of resting spores of *S. endobioticum* is not evaluated because the positive control is prepared with a high concentration of inoculum in close proximity to the sprouts. As it is a qualitative result no quantification is needed. A test is considered valid when tubers planted in known wart-infested soil produce warts (positive control). Tubers planted in the negative control should not produce warts. Temperature and humidity conditions in the glasshouse should be recorded. When the person carrying out the test has limited experience in identifying warts caused by *S. endobioticum*, any warts produced in test samples should be examined microscopically for the presence of summer sporangia and/or resting spores.

Cultivars known to be susceptible to pathotype 1(D1) (and hence to all other pathotypes) include Deodara, Evora, Morene, Tomensa, Maritiema and Arran Chief.

- Pot test

  Samples of soil are placed in pots (between 5 and 10 L) and each is planted with at least one tuber of a cultivar highly susceptible to all pathotypes (see above). The number of replicates depends on the amount of soil in the bulk sample collected from the field; after taking apart soil for direct examination (section 3.3.1), the rest of the soil is used for the pot test. The number of pots used also depends on the size of the pots. Pot tests should preferably be carried out in a greenhouse, where the pathogen can be contained more easily (closed system). Alternatively, pots may be placed in the open air provided that adequate containment measures are in place. In the greenhouse, pots are kept at 16–18°C and the soil is kept moist. Lighting conditions should be suitable for potato growth. Sprouts are cut back when they reach a height of approximately 60 cm in order to induce sprouting of additional eyes and stolon formation. After approximately 100 days, when new tubers have formed, plants are lifted and examined for warts.

  Performance criteria available

  In a test performed by the Dutch NPPO, 50% of tubers of cv. Maritiema developed warts with pathotypes 1(D1) and 6(O1) at an inoculum density of 1 resting spore per gram of soil, and >90% of tubers developed warts at a density of 5 resting spores per gram of soil (Baayen *et al.*, 2005). In the United Kingdom, cv. Arran Chief grown in pots in a glasshouse demonstrated 87% infection with 0.6 resting spores per gram of soil and 27% with 0.2 resting spores per gram of soil (Browning, 1995).

4. Identification

4.1. Morphological identification of summer sporangia and resting spores

4.1.1. Morphological characteristics of summer sporangia and resting spores

Plant material with warts can be examined for the presence of summer sporangia and/or resting spores using a stereomicroscope (Fig. 2E–G) (Walker, 1983). When necessary, microscopic slices of wart tissue are made, and examined under a light microscope at ×100 to ×400 magnification. Thin-walled, transparent summer sporangia are formed in young warts during the growing season. Resting spores are found during the growing season and in decomposing warts; they are embedded in the host tissue, filling the host cell almost completely.

Resting spores are aseptate, golden brown and thick-walled (triple wall) with the outer wall furrowed, prominently ridged and irregularly thickened, 25–75 µm (mean 50 µm) in diameter. They are spherical to ovoid in shape. In soil solutions (direct examination under a microscope), a high level of expertise is required to distinguish resting spores from surrounding soil particles and organic material (Fig. 7). When warts decay, the resting spores initially carry some attached host cell tissue. Eventually, the attached host cells disintegrate and the resting spores are left with a characteristic angular appearance in median view as the last sign of the presence of the former host cell wall. In surface view, the remaining host tissue typically appears as characteristic ridging. This, and other morphological characters, distinguishes the resting spores of *S. endobioticum* from those of other terrestrial *Synchytrium* species that could be encountered in potato fields as a result of weed infection (Pratt, 1974a,b) (Fig. 8).

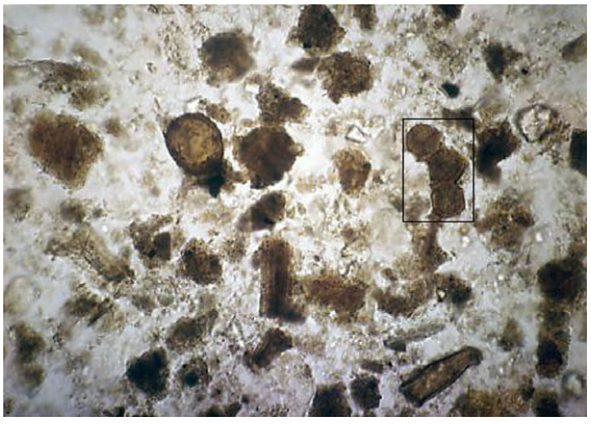


Fig. 7 Soil float after wet sieving illustrating the difficulty of direct examination of sieved soil for resting spores of *Synchytrium endobioticum.* Three resting spores visible in the rectangle. Centre left structure is most probably not resting spores of *S. endobioticum*. Soil particles elsewhere (courtesy, SASA, GB).

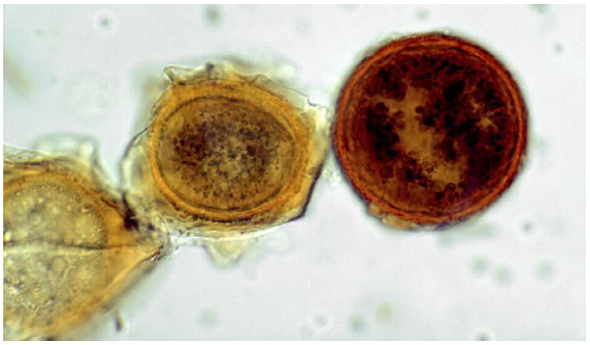


Fig. 8 Resting spores of *Synchytrium endobioticum* (left) and *Synchytrium succisae* (right) (courtesy, SASA, GB).

4.1.2. Possible confusions

Some wild plant species found in potato fields, such as *Taraxacum officinale,* may be infected by *Synchytrium* species other than *S. endobioticum*. None of these combines the unique features of resting spores of *S. endobioticum* (golden brown resting spores with a thick wall, an angular appearance and strong ridges, size 25–75 µm). Care should be taken that pollen grains and spores of *Endogone* spp. found in the soil are not confused with resting spores of *S. endobioticum*.

4.2. Molecular identification

The identity of summer sporangia or resting spores observed under the microscope can be confirmed by conventional PCR (Lévesque *et al.*, 2001; van den Boogert *et al.*, 2005), and real-time PCR (van Gent-Pelzer *et al.*, 2010; Smith *et al.*, 2014). An international test performance study (TPS) was organized under the Euphresco SENDO project to generate validation data for three molecular *S. endobioticum* detection and identification tests (van den Boogert *et al.*, 2005; van Gent-Pelzer *et al.*, 2010; Bonants *et al.*, 2015). Two TPS rounds were organized focusing on different test matrices: wart material and resting spore suspensions. At the time of the TPS design, the test developed by Smith *et al.* (2014) was not available to the TPS coordinators. For this reason, this test was not included in the Euphresco SENDO project. The tests included in the Euphresco SENDO project are described in Appendices 2, 3 and 6, respectively, the real-time PCR test of Smith *et al.* (2014) is described in Appendix 4. It should be noted that these tests will detect both viable and non-viable resting spores. Apart from identification of resting spores of *S. endobioticum*, these tests can successfully be performed on potato wart material. Conventional PCR is based on the internal transcribed spacer of the ribosomal DNA region (ITS) with the forward primer annealing to ITS1 and the reverse primer annealing to ITS2. The real-time PCR of van Gent-Pelzer *et al.* (2010) is based on ITS2. The real-time PCR of Smith *et al.* (2014) that is described here is based on small subunit (SSU) ribosomal DNA (18S rDNA). The real-time PCR developed by Bonants *et al.* (2015) is based on a pathotype 1(D1)-associated single nucleotide polymorphism (SNP) identified using Complexity reduction of polymorphic sequences (CRoPS) analysis. When using potato wart material, an internal positive isolation control targeting the plant COX gene is used to monitor the effectiveness of DNA isolation.

4.3. Determination of the viability of resting spores

Determination of viability can be made by microscopic examination or bioassay (see section 3.3.2). Details on microscopic examination are given below.

Fresh resting spores of *S. endobioticum* generally have homogeneous, granular, greyish contents. Upon germination,

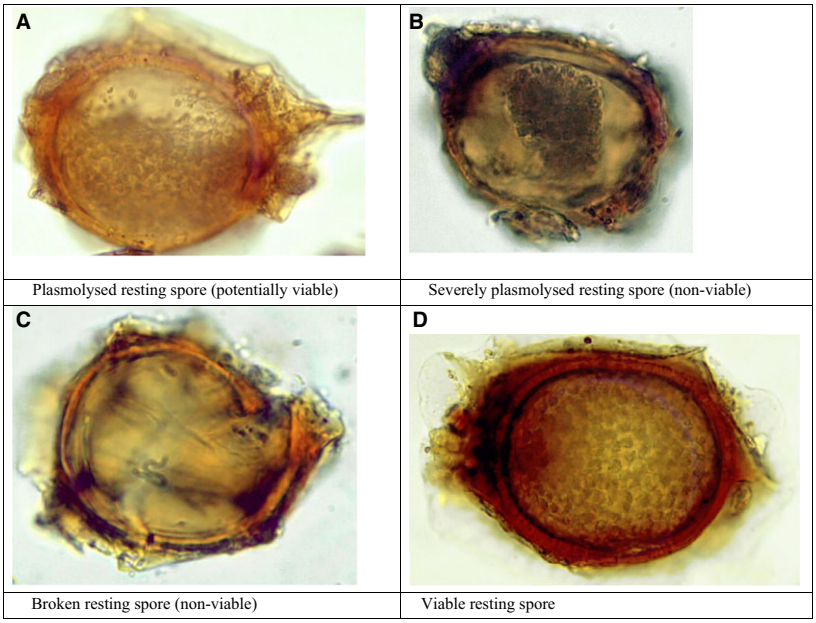


Fig. 9 Resting spores of *Synchytrium endobioticum* in various stages of plasmolysis (A, B) and of a broken resting spore (C) compared to a viable resting spore (D) (courtesy SASA).

resting spores become empty and disintegrate. There is considerable dispute about whether the viability of resting spores can be assessed by light microscopic examination of their contents and/or plasmolysis of these contents. It is generally agreed that resting spores with incomplete, heterogeneous contents may be difficult to identify as dead or alive. It is also agreed that the use of vital staining and UV fluorescence techniques does not solve this difficulty. Distinguishing between live and dead resting spores should therefore be restricted to cases where the features observed allow for unambiguous discrimination, and to experts with many years of experience with *S. endobioticum*. In case of doubt, resting spores should be considered viable.

Pictures of resting spores in various stages of plasmolysis and of a broken resting spore compared to a viable resting spore are shown in Fig. 9.

4.4. Pathotype identification

Pathotype identification can be performed as described in Appendix 5. Pathotype 1(D1) can be distinguished from at least pathotypes 2(G1), 6(O1) and 18(T1) by real-time PCR (Bonants *et al.*, 2015), as described in Appendix 6.

5. Reference material

Reference compost of pathotypes 1(D1), 2(G1), 6(O1) and 18(T1) can be obtained from:

The Netherlands Plant Protection Service, Wageningen, the Netherlands

Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Germany (see Further information below).

J. Przetakiewicz, Plant Breeding and Acclimatization Institute, National Research Institute, Department of Plant Pathology, Laboratory of Quarantine Organisms, Poland

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7. Performance criteria

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

8. Further information

Further information on this organism can be obtained from:

G. C. M. van Leeuwen, Netherlands Plant Protection Service, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands (e-mail: g.c.m.van-leeuwen@nvwa.nl);

K. Flath, Julius Kühn-Institut (JKI), Institute for Plant Protection of Field Crops and Grassland, Stahnsdorfer Damm 81, 14552 Kleinmachnow, Germany (e-mail: kerstin.flath@jki.bund.de)

J. Przetakiewicz, Plant Breeding and Acclimatization Institute – National Research Institute, Department of Plant Pathology, Laboratory of Quarantine Organisms, Radzikow, 05-870 Blonie, Poland (e-mail: j.przetakiewicz@ihar.edu.pl)

9. 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

10. 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: R. P. Baayen, Plant Protection Service, Wageningen (NL); H. Stachewicz, Julius Kühn-Institute (formerly Biologische Bundesanstalt für Land- und Forstwirtschaft, BBA), Kleinmachnow (DE). It has been revised by G. C. M. van Leeuwen, M. Westenberg and B. T. L. H. van de Vossenberg, Netherlands Plant Protection Service, National Reference Centre, Wageningen (NL); K. Heungens, Institute for Agricultural and Fisheries Research (ILVO), Plant Unit – Crop Protection Research Area, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium; A. Schlenzig, SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, United Kingdom.
It was reviewed by the European Mycology Network and the Panel on Diagnostics in Mycology.

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Appendix 1 – Buffers and suspensions

Saturated calcium chloride solution

To make $CaCl_2$ solution with s.g. of 1.4, make a 40% (w/w) solution. This can be done by adding 530 g of $CaCl_2H_2O$ to 470 g of H_2O.

Kaolin suspension (for zonal centrifuge, section 3.3.1.3)

Suspend 200 g of kaolin in 1 L of tap water.

Lactoglycerol

1:1:1 mixture of lactic acid, glycerol and water (e.g. 350 mL lactic acid, 350 mL glycerol and 350 mL distilled water).

Appendix 2 – Detection of *S. endobioticum* in potato wart material and resting spore suspensions using conventional PCR

1. General information

1.1 Detection of *S. endobioticum* in potato warts and resting spore suspension obtained from soil or warts using conventional PCR.

1.2 The conventional PCR was first published by Lévesque *et al.* (2001), but described in detail by van den Boogert *et al.* (2005). The PCR reaction mix was updated by NPPO-NL in 2013 and validated

in an international test performance study organized in the framework of the Euphresco SENDO project.

1.3 Primers F49 (5′-CAACACCATGTGAACTG-3′) and R502 (5′-ACATACACAATTCGAGTTT-3′) amplify 472 bp of the ITS region of the ribosomal DNA.

1.4 Amplification is performed in a thermal cycler with heated lid (e.g. T100 thermal cycler, Bio-Rad).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Potato wart material (max. 100 mg) or resting spore suspensions (10 µL) are extracted using the Plant Tissue Mini Protocol from the DNeasy® Plant Mini Kit (Qiagen) and eluted in 50 µL AE buffer. Appendix 4 describes an alternative DNA extraction method for resting spore suspensions.

2.1.2 After DNA extraction, no additional DNA clean-up is required. Extracted DNA should be used immediately or stored, preferably at approximately −20°C, until use.

2.2 Conventional PCR

2.2.1 Master mix conventional PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	13.8	N.A.
Colourless GoTaq Flexi buffer (Promega)	5×	5.0	1×
$MgCl_2$ (Promega)	25 mM	1.5	1.5 mM
dNTPs (Promega)	10 mM each	0.5	0.2 mM
Primer F49	10 µM	1.5	600 nM
Primer R502	10 µM	1.5	600 nM
GoTaq DNA Polymerase (Promega)	5 U μL^{-1}	0.2	1 U
Subtotal		24.0	
Genomic DNA extract		1.0	
Total		25.0	

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

2.2.2 PCR conditions: 2 min at 95°C, 35× (30 s at 95°C, 30 s at 57°C, 30 s at 72°C), 5 min at 72°C, quick cooling to room temperature.

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 extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* (e.g. pathotype 1(D1)) infected potato wart material (max. 100 mg).
- 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 undiluted DNA extracted from *S. endobioticum* (e.g. pathotype 1 (D1)) infected potato wart material (max. 100 mg).

3.2 Interpretation of results

In order to assign results from this test, the following criteria should be adopted.

Verification of the controls:

- NIC and NAC should produce no amplicons
- PIC and PAC should produce amplicons of 472 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 472 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

Performance criteria for analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, repeatability and reproducibility (= accuracy) were determined under the Euphresco SENDO project.

4.1 Analytical sensitivity data

Tenfold dilution series prepared from seven DNA extracts obtained from potato wart tissue diluted in DNA of healthy potato resulted in a limit of detection at a relative infection rate (RIR) of 1%. Undiluted naturally infected potato wart tissue is regarded as having RIR 100%. Lower relative concentrations of the pathogen resulted in lower success rates: 0.1% RIR = 57% success, 0.01% RIR = 14% success. When using the matrix potato warts, the van den Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010), and Bonants *et al.* (2015) tests performed equally well.

Tenfold dilution series were prepared from at least 10 resting spore suspensions obtained from potato warts starting at 500 spores μL^{-1}. At this concentration, a success rate of 82% was obtained. Lower concentrations resulted in lower success rates: 50 spores μL^{-1} = 80% success, and 5 spores μL^{-1} = 40% success. When using the matrix resting spores, the van Gent-Pelzer *et al.* (2010) test outperformed the van den Boogert *et al.* (2005) and Bonants *et al.* (2015) tests.

4.2 Analytical specificity data

Inclusivity: fifteen wart samples including *S. endobioticum* strains of five different pathotypes [four 1(D1), one 2(G1), one

6(O1), eight 18(T1), and one 38 (Nevsehir)] were tested at 100% RIR. All samples produced positive results.

Exclusivity: no data, no other *Synchytrium* species were tested.

4.3 Data on repeatability

Biological duplicates and triplicates of positive and negative samples tested by TPS partners were used to calculate the overall repeatability of the van den Boogert *et al.* (2005) test. For the matrix potato wart, results of 13 partners covering 52 repeatability samples (100% RIR, and healthy potato) were analysed. TPS participants obtained repeatable results in 94% of all potato wart samples tested. For the matrix resting spores, results of 14 partners covering 28 repeatability samples (500 spores μL^{-1} and molecular-grade water) were analysed. TPS participants obtained repeatable results in 64% of all resting spore samples tested. The lower repeatability for resting spore suspensions can be explained by the limit of detection for this test.

4.4 Data on reproducibility (= accuracy), diagnostic sensitivity and diagnostic specificity

For the matrix potato warts, TPS participants generated data for 144 samples covering infected and healthy samples. The overall accuracy (equal to repeatability given the TPS set-up) of the van den Boogert *et al.* (2005) test was 97.2%. Diagnostic sensitivity (% correctly identified positive samples) and diagnostic sensitivity (% correctly identified negative samples) obtained were, respectively, 96.3% and 100%.

For the matrix resting spores, the obtained percentage accuracy, percentage diagnostic sensitivity and percentage diagnostic specificity were 73.9%, 68.0% and 100%, respectively.

Appendix 3 – Detection of *S. endobioticum* in potato wart material and resting spore suspension using real-time PCR (test 1 – van Gent-Pelzer *et al.*, 2010)

1. General information

1.1 Detection of *S. endobioticum* in potato warts and resting spore suspension using real-time PCR developed by van Gent-Pelzer *et al.* (2010).

1.2 The test is designed to amplify 84 bp of the ITS2 sequence of *S. endobioticum* and 79 bp of the cytochrome oxidase subunit 1 (COX) of plant DNA as an internal control.

1.3 Primers and probes

Forward primer: Sendo ITS2F (5′-TTTTTACGCTCACTTTTTTTAGAATGTT-3′).

Reverse primer: Sendo ITS2R (5′-CTGCCTCACACACCACATACA-3′).

Sendo probe2 (5′-AATTCGAGTTTGTCAAAAGGTGTTTGTTGTGG-3′).

FAM label and Eclipse Dark Quencher (EDQ); forward primer COX F (5′-CGTCGCATTCCAGATTATCCA-3′); reverse primer COX RW (5′-CAACTACGGATATATAAGRRCCRRAACTG-3′).

Probe COXSOL 1511T (5′-AGGGCATTCCATCCAGCGTAAGCA–3′) Yakima Yellow label and Black Hole Quencher 1 (BHQ1).

1.4 Amplification is performed in a real-time PCR thermal cycler with heated lid (e.g. CFX96, Bio-Rad).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Potato wart material (max. 100 mg) or resting spore suspensions (10 µL) are extracted using the Plant Tissue Mini Protocol from the DNeasy® Plant Mini Kit (Qiagen) and eluted in 50 µL of AE buffer. Appendix 4 describes an alternative DNA extraction method for resting spore suspensions.

2.1.2 After DNA extraction, no additional DNA clean-up is required. Extracted DNA should either be used immediately or stored until use, preferably at approximately −20°C.

2.2 Real-time PCR (van Gent-Pelzer *et al.*, 2010)

2.2.1 Two simplex reactions are prepared; one for *S. endobioticum* detection and one for amplification of the plant COX gene as an internal control.

2.2.2 Master mix real-time PCR *S. endobioticum* detection.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.25†	N.A.
2× Premix Ex Taq (TaKaRa)	2×	15.0	1×
ROX Reference Dye/Dye II (TaKaRa)	Use when needed‡		
Sendo ITS2F	(10 µM)	0.75	250 nM
Sendo ITS2R	(10 µM)	0.75	250 nM
Sendo probe 2	(10 µM)	0.25	83 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

*Molecular grade water should be used. Alternatively, sterile (autoclaved or 0.45 µm filtered), purified (deionized or distilled) and nuclease-free water can be used.

†The volume of molecular-grade water is reduced to 9.65 µL when ROX Reference Dye/Dye II is used.

‡ROX reference dye/dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL of ROX Reference Dye (50×), final concentration 1×. For the AB 7500 Real-Time PCR System use 0.6 µL of ROX Reference Dye II (50×), final concentration 1×. When ROX Reference Dye or Dye II is used, reduce the volume of molecular-grade water to 0.6 µL per reaction.

2.2.3 Master mix real-time PCR for plant DNA amplification

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.5†	N.A.
2× Premix Ex Taq (TaKaRa)	2×	15.0	1×
ROX Reference Dye/Dye II (TaKaRa)	Use when needed‡		
COX F	(10 µM)	0.6	200 nM
COX RW	(10 µM)	0.6	200 nM
COX SOL 1511T	(10 µM)	0.3	100 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

*Molecular-grade water should be used. Alternatively, sterile (autoclaved or 0.45 µm filtered), purified (deionized or distilled) and nuclease-free water can be used.

†The volume of molecular-grade water is reduced to 9.9 µL when ROX Reference Dye/Dye II is used.

‡ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL of ROX Reference Dye (50×), final concentration 1×. For the AB 7500 Real-Time PCR System use 0.6 µL of ROX Reference Dye II (50×), final concentration 1×. When ROX Reference Dye or Dye II is used, reduce the volume of molecular-grade water to 0.6 µL per reaction.

2.2.4 PCR conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* [e.g. pathotype 1(D1)] infected potato wart material (max. 100 mg).
- 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.
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification: amplification of undiluted and 100× diluted DNA extracted from *S. endobioticum* [e.g. pathotype 1(D1)] infected potato wart material (max. 100 mg).

In addition to the external positive controls (PIC, PAC1 and PAC2), an internal positive isolation control is used to monitor each individual sample separately (specific amplification of the plant COX gene). The use of this internal control is not necessary in the case of *S. endobioticum* detection in resting spore suspension.

3.2 Interpretation of results

When working with potato wart material (NPPO NL) or with soil extracts after zonal centrifugation (ILVO), late Ct values can be expected. To avoid false positive results, a cycle cut-off value for *S. endobioticum* is set at 30. This cut-off value is obtained using false positive Ct values obtained for healthy potato by TPS participants. Three standard deviations were subtracted from the mean false positive Ct value resulting after rounding down to the nearest whole number, in a cycle cut-off value of 30. No cut-off value is used when analysing a resting spore suspension. It should be noted that samples of zonal centrifuge extracts of some soils resulted in late Ct values when processed with this test. These samples were consistently negative (no exponential curve) with the test described in Appendix 4, suggesting a higher specificity.

The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time. To assign results from real-time PCR-based tests the following criteria should be adopted:

Verification of the controls

- NAC should give no amplification curve for *S. endobioticum* and the plant COX gene (when testing wart material)
- NIC should give no amplification curve or a late amplification curve with a Ct > 30 for wart material, or no exponential amplification curve for resting spores (to be adapted for some soils, see comment above) for *S. endobioticum*. When testing wart material, the plant COX gene should produce an exponential amplification curve
- PIC, PAC1 and PAC2 should produce an exponential amplification curve, and a Ct < 30 when testing wart material, and when testing resting spores it should give an exponential amplification curve (to be adapted for some soils see comment above) for *S. endobioticum*. When testing wart material, an exponential amplification curve should be produced for the plant COX gene.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve, and a Ct value below 30 when testing wart material, or if it produces an exponential amplification curve (when testing resting spores, to be adapted for some soils see comment above) for *S. endobioticum*
- A test will be considered negative if it produces no exponential amplification curve or it should give a late amplification curve with a Ct > 30 when testing wart material or no exponential amplification curve when testing resting spores (to be adapted for some soils see comment above) for *S. endobioticum.* When testing wart material, an

exponential amplification curve should be produced for the plant COX gene
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Performance criteria for analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, repeatability and reproducibility (= accuracy) were determined under the Euphresco SENDO project.

4.1 Analytical sensitivity data

Tenfold dilution series prepared from seven DNA extracts obtained from warted potato tissue diluted in DNA of healthy potato resulted in a limit of detection at a relative infection rate (RIR) of 1% with an average Ct value of 27.1 (SD = 2.0). Undiluted naturally infected warted potato is regarded as having a 100% RIR. Lower relative concentrations of the pathogen resulted in lower success rates: 0.1% RIR = 43% success. Relative concentrations ≤0.01% resulted in Ct values that exceeded the cycle cut-off value of 30. When using the matrix potato warts, the van den Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010) and Bonants *et al.* (2015) tests performed equally well.

Tenfold dilution series were prepared from at least 10 resting spore suspensions obtained from potato warts starting at 500 spores μL^{-1}. At 500 and 50 spores μL^{-1}, a success rate of 100% was obtained. Lower concentrations resulted in lower success rates: 5 spores μL^{-1} = 90% success and 0.5 spores μL^{-1} = 40% success. When using the matrix resting spores, the van Gent-Pelzer *et al.* (2010) test outperforms the van den Boogert *et al.* (2005) and Bonants *et al.* (2015) tests.

4.2 Analytical specificity data

Inclusivity: fifteen wart samples covering *S. endobioticum* strains of five different pathotypes [four 1(D1), one 2(G1), one 6(O1), eight 18(T1), and one 38(Nevsehir)] were tested at 100% RIR. All samples produced positive results.

Exclusivity: no data; no other *Synchytrium* species were tested.

4.3 Data on repeatability

Biological duplicates and triplicates of positive and negative samples tested by TPS partners were used to calculate the overall repeatability of the van Gent-Pelzer *et al.* (2010) test. For the matrix potato wart, results of 13 partners covering 52 repeatability samples (100% RIR and healthy potato) were analysed. TPS participants obtained repeatable results in 94% of all potato wart samples tested. For the matrix resting spores, results of 12 partners covering 24 repeatability samples (500 spores μL^{-1} and molecular-grade water) were analysed. TPS participants obtained repeatable results in 83% of all resting spore samples tested.

4.4 Data on reproducibility (= accuracy), diagnostic sensitivity and diagnostic specificity

For the matrix potato warts, TPS participants generated data for 144 samples covering infected and healthy samples. The overall accuracy (equal to repeatability given the TPS set-up) obtained for the van Gent-Pelzer *et al.* (2010) test was 97.9%. Diagnostic sensitivity (% of correctly identified positive samples) and diagnostic sensitivity (% of correctly identified negative samples) were 97.2% and 100%, respectively.

For the matrix resting spores, percentage obtained accuracy, diagnostic sensitivity and diagnostic specificity were, respectively, 85.4%, 76.7% and 100%.

Appendix 4 – Detection of *S. endobioticum* in potato wart material and soil-extracted resting spore suspension using real-time PCR (test 2 – based on Smith *et al.*, 2014)

1. General information

1.1 Detection of *S. endobioticum* in potato warts or in soil suspensions containing resting spores based on a real-time PCR method developed by Smith *et al.* (2014).

1.2 Two tests are described in Smith *et al.* (2014). The test described here is the one designed to amplify 143 bp of the rDNA SSU (18S) sequence of *S. endobioticum*. Unlike the test described in Appendix 3 this test was not the subject of a European inter-laboratory test performance study; it targets a different gene and has advantages in terms of specificity and sensitivity. It was validated (with minor differences in terms of Taqman label and type of master mix) in at least one European laboratory.

1.3 Primers and probes

Forward primer Se18S_RTF1: (5′-CTCTGGTTGAG CTCCATTTAC-3′).

Reverse primer Se18S_RTR2 (5′-CCTATTCTAT-TATTCCATGCTGTA-3′).

Taqman probe Se18S_TM1 (5′-TATCCTGGTTCCC-CACAGGCACTC-3′) with 5′ 6-FAM label and 3′ BHQ1 quencher. Original probe labels (not validated) were 5′ Cy5 or Quasar 670 label and 3′ Iowa Black RQ or BHQ2 quencher.

1.4 Amplification is performed in a real-time PCR thermal cycler with heated lid (e.g. Applied Biosystems 7900HT).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 See Appendix 3 for extraction of DNA from wart material. For extraction of DNA from soil suspensions containing resting spores, the PowerSoil DNA Isolation Kit from MoBio (now DNeasy Powersoil Kit from Qiagen) resulted in significantly higher target DNA detection than the method used in Appendix 3. This kit is similar to the (currently no longer available) Ultra-Clean Soil DNA extraction kit from MoBio, which was used successfully for DNA extraction

of extracted resting spores in van den Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010) and (partially) in Smith *et al.* (2014).

When analysing soil suspensions or soil extracts containing resting spores (e.g. obtained using the zonal centrifuge) the particles can be concentrated on nylon filters as explained in section 3.3.1. Each nylon filter is placed in one of the kit's 'Power bead' tubes, extraction buffer is added and samples are vortexed for 30 min instead of the prescribed 10 min, after which the remainder of the protocol is followed.

2.1.2 After DNA extraction, no additional DNA clean-up is required. Either use extracted DNA immediately or store DNA until use, preferably at approximately −20°C.

2.2 Real-time PCR (Smith *et al.*, 2014)

2.2.1 A simplex reaction is prepared for *S. endobioticum* detection. When testing wart material, an internal control for amplification of the plant COX gene can be used as described in Appendix 3 (section 2.2.3). For soil suspensions containing resting spores (as well as for wart material) an internal reaction control can be used as described in Smith *et al.* (2014). This internal reaction control consists of an artificial 184 bp target supplied at 50 copies per reaction. Its amplicon is detected based on Sybr Green and should produce a Ct value below a threshold value in order to exclude false negatives. For details see Smith *et al.* (2014).

2.2.2 Master mix*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration†
Molecular-grade water‡	N.A.	5.6	N.A.
Maxima Probe/ROX qPCR master mix (Thermo Fisher)§	2×	10.0	1×
Se18S_RTF1	10 µM	1	500 nM
Se18S_RTR2	10 µM	1	500 nM
Se18S_TM1	10 µM	0.4	200 nM
Subtotal		18.0	
DNA extract		2.0	
Total		20.0	

*The mix was modified from the one in the original article in terms of final volume (smaller), master mix brand, absence of skim milk and absence of the internal control.

†Primers and probe were also tested at half concentration each, producing similar results. This adjustment may be preferred if many samples are to be processed.

‡Molecular-grade water should be used. Alternatively, sterile (autoclaved or 0.45 µm filtered), purified (deionized or distilled) and nuclease-free water can be used.

§Similar results were obtained with Takara Ex Taq master mix (as used in Appendix 3).

2.2.3 PCR conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg) when the sample consists of wart material; DNA from soil (extract) that does not contain *S. endobioticum* resting spores when the sample consists of resting spores recovered from soil.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum*-infected potato wart material (max. 100 mg) when the sample consists of wart material; DNA extraction from a known quantity of resting spores that were spiked in soil extract (= the material obtained after using the sieving or the zonal centrifugation method). This quantity can for example be 20 resting spores per soil extract, which is 10 times the limit of detection obtained during the validation.
- 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.
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification: amplification of undiluted and 100× diluted DNA extracted from *S. endobioticum*-infected potato wart material (max. 100 mg). Given the sensitivity of the test, it may be appropriate to use a higher dilution for PAC2 (e.g. 1000-fold diluted DNA from wart material). The PAC controls can also be DNA extracted from known amounts of resting spores (e.g. from 2000 and from 20 resting spores). DNA extraction from resting spores is significantly more efficient when they are present in a background of soil extract, obtained after applying the zonal centrifuge method, instead of pure water.
- In addition to the external positive controls (PIC, PAC1 and PAC2), an internal positive isolation control can be used to monitor each individual sample separately. For details, see section 2.2.1. of this appendix.

3.2 Interpretation of results

To assign results from real-time PCR-based tests the following criteria should be adopted:

Verification of the controls

- NIC and NAC should give no amplification. PIC should give an exponential amplification curve. For 20 resting spores per DNA extract, the average Ct value was approximately 31.5 during the validation

- PAC1 and PAC2 should give exponential amplifications curves. For undiluted DNA (PAC1) the Ct value was under 19 during the validation but can differ for 2 Cts given differences in the amount of target DNA in wart material. For 100× diluted DNA (PAC2), the Ct value was under 26 during the validation
- For interpretation of the plant COX gene internal control (= an optional control when using wart tissue as the sample) see Appendix 3.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve and a Ct value <35, which corresponds to the approximate Ct value for a single resting spore during our validation. This threshold needs to be adjusted according to the Ct value obtained for a single spore with the laboratory's own PCR equipment, master mix and fluorescence threshold used. Samples with Ct values of 35–40 may be positive but should be re-analysed, as they contain very low levels of pathogen DNA. Samples consistently producing Ct values ≤40 are considered positive given the specificity of the test and the fact that very old resting spores with partially degraded DNA may be present in soil samples
- A test will be considered negative if it produces no exponential amplification curve and/or a Ct value >40
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

Based on analyses of dilution series of resting spores, spiked in zonal centrifuge extract of *Synchytrium*-free soil, the method consistently detected down to 2 resting spores per zonal centrifuge extract, the lowest number tested. Examples of corresponding Ct values during the validation tests were 33.8 ± 0.9 and 34.1 ± 0.1 (2 biological replicates per test).

The limit of detection was not determined on wart tissue, but given Ct values of approximately 19 for non-diluted wart tissue, detection should theoretically still be reliable in 10^5-diluted DNA from wart material.

The Ct of 100 plasmidic copies of the target (the smallest amount tested) was approximately 33.

The test resulted in 1.6 ± 0.2 lower Ct values than the method in Appendix 3 when processing the same DNA extract from 200 resting spores (3 independent tests, minimum 2 biological replicates per test).

4.2 Analytical specificity data

Specificity was not part of the validation, but was tested extensively by the original authors of the test (Smith *et al.*, 2014): they found the test to be fully specific for *S. endobioticum* when tested on 12 *Synchytrium* species. Samples of zonal centrifuge extracts of some soils (and not others) resulted in late Ct values when processed with the method described in Appendix 3. These samples were consistently negative (Ct > 40) with this method (Appendix 4), suggesting a higher specificity. Although not tested, a similar difference in specificity is expected for the matrix potato (wart) tissue, given the reported late Ct values for this matrix when processed with the method described in Appendix 3.

4.3 Data on repeatability

The average coefficient of variance of the Ct was 1.5%, 2.2%, 0.6% and 0.3% between replicate analyses (DNA extraction + real-time PCR) in the same experiment for 2, 20, 200 and 2000 resting spores, respectively. This is based on 2 replicates within each experiment and the average of two (2 and 20 resting spores) or three (200 and 2000 resting spores) experiments.

4.4 Data on reproducibility

The average coefficient of variance of the Ct was 1.4%, 0.4%, 0.4% and 0.6% based on the average Ct values obtained in two (2 and 20 resting spores) or three (200 and 2000 resting spores) experiments when analysing 2, 20, 200 or 2000 resting spores, respectively.

4.5 Other performance criteria

No data on diagnostic sensitivity and diagnostic specificity is available.

The method is robust, given the allowed changes in primer and probe concentration (see section 2.2.2.), reaction volume, master mix brand and probe labelling.

4.6 Other information

Zonal centrifugation followed by real-time PCR was also performed with a naturally infested soil sample from a recent outbreak. Four subsamples were processed and yielded an average of 2912 resting spore equivalents per gram of soil, which indicates an exceptionally high infestation level. The standard deviation of the Ct after separate zonal centrifugation, DNA extraction and real-time PCR was 0.42.

Appendix 5 – Pathotype identification

Pathotype identification of new findings of *S. endobioticum* is possible with the Glynne–Lemmerzahl method (Glynne, 1925; Lemmerzahl, 1930; Noble & Glynne, 1970) using a set of differential cultivars. The most important pathotypes in the western part of the EPPO region are 1(D1), 2(G1), 6 (O1) and 18(T1), although pathotype 8(F1) has been recently detected. In the rest of the EPPO region other pathotypes also occur, for example in Turkey pathotype 38 (Nevşehir) is present (Cakir *et al.*, 2009).

The most important pathotypes in the western part of EPPO region can be differentiated using differential potato cultivars as indicated in Table 1. The differential cultivars are partly grouped; it is only necessary to include one cultivar of each group in a test for pathotype identification.

The Glynne–Lemmerzahl method is rapid (warts are formed within a few weeks) but requires, as inoculum, warts containing summer sporangia (see section 1 below).

Pathotype 1(D1) can be discriminated from pathotypes 2 (G1), 6(O1) and 18(T1) by real-time PCR (Bonants *et al.*, 2015) (see Appendix 6).

Methods to produce inoculum are described below. These are safer than field tests.

1. Production of fresh warts from old warts or resting spores

Fresh warts can be produced with the Spieckermann method (see 1.1 below), the Potocek tube test (see 1.2 below) or with a method used in SASA (see 1.3). Alternatively, warts can be collected directly in the field, but then these should be fresh (green/white coloured warts).

1.1 Spieckermann method (production of fresh warts from old ones)

In the Spieckermann method (Spieckermann & Kothoff, 1924), inoculum consisting of resting spores in any type of sand is spread over tuber blocks/eye pieces. After infection of the sprouts, warts are formed on susceptible cultivars.

1.1.1 Preparation of the compost (mixture of resting spores and sand)

To prepare compost, warts should be used within 14 days after harvest and, in the meantime, stored at 4°C to prevent rotting. Leaves, stems and normal tuber parts are removed from the warts (adherent soil need not be removed) and the warts are cut into approximately 1 cm pieces or slices. The pieces and slices are well mixed with clean sand (approximately 3 kg of sand per kg of warts) and incubated at a temperature of 18–25°C. The mixture is moistened 2–3 times a week with tap water. The mixture is thoroughly mixed 2–3 times a week during the first 2 months, and then weekly during the next 2 months. After 4 months, the mixture is no longer stirred or moistened but is slowly air dried at the same temperature for an additional 2 months. After a total of 6 months the compost should be dry enough for storage in air-tight boxes or bags (collection). When stored at 10–18°C under dry conditions the compost can be used for 10–20 years. Care should be taken to avoid cross-contamination of composts made of different isolates. If speed is required (pathotype identification), the compost can be used after a period of 4 months to inoculate susceptible cultivars (see 3.3.2 above, Bioassays) in order to produce fresh warts. The resulting concentration in the compost is well above the required concentration for successful infection.

1.1.2 Production of fresh warts

Plugs of potato tissue (approximately 2 × 2 × 2 cm) with at least one main eye are cut out from tubers of a susceptible cultivar (see 3.3.2 above, Bioassays) stored at 4°C. Subsequently, these are stored for a minimum of 24 h in a closed box/bag at 8–10°C (fridge) to stimulate wound healing. The plugs are then placed in rows in disinfected boxes (for example 25 × 40 × 5 cm) containing a moistened, approximately 1 cm thick, formalin-free, softboard plate. The distance between rows is approximately 5 mm. The plugs are moistened with a fine mist of water, after which 1.0–1.5 g of inoculum compost per plug is placed on top of the eye. The inoculated plugs are again moistened, and placed in a controlled environment at 16–18°C in the dark (boxes may be closed with a lid to avoid quickly drying out). The relative humidity in the controlled environment should be high (a minimum of 85–90%). During incubation, the boxes with the plugs are moistened daily with a fine water mist, just sufficient to keep them moist (not wet). After approximately 10–15 days, the main sprouts (by then 6–8 cm long) are cut down to 1–2 cm. The side sprouts are then cut down to 2–3 cm after another 8–10 days, and twice more after sprouts have again attained a length of 8–10 cm. The first small warts usually appear after 4–5 weeks of incubation.

1.2 Potocek's tube test (production of fresh warts from resting spores in soil)

Method A (Potoček *et al.*, 1991)

Thirty tubers of a highly susceptible cultivar (see 3.3.2 above, Bioassays) with their buds out of dormancy should be used for the testing of each soil sample. Soil samples are placed in conical plastic tubes (3–4 cm in diameter at the upper end, 3.5–4.5 cm at the lower end and 8 cm long) attached to the crown of test tubers by a simple system of clips and elastic bands. Sprouts are allowed to grow up through the soil for 5–7 weeks with appropriate watering. Any sprouts that grow quickly through the soil should be cut to stimulate the growth of additional sprouts. The soil is then removed, and fresh (whitish/green) warts are collected on the sprouts.

Method B (Przetakiewicz, 2015)

Infested soil is replaced by concentrated inoculum. A high inoculum density is reached by centrifugation of a spore suspension, obtained after extraction from infested

Table 1. Differential potato cultivars for the identification of pathotypes 1(D1), 2(G1), 6(O1), and 18(T1) of *S. endobioticum.* Results given here are mostly based on results from the Glynne–Lemmerzahl method

Cultivar group	Resistance	1(D1)	2(G1)	6(O1)	18(T1)
Tomensa/Evora/Deodara	None	+	+	+	+
Irga/Producent	1	–	+	+	+
Talent	1	–	–*	–*	+
Saphir	1, 6, 18	–	+	–	–
Ikar/Gawin/Karolin/Belita	1, 2, 6, 18	–	–	–	–

*Slightly susceptible reaction: non-necrotic sori fields are observed but not full wart formation.
+, wart formation; –, no wart formation.

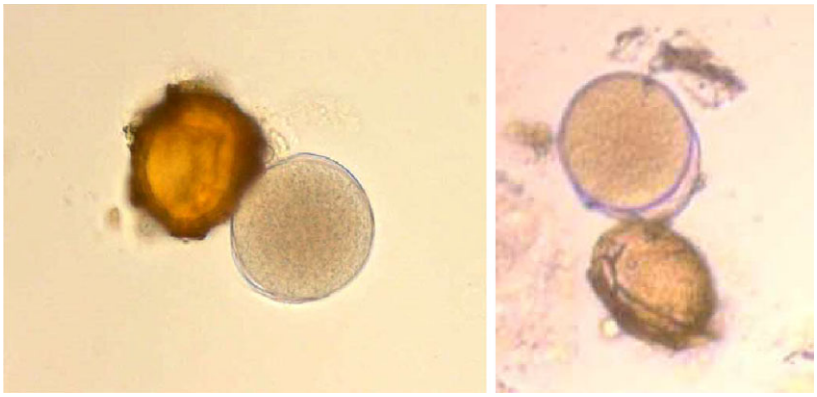

Fig. 10 Photos of germinating resting spores of *Synchytrium endobioticum* (courtesy SASA).

soil by wet sieving (see 3.3.1 above, Direct examination) and decanting.

A plastic ring (2 cm high and 3 cm in diameter) is fixed on the top of a tuber using warm paraffin or Vaseline. A highly susceptible potato cultivar (see 3.3.2 above, Bioassays) should be used. Each ring is filled with sterile river sand up to a depth of 1.5 cm. One millilitre of the concentrated inoculum is then pipetted into each of the rings. The inoculated tubers with rings are moistened and placed in a plastic box at 16–18°C in the dark. The sand in the rings is moistened daily with distilled water to maintain sufficient moisture. During incubation the sprouts are cut down at the upper edge of the rings to stimulate the growth of additional sprouts. If no potato wart symptoms are observed, the sprouts are cut down again. After a period of 4–6 weeks fresh (whitish/green) warts are collected on the sprouts.

1.3 SASA method

1.3.1 Production of inoculum

Old (brown) wart tissue is broken into smaller pieces and air dried at room temperature until it becomes hard. The hard tissue is ground using a planetary ball mill for 5 min at 300 r.p.m. Grinding is repeated if necessary, allowing material to cool down between the intervals.

Grinding by hand using a mortar and pestle is possible but difficult as the dried wart tissue is extremely hard.

The ground material is dry-sieved, collecting the fraction from 25 to 75 µm, and then extracted using the chloroform method of Pratt described in 3.3.1.1. Dry resting spores can be stored for at least 5 years at room temperature.

1.3.2 Production of fresh warts

Approximately 10 mg of extracted resting spores is sprinkled onto the surface of 10 mL sterile distilled water in a small plastic Petri dish and incubated in the dark at 20°C for 21 days. After 21 days, the spores are examined under a microscope to ensure germination has started (Fig. 10). If germination has not started the spores should be incubated further and examined daily.

Potato tubers with small sprouts about 1–2 mm long are placed in transparent plastic boxes lined with damp tissue paper with the marked sprouts facing up. The sprouts are ringed with melted Vaseline using a syringe. The ring must be unbroken and high enough to hold the spore suspension without leaking.

The 10 mL of germinating resting spores is diluted further to 20 mL with sterile water and placed within the rings using a pipette or a squeeze bottle until the sprout is completely submerged in spore suspension. The plastic boxes are covered with lids and incubated for 4 days at 10°C, after which the boxes are opened, the inoculum and Vaseline ring is removed and the box is moved to a misted glasshouse at 15–18°C (16 h light).

Small fresh warts start to develop 3–4 weeks after inoculation.

2. Glynne–Lemmerzahl method

When only a very few fresh warts are available, wart tissue should first be multiplied on highly susceptible cultivars (see 3.3.2 above, Bioassays) following the Glynne–Lemmerzahl method. With this method, multiple series of tests can be carried out in relatively short periods of time, under optimal conditions for infection and incubation (preferably in the period from October to May). For pathotype identification, enough fresh wart material should be available to perform a complete test including all differential cultivars (Table 1).

It is recommended to perform three independent tests (resulting in unambiguous pathotype identification). For each test, 10 eye plugs are used per differential cultivar (Table 1).

Sprouts 1–2 mm in length on entire tubers of the differential cultivars (Table 1) or eye plugs (approximately 27 × 27 mm) cut out from these tubers, are ringed with melted Vaseline, using a syringe without a needle. If eye plugs are used, these are first stored for a minimum of 24 h in a closed box at 8–10°C (in a refrigerator) to stimulate wound healing. Pieces of fresh wart tissue (white to light brown in colour) are placed inside the Vaseline rings together with some distilled water. Only the uncut surface of the wart inoculum should be put into contact with the water. Each piece of fresh wart tissue should weigh 2–3 g; larger warts should be cut into small pieces of 2–3 g. After incubation for 48 h at 8–12°C the wart tissue and Vaseline rings are removed. The wart

inoculum can be re-used 3–4 times. The tubers or eye fields are then moistened with tap water, covered with a layer of 1–2 cm of moist peat or sand, or alternatively damp cotton wool, and incubated at 16–18°C. Evaluation of the reaction types produced is done after 25–30 days. The covering layer of peat, sand or cotton wool should be frequently moistened with tap water during the entire incubation period (23–25 days) in order to promote wart formation.

When even a single wart with resting spores has been formed the test should be rated as '+' (Table 1). For a test to be considered successful, at least 50% of the reactions of the eye pieces of the susceptible differential cultivar should be rated as '+' (Table 1).

Specific expertise is needed to identify pathotypes other than those mentioned in Table 1, and it is recommended to contact specialist laboratories.

In particular, it should be noted that pathotype 8(F1) has not been included in the table as distinction from 18(T1) is difficult. In case of doubt, specialist laboratories should be contacted (see section 8, Further information).

Differential cultivars are available as follows:

Deodara	Netherlands Plant Protection Organization, NPPO-NL, PO Box 9102, 6700 HC Wageningen, the Netherlands. Laboratory of Potato Gene Resources and Tissue Culture, Plant Breeding and Acclimatization Institute, 76-009 Bonin, Poland; SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, UK
Evora	HZPC, Edisonweg 5, 8501 XG Joure, the Netherlands; https://www.hzpc.com/about/our-worldwide-locations
Irga	Pomorsko Mazurska, Hodowla Ziemniaka Sp. z o.o., Strzekęcino 11/76-024 Świeszyno, Poland; pmhz@pmhz.pl
Ikar	Pomorsko Mazurska, Hodowla Ziemniaka Sp. z o.o., Strzekęcino 11/76-024 Świeszyno, Poland; pmhz@pmhz.pl
Tomensa	H. Böhm, Nordkartoffel Zuchtgesellschaft, Postfach 1380, 21303 Lüneburg, Germany; Laboratory of Potato Gene Resources and Tissue Culture, Plant Breeding and Acclimatization Institute, 76-009 Bonin, POland; SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, UK
Producent	Netherlands Plant Protection Organization, NPPO-NL, PO Box 9102, 6700 HC Wageningen, the Netherlands; Laboratory of Potato Gene Resources and Tissue Culture, Plant Breeding and Acclimatization Institute, 76-009 Bonin, POland; SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, UK
Saphir	Laboratory of Potato Gene Resources and Tissue Culture, Plant Breeding and Acclimatization Institute, 76-009 Bonin, Poland; SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, UK
Talent	NORIKA Nordring-Kartoffelzucht- und Vermehrungs, Parkweg 4, 18190 Sanitz/ OT Groß Lüsewitz, Germany; info@norika.de
Karolin	Laboratory of Potato Gene Resources and Tissue Culture, Plant Breeding and Acclimatization Institute, 76-009 Bonin, Poland; SASA, 1 Roddinglaw Road, Edinburgh EH12 9FJ, UK
Gawin	Pomorsko Mazurska, Hodowla Ziemniaka Sp. z o.o., Strzekęcino 11/76-024 Świeszyno, Poland; pmhz@pmhz.pl

Appendix 6 – Identification of *S. endobioticum* pathotype 1(D1) using real-time PCR

1. General information

1.1 Detection of *S. endobioticum* pathotype 1(D1) in potato warts and resting spore suspension using real-time PCR developed by Bonants *et al.* (2015) and optimized by HLB, Wijster, the Netherlands.

1.2 The test is designed to specifically amplify 122 bp of a region containing a pathotype 1(D1)-specific SNP identified by CRoPS analysis. This analysis included strains from pathotypes 1(D1), 2(G1), 6 (O1) and 18(T1). Note that in rare cases some pathotype 1(D1) strains fail to produce a specific signal. When using potato wart material, an internal positive control targeting the plant COX gene is used to monitor the effectiveness of DNA isolation.

1.3 Forward primer: Fw_L1+ 2 (5′-CTAAGCGGATT-GATTACCG-3′)
Reverse primer: Rv_L1+1 (5′-GGGCCTGAAA-CACTAGAG-3′)
Probe: probe_sendo_P1_L1 VIC (5-ACCAAT-GACGGGCTAC-3) Yakima Yellow label and BHQ1 quencher
Forward primer: COX F (5′-CGTCGCATTCCA-GATTA TCCA-3′)
Reverse primer: COX RW (5′-CAACTACGGATA-TATAAGRRCCRRAA CTG-3′)
Probe: COXSOL 1511T (5′-AGGGCATTCCATC CAGCGTAAGCA–3′) Yakima Yellow label and BHQ1 quencher.

1.4 Amplification is performed in a real-time PCR thermal cycler with heated lid (e.g. CFX96, Bio-Rad).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Potato wart material (max. 100 mg) is extracted using the Plant Tissue Mini Protocol from the DNeasy® Plant Mini Kit (Qiagen) and eluted in 50 µL AE buffer.

2.1.2 After DNA extraction, no additional DNA clean-up is required. Either use extracted DNA immediately or store it at −20°C until use.

2.2 Real-time PCR (Bonants *et al.*, 2015)

2.2.1 Two simplex reactions are prepared; one for *S. endobioticum* pathotype 1(D1) identification, and one for amplification of the plant COX gene as internal control.

2.2.2 Master mix real-time PCR *S. endobioticum* pathotype 1(D1)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.25†	N.A.
2× Premix Ex Taq (TaKaRa)	2×	15.0	1×
ROX Reference Dye/Dye II (TaKaRa)	Use when needed‡		
FW_L1+2	(10 µM)	0.75	250 nM
RV_L1+1	(10 µM)	0.75	250 nM
Probe_sendo_P1_L1VIC	(10 µM)	0.25	83 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

*Molecular grade water should be used. Alternatively, sterile (autoclaved or 0.45 µm filtered), purified (deionized or distilled) and nuclease-free water can be used.
†The volume of molecular-grade water is reduced to 9.15 µL when ROX Reference Dye/Dye II is used.
‡ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50×), final concentration 1×. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50×), final concentration 1×. When ROX Reference Dye or Dye II is used, reduce the volume of molecular-grade water by 0.6 µL per reaction.

2.2.3 Master mix real-time PCR for plant DNA amplification

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.5†	N.A.
2× Premix Ex Taq (TaKaRa)	2×	15.0	1×
ROX Reference Dye/Dye II (TaKaRa)	Use when needed‡		
COX F	(10 µM)	0.6	200 nM
COX RW	(10 µM)	0.6	200 nM
COXSOL 1511T	(10 µM)	0.3	100 nM
Subtotal		27.0	
Genomic DNA extract		3.0	
Total		30.0	

*Molecular-grade water should be used. Alternatively, sterile (autoclaved or 0.45 µm filtered), purified (deionized or distilled) and nuclease-free water can be used.
†The volume of molecular-grade water is reduced to 9.9 µL when ROX Reference Dye/Dye II is used.
‡ROX Reference Dye/Dye II is used for normalization of the fluorescent signal when working with Applied Biosystems (AB) real-time PCR instruments. For AB 7000/7700/7900HT and 7300 Real-Time PCR Systems, use 0.6 µL ROX Reference Dye (50×), final concentration 1×. For the AB 7500 Real-Time PCR System use 0.6 µL ROX Reference Dye II (50×), final concentration 1×. When ROX Reference Dye or Dye II is used, reduce the volume of molecular-grade water by 0.6 µL per reaction.

2.2.4 PCR conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: DNA extraction from healthy potato material (max. 100 mg).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: DNA extraction from *S. endobioticum* pathotype 1(D1)-infected potato wart material (max. 100 mg).
- 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.
- Two positive amplification controls (PAC1 and PAC2) to monitor the efficiency of the amplification of *S. endobioticum* pathotype 1(D1): amplification of undiluted and 100× diluted DNA extracted from *S. endobioticum* 1 (D1)-infected potato wart material (max. 100 mg).
- In addition to the external positive controls (PIC, PAC1 and PAC2), an internal positive isolation control is used to monitor each individual sample separately (specific amplification of plant COX gene). The use of this internal control is not necessary in the case of detection of *S. endobioticum* pathotype 1(D1) in a resting spore suspension.

3.2 Interpretation of results

To assign results from real-time PCR-based tests the following criteria should be followed:

Verification of the controls

- NIC and NAC should give no amplification curve
- The Internal positive control curves should be exponential
- PAC1 and PAC2 *S. endobioticum* 1(D1) should produce an amplification curve, for both *S. endobioticum* 1(D1) and the plant COX gene
- PIC *S. endobioticum* 1(D1) should produce an amplification curve for both *S. endobioticum* 1(D1) and the plant COX gene.

When these conditions are met

- A test will be considered positive for *S. endobioticum* pathotype 1(D1) if it produces an exponential amplification curve
- A test will be considered negative if it produces no exponential amplification curve for *S. endobioticum* 1(D1). When testing wart material an exponential curve should be produced for the plant COX gene. It should be noted

that pathotype 1(D1) SNP is not present in all pathotype 1(D1) strains. A negative result can be the result of a pathotype 1(D1) strain lacking the associated SNP
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Performance criteria for analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, repeatability and reproducibility (= accuracy) were determined under the Euphresco SENDO project.

4.1 Analytical sensitivity data

Tenfold dilution series prepared from three DNA extracts obtained from potato wart tissue diluted in DNA of healthy potato resulted in a limit of detection at a relative infection rate (RIR) of 1% with an average Ct value of 35.0 (SD = 2.2). Undiluted naturally infected potato wart tissue is regarded as having 100% RIR. Lower relative concentrations of the pathogen resulted in lower success rates: 0.1% and 0.01% RIR = 33% success. When using the matrix potato warts, the van den Boogert *et al.* (2005), van Gent-Pelzer *et al.* (2010) and Bonants *et al.* (2015) tests performed equally.

Tenfold dilution series were prepared from at least five resting spore suspensions obtained from potato warts starting at 500 spores μL^{-1}. At 500 spores μL^{-1} a success rate of 40% was obtained. Lower concentrations gave negative results. When using the matrix resting spores, the van Gent-Pelzer *et al.* (2010) test outperforms the van den Boogert *et al.* (2005) and Bonants *et al.* (2015) tests. The Bonants test is not recommended for detection of pathotype 1(D1) in resting spore suspensions at concentrations ≤500 spores μL^{-1}.

4.2 Analytical specificity data

Fifteen wart samples covering *S. endobioticum* strains of five different pathotypes [four 1(D1), one 2(G1), one 6 (O1), eight 18(T1) and one 38(Nevsehir)] were tested at 100% RIR. No other *Synchytrium* species were tested. Pathotype 1(D1) strains from Netherlands, and Ireland tested positive in the pathotype 1(D1) test. One pathotype 1 (D1) strain from Sweden resulted in a negative result. The same was observed by Bonants *et al.* (2015), and they concluded that the pathotype 1(D1)-associated SNP is not present in all pathotype 1(D1) strains. Pathotype 1(D1) strains from the Netherlands and Germany tested by Bonants *et al.* (2015) all possess the associated SNP.

4.3 Data on repeatability

Biological duplicates and triplicates of positive and negative samples tested by TPS partners were used to calculate the overall repeatability of the Bonants *et al.* (2015) test. For the matrix potato wart, results of 13 partners covering 52 repeatability samples (100% RIR and healthy potato) were analysed. TPS participants obtained repeatable results in 98% of all potato wart samples tested. For the matrix resting spores, results of seven partners covering 14 repeatability samples (500 spores μL^{-1} and molecular-grade water) were analysed. TPS participants obtained repeatable results in 29% of all resting spore samples tested. The Bonants test is not recommended for detection of pathotype 1(D1) in resting spore suspensions at concentrations ≤500 spores μL^{-1}.

4.4 Data on reproducibility (= accuracy), diagnostic sensitivity and diagnostic specificity

For the matrix potato warts, TPS participants generated data for 144 samples covering infected and healthy samples. The overall accuracy (equal to repeatability given the TPS set-up) obtained for the Bonants *et al.* (2015) test was 96.5%. Diagnostic sensitivity (% of correctly identified positive samples) and diagnostic sensitivity (% of correctly identified negative samples) were 95.4% and 100%, respectively.

For the matrix resting spore percentage accuracy, diagnostic sensitivity and diagnostic specificity obtained were 61.4%, 45.7% and 77.1%, respectively. The test is not recommended for detection of pathotype 1(D1) in resting spore suspensions at concentrations ≤500 spores μL^{-1}.