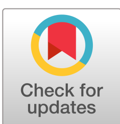
Bulletin OEPP/EPPO Bulletin (2020) **50** (3), 364–371 ISSN 0250-8052. DOI: 10.1111/epp.12688



European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 3/88 (1)

Phytosanitary procedures

PM 3/88 (1) Testing of potato varieties to assess resistance to *Synchytrium endobioticum*

Specific scope

This Standard describes the testing of potato varieties to assess resistance to potato wart disease caused by the fungus *Synchytrium endobioticum*. It should be used to fulfil requirements mentioned in PM 9/5 *National regulatory control system for Synchytrium endobioticum.*

Specific approval

First approved in 2020–09.

1. Introduction

The tests described in this Standard are intended to be used in official schemes for testing new potato varieties for resistance to *Synchytrium endobioticum*. This Standard makes it possible to compare results obtained from the different tests.

Molecular analyses are being developed and could help in the assessment of resistance but are not included in this Standard since they are currently not used in routine testing (Przetakiewicz & Plich, 2017; Prodhomme *et al*., 2019).

2. Definition of resistance

Resistance is assessed against specific pathotypes. A resistant variety is defined as one which reacts to a pathotype of *S. endobioticum* in such a way that secondary infection is prevented (i.e. that no resting spores are produced).

The degree of this resistance should be quantified and all the varieties ranked according to a standard scoring notation (see Table 1), indicating their potential use for control measures in relation to local conditions. Tests should be done for the pathotypes present in the country or region where the potato varieties should be used. The scale to assess resistance has five scores for all methods. Resistant varieties are those that are scored 1, 2, 3; slightly susceptible varieties are scored 4 and extremely susceptible varieties are scored 5 (Langerfeld & Stachewicz, 1994). The decisive criterion between scores 3 and 4 is the presence of resting spores and may require the use of microscopy: if resting spores are observed the score should be 4, if there are no resting spores, the score should be 3 (see Table 1).

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Table 1. Score and reaction types on individual sprout and classification, with description and illustration, based on Langerfeld & Stachewicz (1994) (pictures courtesy of J. Przetakiewicz, IHAR-PIB, PL)

Score	Resistance group	Reaction type	Illustration	Description
1	R1	Extremely resistant		Early defence necrosis; no visible sorus formation
2	R1	Resistant		Late defence necrosis; sorus formation partially visible, sori immature or necrotic before maturity
3	R2	Weakly resistant	Sori with visible summer sporangia under microscope	Very late defence necrosis; single ripe sorus or sorus fields developed, but completely surrounded by necrosis; up to five non-necrotic summer sori permitted, clear necrosis in other zones of the same tuber piece. No formation of tumours or resting spores. To decide between groups 3 and 4, it may be necessary to prepare thin slides of infected tissue: if there are no resting spores, the score should be 3.

(continued)

Table -0001 *(continued)*

Score	Resistance group	Reaction type	Illustration	Description
4	S1	Slightly susceptible	Resting (winter) sporangia in varying degrees of ripeness under microscope	Scattered infections; sori or sorus fields non-necrotic, few in number; late necrosis can be present on other infection sites on the sprout; the sprout can be slightly malformed (thickened). Resting (winter) sporangia are present. To decide between groups 3 and 4, it may be necessary to prepare thin slides of infected tissue: if resting spores are present, the score should be 4.
5	S2	Extremely susceptible	Symptoms as seen 4 weeks after inoculation using the Glynne-Lemmerzahl method	Dense infection fields, numerous ripe non-necrotic sori and sorus fields, fields with dense non-necrotic infection sites, predominant tumour formation.

3. Test procedure

(a) Number of tubers to be tested: a minimum of 40 tubers or eye plugs divided into at least two replicates (e.g. 20 tubers and two replicates, or 10 tubers in replicate one and then 30 tubers in replicate two) should be tested to demonstrate resistance.
(b) Tests are generally done over 2 years. However, where the results of tests in the first year indicate that a variety is extremely susceptible to a pathotype, there is no requirement to repeat these tests in a second year.
(c) Before a testing seasons starts the inoculum should be tested for purity (i.e. pathotype determination) using the differential varieties mentioned in EPPO Diagnostic Standard PM 7/28 *Synchytrium endobioticum*.
(d) A positive control (extremely susceptible variety for the pathotype to be tested) should be included in the test for resistance to check that the inoculation worked properly. See details in Appendices 1–3. Varieties such as Deodara, Evora, Morene, Tomensa, Eerstling (synonyms: Duke of York, Erstling or Midlothian Early) can be used as they are susceptible to all known pathotypes.
(e) There are currently three different test methods used in the EPPO region: Glynne-Lemmerzahl, Science & Advice for Scottish Agriculture (SASA), and Spieckermann. See Appendices 1–3 respectively for details, and Section 5 for criteria to decide on the method to use.
(f) Pot tests and field tests are also sometimes used in some parts of the EPPO region but they are not recommended as part of this Standard, which focuses on official resistance testing and does not address field resistance.

4. Assessment

All tubers should be assessed and be given a score from 1 to 5 as described in Table 1.

Training of assessors is important to have reliable assessments over a testing period and over the years. For example, blind tests may be organized at the start of the testing period for the team of assessors (or different authorized organizations performing the tests) to ensure consistency between the assessments.

Different tubers from the same potato variety will display a range of reactions and scores. The results of the tested tubers of each variety are accumulated and assigned into one of the three categories below (Table 2).

Table 2. Guidance for resistance assessment of a tested potato variety

Final scoring	Conditions	Comments
Resistant variety	All tubers in all replicates score 1 to 3	
Slightly susceptible variety	At least one tuber scores 4	No tuber should score 5. If only one scores 4, the testing may be repeated to check that it was not resulting from impurity in the variety lot. If all tubers score 1–3 in the new replicate, then the variety can be considered resistant.
Extremely susceptible variety	At least one tuber in at least one replicate scores 5	

5. Criteria to decide on the test method to use

To decide on the test method to use when establishing testing for resistance, the following criteria may be considered:

- available space (more space is needed when using entire tubers than eyes plugs);
- the time for incubation is 4 weeks for the Glynne-Lemmerzahl and SASA methods, and 8 weeks for the Spieckermann test method;
- maintenance of inoculum: a supply of fresh warts (as required for the Glynne-Lemmerzahl method) has to be maintained throughout the year, whereas resting spores as inoculum can be stored over several years without maintenance.

6. Enquiries

Further questions may be addressed to J. Przetakiewicz (IHAR-PIB, PL) and G. van Leeuwen (NVWA, NL).

Acknowledgments

This Standard was first drafted by K. Flath and F. Chilla (DE), G. van Leeuwen (NL), A. Schlenzig (GB) and J. Przetakiewicz (PL) and further reviewed by the Panel on Phytosanitary Measures for Potato.

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Appendix 1—Glynne-Lemmerzahl method

The Glynne-Lemmerzahl method is the official test procedure for resistance assessment of potato varieties to *Synchytrium endobioticum* in Poland and Germany.

Laboratory tests begin in late autumn and continue until late spring. Potato tubers are stored at low temperatures (4°C) during the testing season to inhibit the development of sprouts.

Material/Reagents

- Monceren (Bayer CropScience, active substance: pencycuron) or Miedzian® Extra 350SC (active substance: copper as copper oxychloride)
- Petroleum jelly, e.g. Vaseline
- Syringe without a needle
- Distilled water
- River sand or peat
- Plastic boxes with lids
- Stereo microscope and light microscope
- Tubers of susceptible varieties for production of inoculum (see EPPO Standard PM 7/28 for details).

1. Tuber preparation

The tubers are washed in tap water if necessary to remove soil. Tubers are incubated at room temperature in darkness or dim light to promote sprouting. They are incubated until sprouts reach 1–2 mm. It is possible to use entire tubers or cut eye plugs (approximately 3 × 3 × 2 cm) containing developing sprouts that are 1–2 mm in length. Subsequently, the sprouts are ringed with warm Vaseline using a syringe without a needle (Fig. 1). The eye fields are stored for a short time or overnight so that the Vaseline solidifies.

Fig. 1 Ringing sprout with Vaseline. Courtesy of J. Przetakiewicz, IHAR-PIB, PL.

2. Inoculation and incubation procedure

To prevent rotting, sprouts can be treated with Monceren (before inoculation) or Miedzian (after inoculation). Entire tubers or eye plugs are placed in a plastic box filled with 1 cm of river sand and Vaseline rings are filled with water to cover the sprout (Fig. 2). Fresh warts are cut into pieces (approximately 1–2 cm in diameter) and placed directly in the water-filled Vaseline rings (Fig. 3). For infection and release of the zoospores, the eye plugs are inoculated with the wart tissues for 2 days at 8–12°C.

If entire tubers are used, encircling the sprout with marker pen beforehand facilitates the recognition of inoculated sprouts after the petroleum jelly has been removed at a later stage.

Fig. 2 Preparing the tubers for inoculation (entire tubers version): rings are filled with water to cover the sprout. Courtesy of J. Przetakiewicz, IHAR-PIB, PL.

Fig. 3 Inoculation: pieces of fresh warts are cut into pieces and placed in the water-filled Vaseline rings. Courtesy of J. Przetakiewicz, IHAR-PIB, PL.

The tubers are regularly sprayed with water and the boxes are covered to maintain a high humidity. After incubation for 2 days the warts are removed and kept for the next infection cycle. Depending on the quality of the warts, it is possible to reuse them up to four times to induce new infections. Subsequently, the eye plugs (or entire tubers) are placed in a new box, covered with peat or sand and cultured at 16–18°C. The boxes are regularly sprayed with water.

3. Assessment

Sprouts are examined for infection 25–30 days after inoculation using a stereo microscope and a light microscope.

Type 4 or 5 reactions should be observed on the positive control on at least 80% of tubers. At least one tuber should score 5.

Appendix 2 – SASA testing method

Material and reagents

For tuber inoculation:
- Petroleum jelly, e.g. Vaseline
- Syringe without needle
- Small squeeze bottles
- Measuring cylinders
- Small Petri dishes (50–60 mm)
- Plastic boxes with lids
- Stereo microscope and light microscope
- Sterile distilled water
- Cooled incubator
- Glasshouse or controlled environment room, ideally with misting

For the inoculum production and spore extraction:
- Planetary ball mill (or mortar and pestle)
- 22 μm and 75 μm sieves and several larger sized sieves, for example 150 and 300 μm
- Sieve shaker
- 50 mL centrifuge tubes with lid
- Centrifuge
- Chloroform
- Fume hood
- Whatman filter hardened n°50

1. Tuber preparation

Tubers are removed from the cold store around 10 days before intended inoculation, washed gently, dried and numbered with a permanent marker pen. Varieties and allocated identification numbers are recorded. Tubers are then stored in the dark at room temperature to induce sprouting.

The highly susceptible variety ‘Morene’ is included in each inoculation to serve as positive control.

2. Germination of resting spores

Conditions to induce germination of resting spores should be set up 21 days prior to inoculation.

Approximately 10 mg of extracted spores (see point 5 below) are sprinkled onto the surface of 10 mL of sterile distilled water in small plastic Petri dishes and incubated in the dark at 20°C for 21 days. If germination (Fig. 4) has not started by then the spores should be incubated further and examined daily.

The content of each Petri dish is diluted with another 10 mL of sterile distilled water for the inoculation.

3. Inoculation and incubation of sprouts

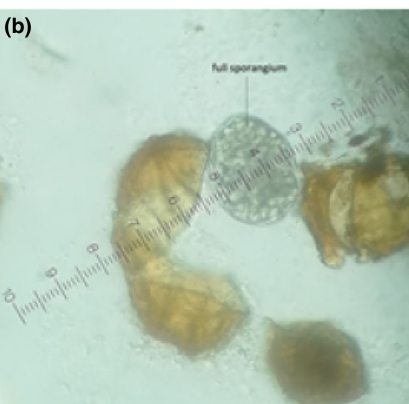
When the sprouts are 1 mm in length, they are ringed with melted Vaseline using a syringe without a needle (Fig. 5). A single sprout or a single cluster of sprouts is ringed on each tuber. Encircling the sprout with marker pen beforehand facilitates the recognition of inoculated sprouts after the Vaseline has been removed at a later stage. The Vaseline ring must be unbroken to hold the spore suspension without leaking and high enough for the suspension to cover the sprout.

The tubers are placed in plastic boxes lined with damp tissue paper with the ringed sprouts facing upwards.

The Vaseline rings are filled with spore suspension using a pipette or a squeeze bottle until the sprout is completely submerged.

The plastic boxes are covered with lids and incubated for 4 days at 10°C in the dark, after which the Vaseline rings are removed and the boxes are placed open in a glasshouse at 15–18°C under periodic misting (3× per day for 30 min).

In cases where the infection failed, for example because the sprout rotted or failed to develop, the tuber can be retested using another sprout.



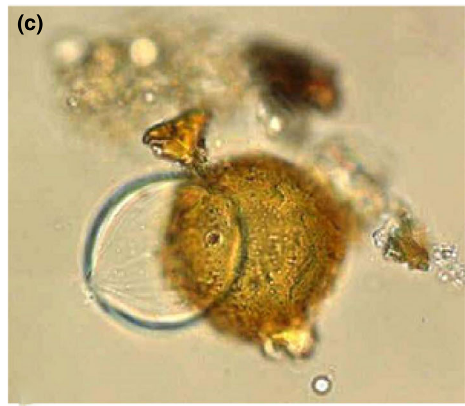


Fig. 4 Germination of resting spores. (A) Germinating resting spore with sorus containing sporangium. (B) Full sporangium after germination from a resting spore. (C) Germinated resting spore with empty sorus attached. (A) and (C) courtesy of A. Schlenzig, SASA; GB; (B) courtesy of L. Bender, Julius Kühn-Institut, Institute for National and International Plant Health, DE.

4. Assessment

Sprouts are examined for infection 3–4 weeks after inoculation using a stereo microscope with 10–15× magnification and a light microscope.

Type 4 or 5 reactions should be observed on the positive control on at least 80% of tubers. At least one tuber scores 5.

5. Inoculum production and resting spore extraction

The positive controls and susceptible tubers from the testing are potted up and grown on in the glasshouse to obtain resting spores for future inoculations. Wart tissue can grow up to fist size on tubers of a very susceptible variety. Once wart tissue turns dark brown or black it can be 'harvested', broken into smaller pieces and air-dried at room temperature until tissue has hardened.

The hard tissue is ground using a planetary ball mill for 5 min at 300 rpm. Repeat grinding if necessary, allowing material to cool down between the intervals.

Ground material is then passed through a stack of sieves with decreasing mesh sizes, first two large sizes (e.g. 300 and 150 µm), then 75 and 22 µm on a sieve shaker.

The 22 µm fraction containing the spores is transferred into Falcon tubes (approx. 1 g per tube) and 25 mL of chloroform is added (in a fume hood) before centrifuging for 20 min at 2000 *g*.

The supernatant contains the spores and is decanted into a new tube to be centrifuged a second and a third time. The pellet contains plant debris and soil and can be discarded.

After the third centrifugation the liquid is left to rest for about 20 min until a distinct layer of spores has formed on the surface. This layer is removed with a cut-off pipette tip and passed through a funnel laid out with filter paper (Whatman "hardened 50") to collect the spores. Allow spores to dry in the fume hood before transferring the extracted spores to glass bottles for storage. The dry spores can be stored at room temperature for at least 5 years.

Fig. 5 Ringing of the sprouts with melted Vaseline before inoculation. Courtesy of A. Schlenzig, SASA; GB.

Appendix 3 – Spieckermann method

Material and reagents

- Knife
- Plastic boxes with lids
- Cardboard plates (to put eye plugs on) (see Fig. 3)
- Inoculum (resting spores mixed with sand) (see PM 7/28, appendix 5, point 1.1.1)
- Stereo microscope (10–25×) and light microscope

1. Tuber preparation

Tubers are removed from the cold store a few days before intended inoculation. Varieties and allocated identification numbers are recorded.

2. Preparation of inoculum

The inoculum consists of resting spores mixed in sand (dry mixture). See EPPO Standard PM 7/28, appendix 5, point 1.1.1 for details.

3. Inoculation and incubation of sprouts

Plugs of potato tissue (approximately 2 × 2 × 2 cm) with at least one main eye are cut out from tubers (note that it is also possible to use entire potato tubers). Subsequently, these plugs are stored for a minimum of 24 h in a closed box/bag at 8–10°C to stimulate wound healing. The plugs are then placed in rows in disinfected boxes. In each tray, a susceptible variety (e.g. Deodara) is included as a positive control.

The distance between rows is approximately 5 mm, and within the row is approximately 1 mm (Fig. 6). The plugs are moistened with a fine mist of water, after which 1.0–1.5 g of inoculum per plug is placed on top of the eye. Inoculated plugs are again moistened and placed in a controlled environment at 16–18°C in the dark. Boxes may have a lid placed on them to avoid eye plugs from drying out. 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 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 on the positive control usually appear after 4–5 weeks of incubation (Fig. 7).

Fig. 6 Tray with cardboard plate inside containing potato eye plugs. Courtesy of G. van Leeuwen, NVWA, NL.

Fig. 7 Tray containing eye plugs of varieties Deodara and Producent, 8 weeks after inoculation. Note: the black-coloured tissue, especially in left-side Deodara, is decaying warts (reaction type 5). Courtesy of G. van Leeuwen, NVWA, NL.

4. Assessment

Scoring of the results takes place 7–8 weeks after inoculation, using a stereo microscope and a light microscope.

Type 4 or 5 reactions should be observed on the positive control on at least 80% of tubers. Type 5 reactions should be observed on the positive control on at least 30% of tubers.