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Diagnostics
Diagnostic

PM 7/60 (2) *Pantoea stewartii* subsp. *stewartii*

Specific scope

This Standard describes a diagnostic protocol for *Pantoea stewartii* subsp. *stewartii.*

Specific approval and amendment

Approved in 2005-09.
Revised in 2016-04.

1. Introduction

Pantoea stewartii subsp. *stewartii* is indigenous to the Americas and has been introduced to other parts of the world with maize seeds. It causes a disease called Stewart's wilt. The principal host is *Zea mays* (maize), especially sweet corn, but dent, flint, flour and pop corn cultivars can also be infected. In the Americas, *Chaetocnema pulicaria* Melsheimer (Coleoptera: Chrysomelidae) is the only known efficient vector and the main overwintering site of the bacterium. Asymptomatic infection of maize with *P. stewartii* subsp. *stewartii* is not known to occur. *Pantoea stewartii* subsp. *stewartii* may be transmitted in seed, and has occasionally been found to be able to overwinter in soil, manure or maize stalks. However, these means of survival are of little importance compared with the insect vectors which harbour the pathogen during their overwintering period, and transfer the bacterium from one plant to another during the growing season. Further information on the biology, distribution and economic importance of the disease is provided by EPPO/CABI (1997) and in the EPPO Global Database (EPPO, 2016).

A flow diagram describing the diagnostic procedure for *P. stewartii* subsp. *stewartii* is presented in Fig. 1.

2. Identity

Name: *Pantoea stewartii* subsp. *stewartii* (Smith, 1898) Mergaert *et al.* (1993).
Synonyms: *Erwinia stewartii* (Smith, 1898) Dye 1963; *Xanthomonas stewartii* (Smith, 1898) Dowson 1939.
Taxonomic position: Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, *Enterobacteriacae*.
EPPO code: ERWIST.
Phytosanitary categorization: EPPO A2 List no. 54; EU Annex II/A1.

3. Detection

3.1. Disease symptoms

The bacterium may be found on/in seeds of maize. No characteristic symptoms are visible on infected seeds. In maize plants, the first phase of the disease, which may affect the plants at the seedling stage, is wilting. The bacterium spreads systemically through the vascular system. If infected during a late growth stage, plants may reach a reasonable size. Leaves develop pale-green to yellow longitudinal streaks, with irregular or wavy margins, which are parallel to the veins and may extend for the length of the leaf (Fig. 2; other pictures can be seen in the EPPO Global Database, https://gd.eppo.int/taxon/ERWIST/photos). These streaks dry out and turn brown. Small water-soaked spots may develop on the husk of the cobs. Bacteria may exude in fine yellowish droplets on the inner face of the husk. Plants that are not killed may produce bleached, dead tassels. Cavities may appear close to the soil in the stalk pith of severely infected plants. *Pantoea stewartii* subsp. *stewartii* is able to penetrate the seed through wounds and via the xylem, and causes infection under the seed coat but is not able to enter the embryo. Sweet corn is particularly susceptible to seed infection. Field and dent corn are more susceptible to another phase, namely leaf blight, usually most apparent after tasselling. Short to long, irregular, pale-green to yellow streaks, which originate from feeding marks of the corn flea beetle (*Chaetocnema pulicaria*),

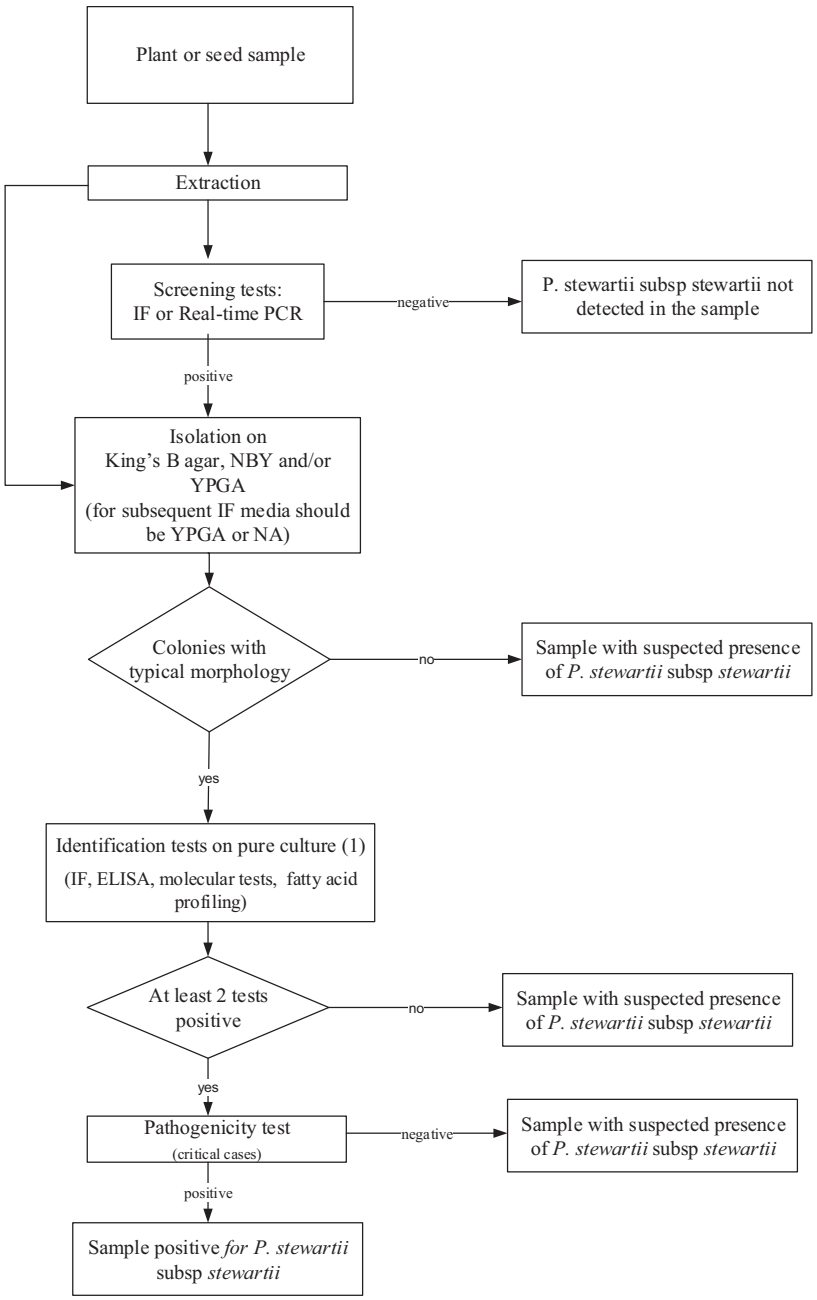

Fig. 1 Flow diagram for the diagnosis of *Pantoea stewartii* subsp. *stewartii* in maize. (1) At least two tests based on different characteristics of the pathogen (e.g. combinations of biochemical, serological or molecular tests) or two molecular tests based on different DNA sequence targets in the genome.

appear along the veins of leaves. Whole leaves sometimes become straw-coloured and die. Diseased and weakened plants are more susceptible to fungal stalk rots.

The disease may be confused with other leaf blights:

- Goss's bacterial wilt and leaf blight (*Clavibacter michiganensis* subsp. *nebraskensis*), which can be very similar to Stewart's wilt
- bacterial leaf blight, *Acidovorax avenae* subsp. *avenae*, which has long narrow stripes or spots with reddish-brown edges; leaves are easily shredded and there may be an associated rot of the upper stalk
- bacterial stripe, *Burkholderia andropogonis*, which has long, narrow, parallel, olive-green to yellow, water-soaked lesions; the upper leaves may be bleached almost white
- leaf blotches and spots and brown stalk rot of maize, which can be caused by *Pantoea ananatis*
- northern corn leaf blight, caused by the fungus *Setosphaeria turcica*, which has large, spindle-shaped, greyish-green to tan spots
- two other fungal diseases – Southern corn leaf blight, *Cochliobolus heterostrophus,* and corn leaf spot,

Fig. 2 Leaf symptoms on maize showing long chlorotic streaks with an irregular margin. Courtesy A. J. Ullstrup (US).

Cochliobolus carbonum – which have well-defined tan to brown spots.

3.2. Sampling

Five to ten leaves, cobs and/or, tassels with typical symptoms are collected from the inspected plot (field) for laboratory examination.

Seed-to-seedling transmission of *P. stewartii* subsp. *stewartii* may occur at low rates in seed lots with >1% kernel infection (Pataky & Ikin, 2003). For seed lots, the usually recommended sample size is 400 seeds per lot corresponding to a 95% probability of detecting an infestation level of 1%.

3.3. Extraction of bacteria from plant samples

Plant parts (leaf, husk, tassel) showing symptoms are surface sterilized in 70% ethanol for 5–10 s, rinsed with sterile water and excised at the leading edge of the lesions. Plant parts are then macerated in a plastic bag with a hand homogeniser (or in a mortar with a pestle) with a few millilitres of sterile phosphate buffer (PB) or comminuted (lightly ground) in a sterile Petri dish with sterile PB. An appropriate aliquot of the extract is transferred into a tube to be used in immunofluorescence (IF) staining. The remaining part of the extract is collected in sterile disposable plastic tubes and used immediately and/or stored at approximately 4–10°C or on ice for future use/reference purposes.

For samples showing symptoms, direct isolation should be performed immediately after maceration.

3.4. Extraction of the bacteria from seeds

The 400-seed sample is divided into subsamples of up to 100 seeds each and put into plastic bags. Seeds treated with any plant protection product should be washed under running tap water until the water runs clear, to remove product from the seed surface (in such cases, the report should mention that the seeds were treated). Sterile PB equal to twice the seed weight is added to the subsamples, and the bag is then closed. The subsamples are incubated in refrigerator at 4–10°C overnight. After incubation, the soaked seeds are shaken on a (rotary) shaker at room temperature for at least 1 h at approximately 200 r.p.m. The soaking liquid from seeds is centrifuged at approximately 10 000 *g* for approximately 10 min at 4–10°C. The pellet is resuspended in 1/10 of the original liquid volume of sterile PB and collected in sterile disposable plastic tubes for further testing, and stored at approximately 4–10°C or on ice. Macerates should be used immediately if at room temperature or within 24 h if stored at 4–10°C. Although it is not recommended, macerates may be stored below −18°C with 10–30% glycerol for several weeks for further testing, but isolation can be impaired.

3.5. Screening tests

IF and real-time PCR have been evaluated in the framework of a Euphresco project (Euphresco, 2010 unpublished report) and are recommended for use as screening tests. The conventional PCR test (PCR A with AGES primers) presented in Appendix 3 has been used in one laboratory for testing seed extracts but cannot be recommended as a screening test without further laboratory validation.

3.5.1. Immunofluorescence cell staining

The plant and seed extracts and their 10× and 100× dilutions in PB are used for indirect IF staining.

Indirect IF staining is preferably done using specific monoclonal antibodies. Those from Linaris (Dossenheim, DE) showed the best performance in the Euphresco test performance study (Euphresco, 2010 unpublished report). The IF test procedure is described in PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009).

Specific information on polyclonal and monoclonal antibodies that can be used and the performance criteria obtained using these antibodies are included in Appendix 2.

3.5.2. Real-time PCR

The pathogen can be detected by real-time PCR performed on the macerates obtained from symptomatic plant parts or after soaking of seeds (Tambong *et al*., 2008). The test is described in Appendix 4.

3.5.3. Isolation of bacteria by dilution plating

As the number of viable cells decreases quickly in macerates, isolation should be done within 24 h. For longer preservation, the suspension should be stored below −18°C with 10–30% glycerol, but this extended storage may impair isolation. The suspensions from symptomatic plant material and seed extracts are serially diluted in 10-fold steps up to 1:10 000 in PB (see Appendix 1). One hundred microlitres of each dilution is spread over the surface on at least one of the following media: King's B supplemented, nutrient-broth yeast extract agar (NBY) and/or yeast peptone glucose agar (YPGA) (see Appendix 1). Plates are incubated at approximately 25–27°C, and examined after 2–5 days for typical colonies of *P. stewartii* subsp. *stewartii*. One to five isolated colonies with typical morphology are used for further analysis. Final reading of the plates should be done after 5 days. Colonies are lemon to orange-yellow or pale-yellow, flat to convex, transparent, with entire edges and slow growing. On King's B agar strains developing colonies with a different appearance have been observed (Fig. 3A).

The cells are Gram-negative, straight short rods (0.4–0.7 × 0.9–1.7 µm). When IF is performed on colonies (see Section 4), colonies should be cultured on YPGA or subcultured on nutrient agar (NA). IF pictures are shown in Fig. 4.

Some laboratories also use a slide agglutination test using their own polyclonal antiserum.

4. Identification

Identification of pure cultures of presumptive *P. stewartii* subsp. *stewartii* isolates should be carried out using at least two tests based on different biological principles. The recommended tests are indicated below.

4.1. Main identification tests

4.1.1. Serological tests

4.1.1.1. Immunofluorescence cell staining. A suspension prepared from 1–3-day-old bacterial growth of presumptive *P. stewartii* subsp. *stewartii* isolates on NA or YPGA is used for indirect IF staining using polyclonal or monoclonal antibodies. The procedure for the IF test is described in PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Specific information on polyclonal and monoclonal antibodies that can be used and performance criteria obtained using these antibodies are included in Appendix 2.

4.1.1.2. Enzyme-linked immunosorbent assay (ELISA) test. An ELISA test for presumptive *P. stewartii* subsp. *stewartii* isolates can be performed using a kit from Agdia (Elkhart, US), following the supplier's instructions. Detailed instructions are provided in PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010b).

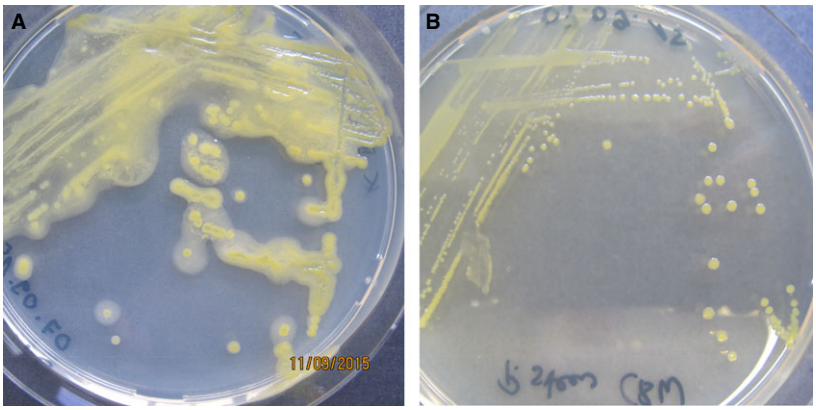

Fig. 3 *Pantoea stewartii* subsp. *stewartii* strain on King's B (A) and NBY (B) medium (typical colony morphology). Courtesy P. Müller, JKI (DE).

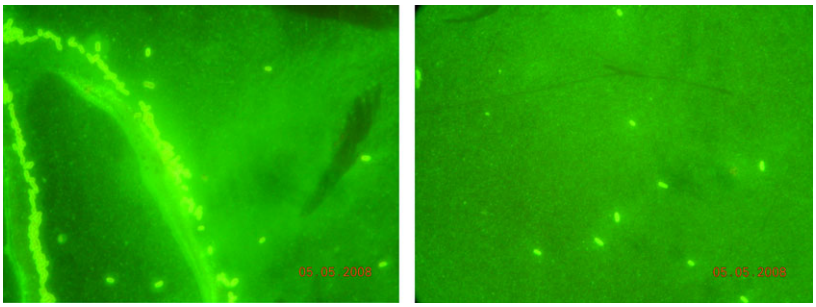

Fig. 4 IF slides showing typical cells of *Pantoea stewartii* subsp. *stewartii*. Courtesy ANSES (FR).

4.1.2. Molecular tests

4.1.2.1. PCR tests. Two conventional PCR test and a real-time PCR test are described in Appendices 3 and 4.

Because of the low sensitivity of the conventional PCR test described in Appendix 4 ($\leq 10^7$ cfu mL^{-1}), a sufficient amount of pure culture should be produced.

Analysis of chromosomal DNA can be performed by pulsed field gel electrophoresis (PFGE) as described by Zhang & Geider (1997) or by rep-PCR following Louws *et al.* (1994). Detailed instructions for rep-PCR are provided in PM 7/100 *Rep-PCR tests for identification of bacteria* (EPPO, 2010a).

4.1.2.2. Barcoding. Multilocus sequence analysis (Brady *et al.*, 2008), based on partial sequences of the *gyrB*, *rpoB*, *atpD* and *infB* genes, have been used to differentiate *P. stewartii* subsp. *stewartii* from the other species of the genus *Pantoea*. *Pantoea stewartii* subsp. *stewartii* can be distinguished from its close relative subsp. *indologenes*, although sequence similarity between the subspecies is high (e.g. up to 99% for *gyrB*).

4.1.3. Fatty acid profiling

Fatty acid analysis is carried out using the MIDI system (Newark, US) and Agilent equipment as described by Janse (1991) and Stead *et al.* (1992).

4.2. Other tests that can provide additional information

Morphological and biochemical tests: the morphological and biochemical properties of *P. stewartii* subsp. *stewartii* isolates are as listed in Table 1.

Table 1. Morphological and biochemical tests for identification of *Pantoea stewartii* subsp. *stewartii* and distinction from similar species

Tests	*Pantoea stewartii* subsp. *stewartii*	*Pantoea stewartii* subsp. *indologenes*	*Pantoea agglomerans*
Motility	Non-motile	Motile (or non-motile)	Motile
Gram staining	–	–	–
Kovac's oxidase test	–	–	–
Acetoin production	–	+	+
Indole production	–	+	–
Nitrate reduction	–	–	+
Aesculin hydrolysis	–	+	+
Growth on *cis*-aconitate	–	+	+
Acid production from			
Maltose	–	+	+
Arbutin	–	+	+
Salicin	–	+	+
Raffinose	+	+	–
Utilization of malonate	–	–	+

4.3. Pathogenicity test

In critical cases when a positive result is obtained, confirmation by pathogenicity test should be performed to confirm infection by *P. stewartiii* subsp *stewartii*. Procedures for pathogenicity tests are given in Appendix 5.

5. Reference strains

NCPPB 2295 (= CFBP 3167), NCPPB 449 (= CFBP 3168).

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (EPPO, 2006).

7. Performance criteria

When performance criteria are available they 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 it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this protocol can be obtained from: J. Németh, National Food Chain Safety Office, Directorate of Plant Protection, Soil Conservation and Agro-environment, Laboratory of Bacteriology, Kodo d. 1., 7634 Pecs, Hungary (tel. 36 72 552246; fax 36 72 255940; e-mail nemethjo@nebih.gov.hu).

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.

11. Acknowledgements

This protocol was originally drafted by: Dr J. Németh, National Food Chain Safety Office Directorate of Plant Protection, Soil Conservation and Agri-environment, Laboratory of Bacteriology, Pécs (HU). Thanks are also due to

Professor J. Pataky, University of Illinois, Urbana (US) for his critical comments on some methods described in this protocol.

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Appendix 1 – Media and buffers

1.1. Preparation of media and buffers

All media and buffers are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

1.1.1. Buffer

Phosphate buffer (50 mM, pH 7.0)

Component	Quantity
Na_2HPO_4·	4.26 g L^{-1}
KH_2PO_4	2.72 g L^{-1}
Distilled water	to 1 L

1.1.2. Media

King's B medium supplemented (King *et al.*, 1954)

Component	Quantity
Proteose peptone	20.0 g L^{-1}
Bacterial glycerol	10.0 mL L^{-1}
K_2HPO_4	1.5 g L^{-1}
$MgSO_4 \cdot 7H_2O$	1.5 g L^{-1}
Microbiological grade agar	15.0 g L^{-1}
Distilled water	to 1 L

200 mg L^{-1} of nystatin (or 200 mg L^{-1} of cycloheximide) should be added after autoclaving.

Yeast peptone glucose agar (YPGA)

Component	Quantity
Difco yeast extract	5.0 g L^{-1}
Difco Bacto peptone	5.0 g L^{-1}
D(+) glucose	10.0 g L^{-1}
Difco Bacto agar	15.0 g L^{-1}
Distilled water	to 1 L

Nutrient-broth yeast extract agar (NBY) (Schaad *et al.*, 2001)

To prepare the medium add all components, except the glucose and $MgSO_4 \cdot 7H_2O$ solutions, to distilled water and bring the volume to 900 mL. Mix thoroughly, gently heat and bring to the boil. Autoclave and cool to 50°C, add the glucose and $MgSO_4 \cdot 7H_2O$ solutions, mix thoroughly and pour on plates.

NBY base

Component	Quantity
Nutrient broth	8.0 g/900 mL
Yeast extract	2.0 g/900 mL
K_2HPO_4	2.0 g/900 mL
KH_2PO_4	0.5 g/900 mL
Microbiological grade agar	12.0 g/900 mL
Distilled water	to 900 mL

D(+) glucose solution

Component	Quantity
D(+)Glucose	5.0 g/50 mL
Distilled water	50.0 mL

Filter sterilize

$MgSO_4$ solution

Component	Quantity
$MgSO_4 \cdot 7H_2O$	0.25 g/50 mL
Distilled water	50.0 mL

Appendix 2 – serological methods

1. IF test

Loewe polyclonal antibodies and Linaris monoclonal antibodies can be used.

1.1. *Performance criteria for detection in seed with the Linaris monoclonal antibody* (EUPH05 *P. stewartii* subsp. *stewartii*; EUPHRESCO Final Report 2010). The test performance study involved 2 laboratories. The samples in the test performance study were spiked seed extracts.

- Analytical sensitivity: 95% of agreement at $<7 \times 10^3$ cfu mL^{-1}
- Diagnostic sensitivity: 92.5%
- Diagnostic specificity: 95%
- Repeatability: 97%
- Accuracy: 93%

1.2. *Performance criteria for detection in seed with the Loewe polyclonal antibody* (EUPH05 *P. stewartii* subsp. *stewartii*; Euphresco, 2010). The test performance study involved 2 laboratories. The samples in the test performance study were spiked seed extracts.

- Analytical sensitivity: 95% of agreement at $<7 \times 10^3$ cfu mL^{-1}
- Diagnostic sensitivity: 75%
- Diagnostic specificity: 33%
- Repeatability: 97%
- Accuracy: 61%

1.3. *Performance criteria for identification* (EUPH05 *P. stewartii* subsp. *stewartii*; Euphresco, 2010)

- Analytical sensitivity: 95% of agreement at 10^3 cfu mL^{-1}
- Diagnostic sensitivity: 100% (Linaris and Loewe)
- Diagnostic specificity: 100% (Linaris), 96.2% (Loewe)
- Repeatability: 100% (Linaris and Loewe)
- Accuracy: 100% (Linaris), 98.2% (Loewe)

2. ELISA test

The commercially available Agdia kit can only be recommended for identification (not for detection) because of the low analytical sensitivity.

2.1. Performance criteria for identification using the Agdia kit (EUPH05 *P. stewartii* subsp. *stewartii*; Euphresco, 2010). The test performance study involved 3 laboratories.

- Analytical sensitivity: 95% of agreement at 10^5–10^6 cfu mL^{-1}
- Diagnostic sensitivity: 100%
- Diagnostic specificity: 100%
- Repeatability: 100%
- Accuracy: 100%

Appendix 3 – Conventional PCR with AGES primers

EUPH05 *Pantoea stewartii* subsp. *stewartii* (Euphresco, 2010). The test was evaluated in the framework of the Euphresco research project (EUPH05 *P. stewartii* subsp. *stewartii*, 2008–2009).

1. General information

1.1 Detection and identification of *P. stewartii* subsp. *stewartii* from seeds and presumptive cultures.

1.2 The nucleic acid source is from presumptive colonies. Plant extracts can also be used as the nucleic acid source (see details on different PCR cycling conditions below) but more validation data is needed for this test to be used as a screening test.

1.3 The primers are designed from the 16S RNA region (developed at AGES, AT).

1.4 The amplicon size is 263 bp

1.5 Oligonucleotides:

PST-1: 5′-CCT CAC ACC ATC GGA TGT G-3′

PST-R: 5′-ATG AGG TTA TTA ACC TCA CCA-3′

1.6 Taq DNA polymerase is sourced from Invitrogen.

2. Methods

2.1 DNA extraction

The bacterial suspension is heated at 95°C for 10 min and cooled on ice. No DNA extraction was performed for seed extracts in the test performance study performed in the framework of Euphresco project. Two laboratories also performed the test with DNA extraction, which seemed to

improve the analytical sensitivity of the test; however, this should be confirmed by further test performance studies (Euphresco, 2010).

2.2 Polymerase chain reaction

2.2.1 Composition of the master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		15.55	–
PCR buffer	10×	2.5	1×
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	10 mM total, 2.5 mM each	0.5	50 µM each
Primer PST-1	20 pmol μL^{-1}	1.25	1 µM
Primer PST-R	20 pmol μL^{-1}	1.25	1 µM
Taq polymerase (Invitrogen Platinum)	5 U μL^{-1}	0.2	0.04 U μL^{-1}
DNA		3	
Total		25	

2.2.2 PCR cycling conditions

95°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s and 72°C for 7 min.

The number of cycles can be increased up to 40 cycles for direct detection of *P. stewartii* subsp. *stewartii* from seed extracts. (NB: Performance criteria indicated under paragraph 4 of this appendix are based on cycling conditions with 30 cycles.) The PCR product is separated in 1.5% agarose gel at 1.5 V cm^{-1}. The DNA fragment is stained in ethidium bromide solution (0.5 µg mL^{-1} in TAE buffer) for 30 min.

3. Essential procedural information

3.1 Controls

- Negative isolation control (NIC): to monitor contamination during nucleic acid extraction. Nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC): to ensure that nucleic acid of sufficient quantity and quality is isolated. Nucleic acid extraction and subsequent amplification of the target organism (for cultures) or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC): molecular-grade water.
- Positive amplification control (PAC): the same as the PIC.

3.2 Interpretation of the results

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of 263 bp.

When these conditions are met:

- A test will be considered positive if it produces amplicons of 263 bp and provided that the NIC and NAC are negative.
- A test will be considered negative if it produces no band or a band of a different size and provided that PIC and PAC are positive.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

These performance criteria are based on colonies as the nucleic acid source and 30 PCR cycles.

4.1 *Performance criteria for detection* (based on a test performance study with 5 laboratories involved).

The samples in the test performance study were spiked seed extracts (EUPH05 *P. stewartii* subsp. *stewartii*; Euphresco, 2010).

- Analytical sensitivity 95% of agreement at 7×10^4 cfu mL^{-1}
- Diagnostic sensitivity: 70%
- Diagnostic specificity: 76%
- Repeatability: 95%

4.2 *Performance criteria for identification* (based on internal validation during EUPH05 *P. stewartii* subsp. *stewartii*; Euphresco, 2010).

- Analytical sensitivity: 95% of agreement at 10^2 cfu mL^{-1}
- Diagnostic sensitivity: 100%
- Diagnostic specificity: 99%

False positive results have been obtained with *P. stewartii* subsp. *indologenes* (please note that this species has different biochemical characteristics from subsp. *stewartii*; see Table 1).

Appendix 4 – PCR modified from Coplin & Majerczak (2002)

1. General information

1.1 This test is based on Coplin & Majerczak (2002).

1.2 The primers are designed from the 16S-23S rRNA/ITS region.

1.3 The amplicon size is 920 bp

1.4 The nucleic acid is sourced from presumptive *P. stewartii* subsp. *stewartii* isolates. Oligonucleotides are:
ES16: 5′ GCG AAC TTG GCA GAG AT-3′
ESIG2c: 5′ GCG CTT GCG TGT TAT GAG-3′

1.5 The Taq DNA polymerase is Platinum Taq DNA polymerase from Invitrogen and Immolase from Bioline. Both are satisfactory.

2. Methods

2.1 DNA extraction

A colony from the suspected culture is suspended in 100 µL of sterile 50 mM phosphate buffer (pH 7.0) in a microvial. The closed vials are heated at 95°C for 10 min and the suspension is then cooled on ice.

Alkalysis may be used to improve isolation of DNA from the bacterial cells [the sample of 100 µL is treated with 50 µL 0.25 M NaOH, heated for 10 min at 95°C, put on ice for 2 min, treated with 50 µL 0.25 M HCl and 25 µL 0.5 M Tris-HCl (pH 8.0) with 0.1% Tween-20, heated for 10 min at 95°C and put on ice for 2 min].

2.2 Polymerase chain reaction

2.2.1 Master mix (with Platinum DNA polymerase)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Platinum PCR* Supermix (Invitrogen)	1.1×	15.5	0.85×
$MgCl_2$	50 mM	0.4	1.0 mM
Primer ES16	20 pmol μL^{-1}	0.8	0.8 µM
Primer ESIG2c	20 pmol μL^{-1}	0.8	0.8 µM
DNA		2.5	
Total		20	

*The composition of the Platinum PCR Supermix is: 22 U mL^{-1} Taq DNA polymerase, 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCL_2$, 4 × 220 µM dNTPs.

PCR cycling conditions: 95°C for 4 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s (for Corbett Research RG-300 thermocycler). The PCR product is separated in 1.5% agarose gel at 1.5 V cm^{-1}. The DNA fragment is stained in ethidium bromide solution (0.5 µg mL^{-1} in TAE buffer) for 30 min.

2.2.2 Master mix (with Immolase polymerase)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		5.9	
ImmoMix (Bioline)*	2×	10	1×
Primer ES16	20 pmol μL^{-1}	0.8	0.8 µM
Primer ESIG2c	20 pmol μL^{-1}	0.8	0.8 µM
DNA		2.5	
Total		20	

*Composition of the 2× ImmoMix (Bioline) is: Immolase DNA polymerase, 134 mM Tris-HCl (pH 8.3 at 25°C), 0.02% Tween 20, 4 × 2 mM dNTPs, 3 mM $MgCl_2$.

PCR cycling conditions: 95°C for 10 min, 25 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 30 s (for Corbett Research RG-300 thermocycler). The PCR product is separated in 1.5% agarose gel at 1.5 V cm^{-1}. The DNA fragment is stained in ethidium bromide solution (0.5 µg mL^{-1} in TAE buffer) for 30 min.

3. Essential procedural information

3.1 Controls

- Negative isolation control (NIC): to monitor contamination during nucleic acid extraction. Nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC): to ensure that nucleic acid of sufficient quantity and quality is isolated. Suspension of a reference strain for nucleic acid extraction and subsequent amplification.
- Negative amplification control (NAC): molecular-grade water
- Positive amplification control (PAC): the same as the PIC.

3.2 Interpretation of the results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC, PAC should produce amplicons of size 920 bp.

When these conditions are met:

- A test will be considered positive if it produces amplicons of 920 bp and provided that the NIC and NAC are negative.
- A test will be considered negative if it produces no band or a band of a different size and provided that PIC and PAC are positive.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1. Performance criteria for identification (based on intra-laboratory validation)

- Analytical sensitivity: $\leq 10^7$ cfu mL^{-1}
- Diagnostic sensitivity: 100%
- Diagnostic specificity: 100%
- Repeatability: 100%

Appendix 5 – Real-time PCR according to Tambong *et al.* (2008)

The test was evaluated in the framework of the Euphresco research project (EUPH05 *P. stewartii* ssp. *stewartii*, 2008–2009).

1. General information

1.1 The protocol was described by Tambong *et al.* (2008).

1.2 The nucleic acid source is symptomatic tissues, seed extracts or suspected colonies.

1.3 The primers are designed from the *cpsD* gene, which plays a role in the pathogenicity and virulence of the pathogen.
1.4 The amplicon size is 103 bp.
1.5 Oligonucleotides:
cps-RT74F: 5′-TGC TGA TTT TAA GTT TTG CTA-3′
cps-177R: 5′-AAG ATG AGC GAG GTC AGG ATA-3′
Probe:
cps-133: 5′-TCG GGT TCA CGT CTG TCC AAC T-3′
The probe (Proligo/Sigma, Woodlands, TX, US) is labelled with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) covalently coupled to the 5′-end and with the blackhole quencher 1 (BHQ-1) at the 3′-end.
1.6 The Taq DNA polymerase is sourced from a Quantitect Probe PCR kit.
1.7 Thermocycler: different equipments were used during the Euphresco test performance study and performed equally.

2. Methods

2.1 DNA extraction
No DNA extraction is required according to Tambong *et al*. (2008) and the Euphresco test performance study.

2.2 Polymerase chain reaction
2.2.1 Composition of the master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		3.4	-
Quantitect Probe PCR kit (containing $MgCl_2$, dNTPs and polymerase) (Qiagen Inc.)	2×	5	1×
Primer cps-RT74F	20 pmol μL^{-1}	0.2	0.4 µM
Primer cps-177R	20 pmol μL^{-1}	0.2	0.4 µM
Probe	10 pmol μL^{-1}	0.2	0.2 µM
DNA		1	
Total		10	

2.2.2 PCR cycling conditions: 95°C for 15 min, 50 cycles 95°C for 1 s, 60°C for 60 s.

3. Essential procedural information

3.1 Controls
The following external controls should be included:
- Negative isolation control (NIC): to monitor contamination during nucleic acid extraction. Nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC): to ensure that nucleic acid of sufficient quantity and quality is isolated. Nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC): molecular-grade water.
- Positive amplification control (PAC): the same as the PIC (pure strain only without matrix).

3.2 Interpretation of the results:
Verification of the controls
- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:
- A real-time PCR test will be considered positive if it produces an exponential amplification curve.
- A real-time PCR test will be considered negative if it does not produce and exponential amplification curve of if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 *Performance criteria for detection in seed* (based on test performance study with 5 laboratories involved) EUPH05 *P. stewartii* ssp. s*tewartii* (Euphresco, 2010)]
- Analytical sensitivity: 95% of agreement at $<7 \times 10^3$ cfu mL^{-1}
- Diagnostic sensitivity: 96%
- Diagnostic specificity: 78%
- Repeatability: 97%
- Accuracy: 90%
- Qualitative reproducibility: 87%

4.2 *Performance criteria for identification* (based on internal validation during EUPH05 *P. stewartii* ssp. s*tewartii*; Euphresco, 2010)
- Analytical sensitivity: 95% of agreement at 10^4 cfu mL^{-1}
- Diagnostic sensitivity: 100%
- Diagnostic specificity: 92.9%
- Repeatability: 100%
- Accuracy: 96.4%

False positive results have been obtained with *P. stewartii* subsp. *indologenes* (please note that this species has different biochemical characteristics from subsp. *stewartii*; see Table 1).

Appendix 6 – Pathogenicity test

The pathogenicity test is carried out on 10 plants by stem inoculation of 8–14-day-old (1–2 leaf stage) plants of a

susceptible cultivar of maize (e.g. 'Jubilee', 'Meritosa', 'Sweet Surprise F_1) grown in a glasshouse. Test plants should be grown at 22–28°C under high relative humidity (70–80%) with appropriate watering to avoid wilting due to water deficiency. Plants are needle inoculated using a sterile hypodermic syringe, with a bacterial suspension (10^7–10^8 cells/mL) prepared in sterile distilled water or phosphate buffer (pH 7) from isolates identified as *P. stewartii* subsp. *stewartii*, incubated for approximately 2 days at 25–28°C on King's B or preferably on NA ('poor' medium). Negative control plants are inoculated with sterile distilled water only. After inoculation, plants are kept in a humid chamber for 1–2 days at 25–28°C. The first disease symptoms (streaking) may appear after 3–5 days, but 7 or more days are necessary for the more typical symptoms (water soaking, yellow pockets of ooze in the vascular tissues) to develop. Wilting symptoms generally appear 14 days after inoculation. In some cases, yellow bacterial ooze forms in the vascular system. Plants should be kept for observation for 20 days.

Re-isolation from symptomatic tissues on media listed previously and checking of the colonies for identification should be performed – some bacteria other than *P. stewartii* subsp. *stewartii* (e.g. *Pantoea agglomerans*) can cause water-soaked symptoms around wounds if stem-inoculated into maize, but without ooze formation.