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EPPO STANDARD ON DIAGNOSTICS

PM 7/110 (2) *Xanthomonas* spp. (*Xanthomonas euvesicatoria* pv. *euvesicatoria*, *Xanthomonas hortorum* pv. *gardneri*, *Xanthomonas euvesicatoria* pv. *perforans*, *Xanthomonas vesicatoria*) causing bacterial spot of tomato and sweet pepper

Specific Scope: This Standard describes a diagnostic protocol for *Xanthomonas* spp. causing bacterial spot of tomato and sweet pepper (*Xanthomonas euvesicatoria* pv. *euvesicatoria*, *Xanthomonas hortorum* pv. *gardneri*, *Xanthomonas euvesicatoria* pv. *perforans*, *Xanthomonas vesicatoria*).[1]

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

Specific approval and amendment: Approved in 2012–09. Revised in 2023–08.

1 | INTRODUCTION

Bacterial spot of *Solanum lycopersicum* was first reported in South Africa and the US (Doidge, 1921; Gardner & Kendrick, 1921), and was first described on *Capsicum annuum* in Florida (Gardner & Kendrick, 1923). The disease has since been observed in areas of all continents where *Solanum lycopersicum* and *Capsicum annuum* are cultivated. For an updated geographical distribution consult EPPO Global Database (EPPO, 2023).

Classification of the bacteria causing leaf spot on both host plants, and therefore their routine identification, have been difficult to resolve. After a number of early revisions, they were classified for some time as *Xanthomonas campestris* pv. *vesicatoria* (Dye, 1978), although several phenotypically and phylogenetically distinct bacterial populations (eventually designated groups A–D) were represented (Dye, 1966; Jones et al., 2004; Stall et al., 1994; Vauterin et al., 1995). Groups A and C were briefly transferred to *Xanthomonas axonopodis* pv. *vesicatoria*, largely on the basis of DNA homology among a large but incomplete collection of xanthomonads (Jones et al., 2000; Vauterin et al., 1995) while group B was clearly separated at species level as *X. vesicatoria*. Group D strains, originally identified in the former Yugoslavia (Šutic, 1957) and including identical strains from Costa Rica, reverted to the species status *X. gardneri* (Jones et al., 2004). A new species, *X. euvesicatoria*, was proposed to distinguish the weakly amylolytic group A strains originally isolated in South Africa (Doidge, 1921) from the starch-degrading group C strains originally isolated in the US (Gardner & Kendrick, 1921), which were designated as *X. perforans*. Based on multilocus analysis, multilocus typing and whole genome sequencing (Barak et al., 2016; Osdaghi et al., 2018; Timilsina et al., 2015; Yaripour et al., 2018; Young et al., 2008), *X. euvesicatoria* and *X. perforans* do not form standalone species and were reclassified as pathovars of the same species as *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans*, respectively (Constantin et al., 2016). In addition, *X. gardneri* was reclassified as *X. cynarae* pv. *gardneri* (Timilsina et al., 2019) and then as *X. hortorum* pv. *gardneri* (Morinière et al., 2020). The bacterial spot pathogens currently fall into three validly described species (*X. vesicatoria*, *X. euvesicatoria* pv. *euvesicatoria*, *X. euvesicatoria* pv. *perforans* and *X. hortorum* pv. *gardneri*) and *X. axonopodis* pv. *vesicatoria* is no longer a valid name (Bull et al., 2010).

The bacteria causing bacterial spot have been disseminated internationally in contaminated commercial seed lots, deposited on the seed surface from infected pulp rather than as internal seed infections (Bashan & Okon, 2011). The bacteria can also spread with movement of infected young plants intended for planting and will survive on tomato volunteers and plant debris. This Standard describes screening tests for infected or contaminated seeds as well as for diagnosis of bacterial spot in symptomatic tomato and pepper plants.

A flow diagram describing the diagnostic procedure for *Xanthomonas* spp. causing bacterial spot of tomato and sweet pepper is presented in Figure 1.

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

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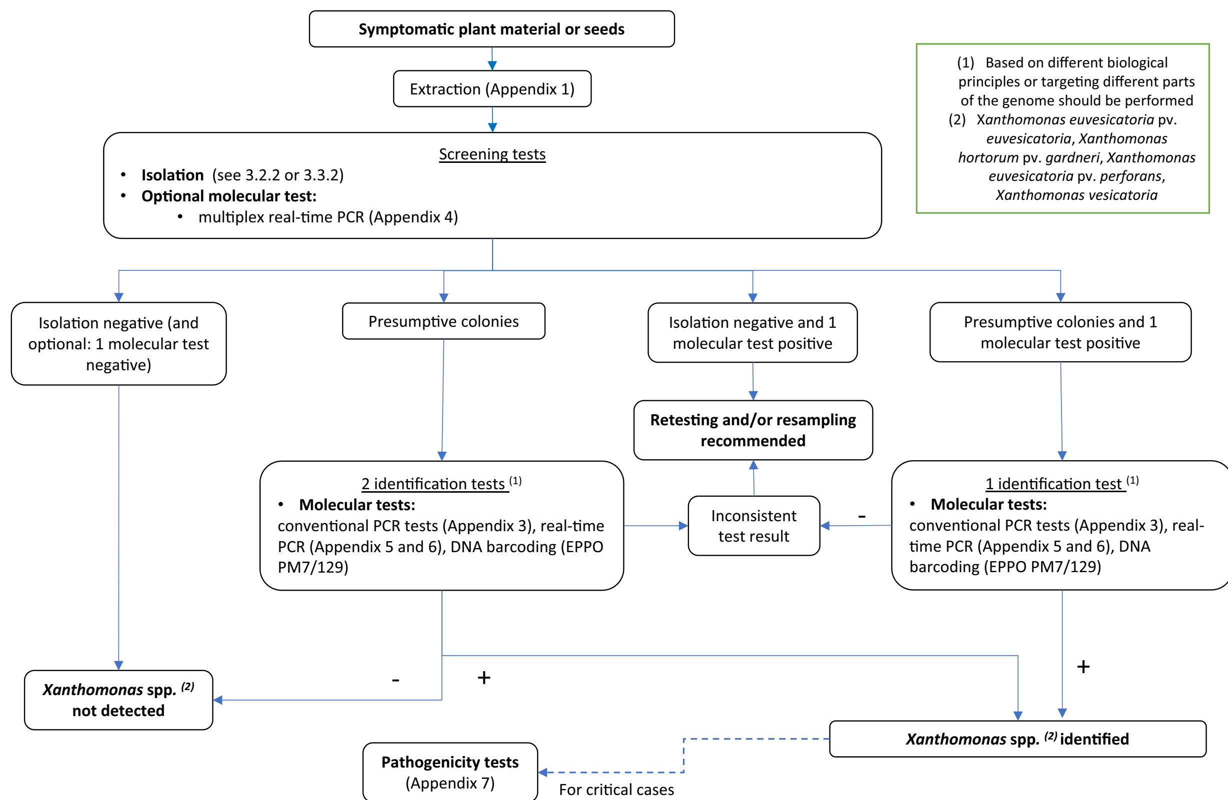

FIGURE 1 Flow diagram for testing tomato and pepper seed and plant samples to detect *Xanthomonas* spp. causing bacterial spot. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

2 | IDENTITY

Name: *Xanthomonas euvesicatoria* pv. *euvesicatoria* Constantin et al. (2016).
Synonyms: *Xanthomonas euvesicatoria* Jones et al. (2004), *Xanthomonas campestris* pv. *vesicatoria*, *X. axonopodis* pv. *vesicatoria*.
Taxonomic position: Domain/empire: Bacteria; Division/phylum: *Proteobacteria*; Class: *Gammaproteobacteria*; Order: *Lysobacterales* (previously known as *Xanthomonadales*); Family: *Lysobacteraceae* (previously known as *Xanthomonadaceae*).
EPPO Code: XANTEU.
Phytosanitary categorization: EPPO A2 no. 390, EU RNQP (Annex IV).

Name: *Xanthomonas euvesicatoria* pv. *perforans* Constantin et al. (2016).
Synonyms: *Xanthomonas perforans* Jones et al. (2004), *Xanthomonas campestris* pv. *perforans*, *X. axonopodis* pv. *vesicatoria*.
Taxonomic position: Domain/empire: Bacteria; Division/phylum: *Proteobacteria*; Class: *Gammaproteobacteria*; Order: *Lysobacterales* (previously known as *Xanthomonadales*); Family: *Lysobacteraceae* (previously known as *Xanthomonadaceae*).
EPPO Code: XANTPF.
Phytosanitary categorization: EPPO A2 no. 392, EU RNQP (Annex IV).

Name: *Xanthomonas hortorum* pv. *gardneri* Morinière et al. (2020).
Synonyms: *Xanthomonas gardneri* (*ex* Šutic, 1957) Jones et al. (2004), *Xanthomonas cynarae* pv. *gardneri* (Timilsina et al., 2019).
Taxonomic position: Domain/empire: Bacteria; Division/phylum: *Proteobacteria*; Class: *Gammaproteobacteria*; Order: *Lysobacterales* (previously known as *Xanthomonadales*); Family: *Lysobacteraceae* (previously known as *Xanthomonadaceae*).
EPPO Code: XANTGA.
Phytosanitary categorization: EPPO A2 no. 391, EU RNQP (Annex IV).

Name: *Xanthomonas vesicatoria* (*ex* Doidge 1920) Vauterin et al. (1995).
Synonyms: *Xanthomonas campestris* pv. *vesicatoria* (Doidge 1920) Dowson 1939.

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Taxonomic position: Domain/empire: Bacteria; Division/phylum: *Proteobacteria*; Class: *Gammaproteobacteria*; Order: *Lysobacterales* (previously known as *Xanthomonadales*); Family: *Lysobacteraceae* (previously known as *Xanthomonadaceae*).
EPPO Code: XANTPF.
Phytosanitary categorization: EPPO A2 no. 157, EU RNQP (Annex IV).

3 | DETECTION

3.1 | Symptoms

3.1.1 | Solanum lycopersicum

On tomato leaves, lesions appear as irregular, water-soaked areas that are green at first, becoming brown and necrotic later. Lesions are frequently surrounded by large chlorotic haloes (Figure 2). Foliar blight can occur when the lesions coalesce. Necrosis of the petioles and canker-like splits can be observed along the stem.

FIGURE 2 Bacterial spot lesions on tomato leaf caused by *X. euvesicatoria* pv. *euvesicatoria* (Courtesy: M. Rosello (Laboratorio de Diagnóstico Fitopatológico, Alicante, ES)).

FIGURE 3 Bacterial spot lesions on tomato leaf caused by *P. syringae* pv. *tomato* (Courtesy: M. Rosello (Laboratorio de Diagnóstico Fitopatológico, Alicante, ES)).

FIGURE 4 (a, b) Typical bacterial spot lesions on the surface of tomato fruits.

On tomato leaves, bacterial speck lesions (caused by *Pseudomonas syringae* pv. *tomato*) look similar initially but are surrounded by a distinct yellow halo (Figure 3).

Lesions on fruits begin as tiny, slightly raised blisters (Figure 4a,b). Subsequently, the spots increase in size and become brownish, scab-like, raised and surrounded by a water-soaked halo. Several lesions can coalesce. On tomato fruits, *P. syringae* pv. *tomato* causes smaller, blackish lesions without a scab-like appearance.

3.1.2 | Capsicum annuum

Lesions on *Capsicum annuum* leaves are of irregular shape and necrotic, in some cases surrounded by a chlorotic halo (Figures 5a,b and 6a–d). When the infection is severe, foliar blight can occur and leaves may fall. On fruits, scab-like, raised, whitish lesions appear (Figure 7a,b).

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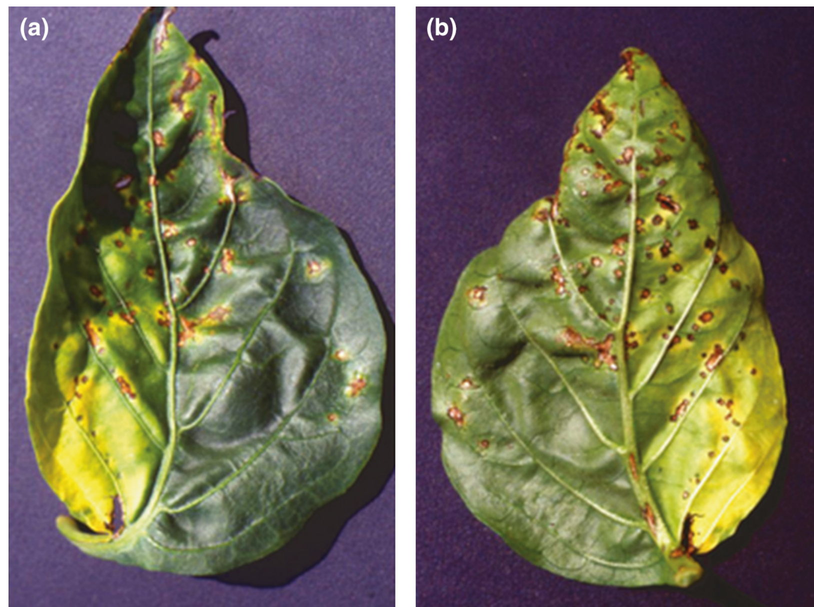

FIGURE 5 (a, b) Bacterial spot lesions on pepper leaf (upper and lower surface).

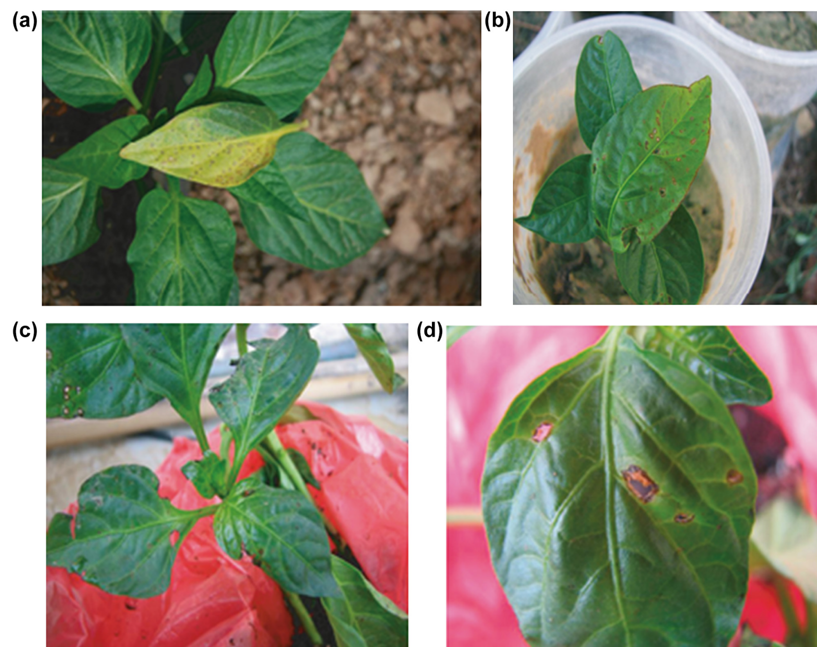

FIGURE 6 (a–d) *Xanthomonas euvesicatoria* pv. *perforans* leaf spot symptoms on pepper. (a) shows small necrotic leaf spots while (b–d) show large necrotic leaf spots with perforated centres.

3.2 | Detection in symptomatic plants

3.2.1 | Test sample requirements

Leaves, petioles or fruits with spots should be washed in tap water and then rinsed with distilled water and blotted dry or lightly surface disinfected (e.g. by wiping with 70% ethanol). Small pieces of tissue bordering necrotic lesions should be crushed in approximately 200–300 µL of sterile physiological saline (SPS = 0.85% NaCl in distilled water) or 1% peptone or 10 mM PBS. The use of sterile water is also suitable. The plant extract can be used for molecular tests or isolation.

3.2.2 | Isolation

The resulting suspension (approximately 10 µL) can be streaked on a suitable non-selective agar medium,

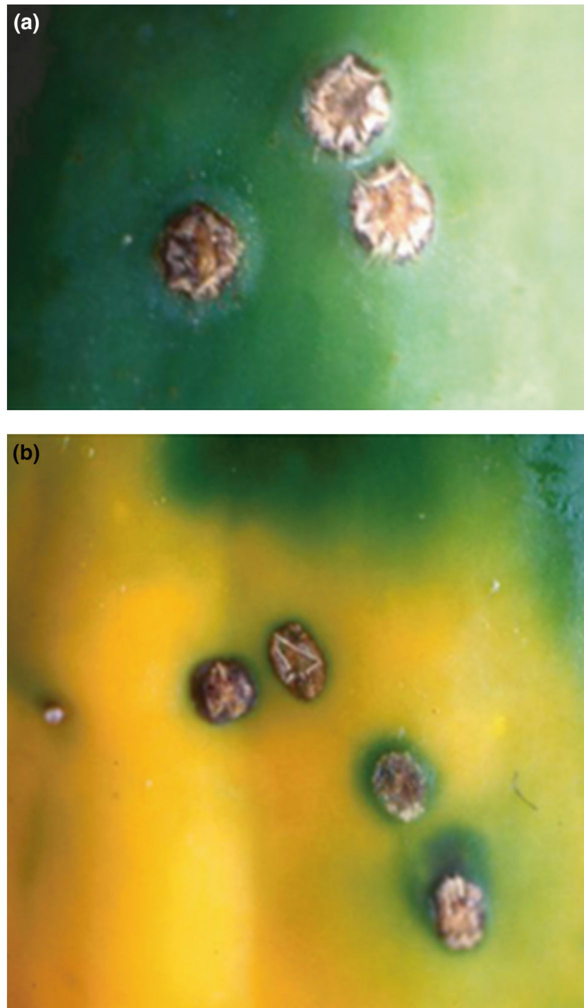

FIGURE 7 (a, b) Typical bacterial spot lesions on the surface of pepper fruit.

e.g. Wilbrink's medium (Koike, 1965), Yeast–glucose–calcium carbonate agar (YGCA) or yeast peptone glucose agar (YPGA) (see Appendix 2) and incubated at 25–28°C. Alternatively, approximately 30–100 µL aliquots of 10-fold dilutions in the same buffer can be plated on semi selective media such as CKTM agar (Sijam et al., 1992) mMXV (Sijam et al., 1991) or modified Tween Medium B (mTMB) agar (McGuire et al., 1986) (see Section 3.3.2 and Appendix 2). Typical yellow colonies appear in 2–5 days. Presumptive colonies may require further purification by re-streaking individual, yellow-pigmented colonies on a fresh non-selective agar medium before further identification is carried out (see Section 4).

3.2.2.1 | *Colony morphology*

On YGCA, colonies are bright yellow, circular, with entire margin, wet and shining, mucoid and raised. On other media, colonies appear pale or bright yellow, circular, mucoid and raised.

3.2.3 | Optional screening tests

3.2.3.1 | *Serological tests*

Few antibodies are commercially available for use in immunofluorescence and ELISA, and no validation data could be retrieved. Consequently, serological tests are not recommended in this protocol.

3.2.3.2 | *Molecular tests*

The multiplex real-time PCR described by Strayer et al. (2016) (see also Appendix 4) can be used for detection in symptomatic plant material.

The conventional duplex PCR tests described by Koenraadt et al. (2009) was used for detection in symptomatic plant material (Nechwatal & Theil, 2020), but due to the lack of validation data, it is not recommended in this protocol for this intended use.

3.3 | Detection in seeds

3.3.1 | Test sample requirements

A minimum sample size of 10 000 tomato or pepper seeds is recommended by the International Seed Federation (ISF) (ISF, 2017) with a maximum subsample size of 10 000 seeds. This sample size should allow detection of down to 0.03% contamination with 95% confidence.

Smaller subsamples (e.g. 5 × 2000 seeds) are recommended if high populations of saprophytic bacteria are likely to mask the potential presence of *Xanthomonas* spp.

In specific cases such as protected cultivations, breeding lines or small seed lots, smaller sample sizes can be tested with this protocol but with a lower confidence of detection, which should be defined for each sample size used. In such case the minimum number of seeds may be lowered to a single sample of 2000 seeds with 95% confidence of detecting down to 0.15% contamination. The tests described have not been validated on treated or pelleted seeds.

Two methods are recommended to extract bacteria from seeds (see Appendix 1). Tenfold dilutions of concentrated extracts are used for isolation.

If the same sample is to be tested for additional pathogens (e.g. *Clavibacter michiganensis* subsp. *michiganensis*), it may be practical to use one extraction method for all bacteria. If using extraction methods optimized for other pathogens, soaking periods exceeding 3 h at room temperature or 16 h at 4°C are likely to favour the growth of saprophytes that might outgrow the xanthomonads.

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3.3.2 | Isolation

Approximately 50–100 μL of the extract and its tenfold dilutions are plated on two semi-selective media, such as CKTM agar (Sijam et al., 1992) mMXV (Sijam et al., 1991) or mTMB agar (McGuire et al., 1986), and incubated at 25–28°C.

Presumptive colonies are then streaked on non-selective media such as YGCA. Other non-selective media can be used such as Wilbrink's medium, nutrient agar (NA), nutrient dextrose (ND) agar, yeast extract–dextrose–calcium carbonate (YDC) agar, nutrient broth–yeast extract (NBY) agar, or adenine-supplemented YPGA (Figure 8) (see Appendix 2).

Per subsample, at least 6 yellow-pigmented colonies similar to those of the positive controls are selected for the identification of putative xanthomonads. If fewer than 6 yellow-pigmented colonies are available all such colonies should be selected.

Colony morphology

On CKTM medium, colonies appear circular, raised, yellow and surrounded by a white crystalline halo (Figure 9). Isolates from tomato usually develop opaque white haloes around the colonies within 3–7 days. On mTMB, *Xanthomonas* colonies are yellow, slightly mucoid, raised and circular (Figure 10). Use of Tween 80 in the medium causes a clear halo to form around the yellow colony in 3–7 days. For further purification, individual yellow-pigmented colonies are recovered after re-streaking on a non-selective agar medium. Identification tests of putative *Xanthomonas* spp. should then be carried out.

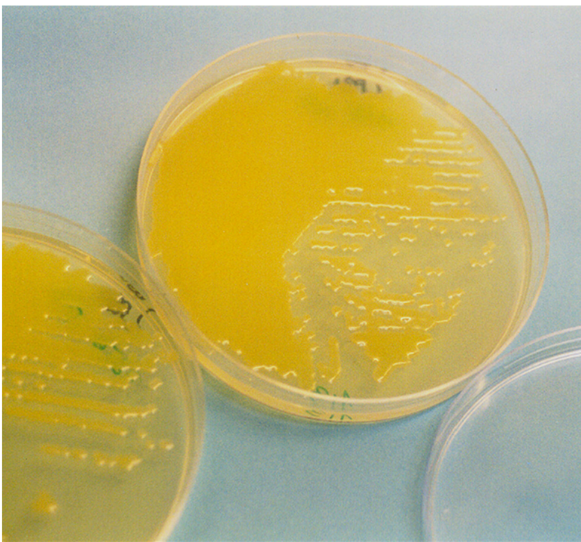

FIGURE 8 *Xanthomonas euvesicatoria* pv. *euvesicatoria* colonies on YPGA after 4 days at 28°C (Courtesy: Rosello M (Diagnostic Laboratory of Phytopathology of Alicante, ES)).

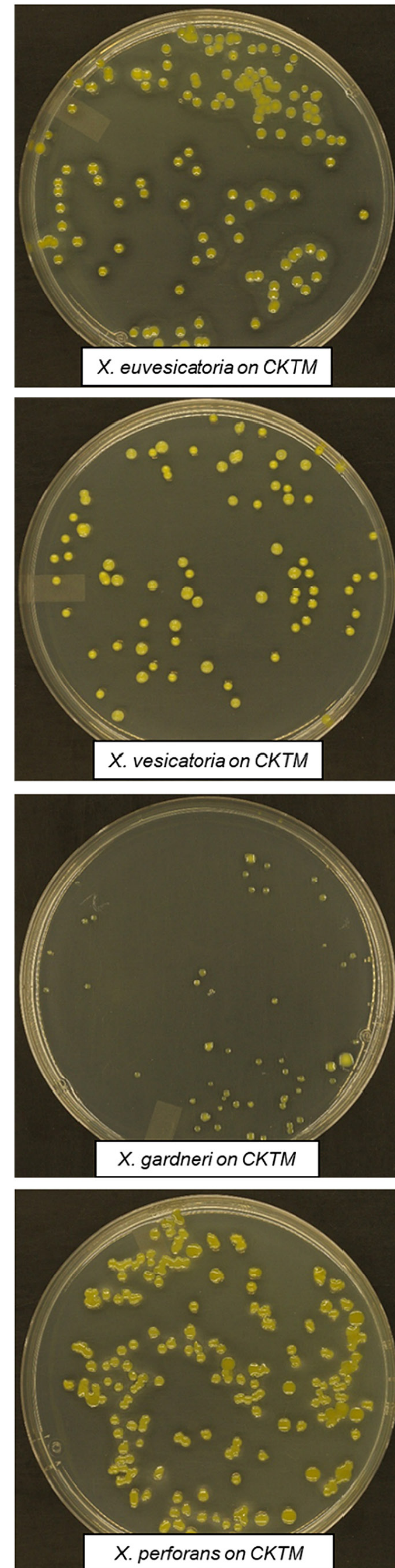

FIGURE 9 *Xanthomonas* spp. colonies on CKTM (Courtesy: ISF).

3.3.3 | Optional screening tests

The multiplex real-time PCR described by Strayer et al. (2016) (Appendix 4) can be used for detection in seed material.

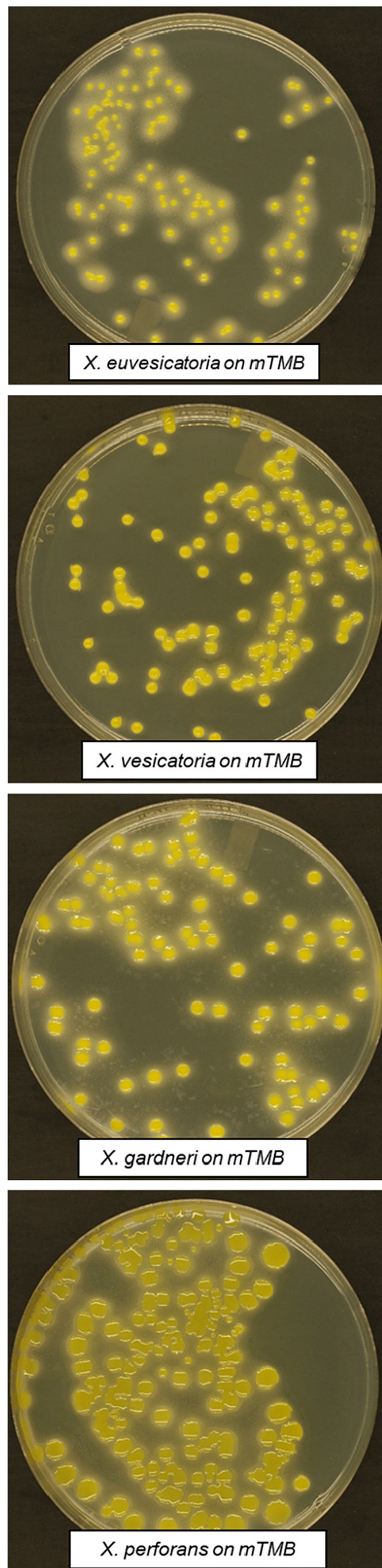

FIGURE 10 *Xanthomonas* spp. colonies on mTMB (Courtesy: ISF).

4 | IDENTIFICATION

Purified isolates of the bacterial spot-causing *Xanthomonas* spp. can be identified using at least two tests based on different biological principles or targeting different parts of the genome. A hypersensitive reaction test may be performed before identification to screen yellow colonies and to avoid performing identification tests on saprophytes (see Section 4.2.3).

4.1 | Molecular tests

4.1.1 | Polymerase chain reaction tests

The tests recommended for the identification of presumptive isolates are listed in the table below:

Test(s)		Target species	Distinction of the 4 species possible
Conventional duplex PCR tests described by Koenraadt et al. (2009) Appendix 3		One test targeting *Xe* and *Xv* and the other one *Xp* and *Xg*	Yes
ISF tests Appendix 5	AFLP derived Taqman real-time PCR	*Xe*, *Xv*, *Xp*, *Xg*	No, unless used as simplex
	XopD Taqman PCR	*Xe*, *Xv*, *Xp*, *Xg*	No
Real-time PCRs (Naktuinbouw, RijkZwaan and Syngenta) Appendix 6	'Xep' test	*Xe*, *Xp*	The 'Xep' real-time PCR test cannot distinguish Xe and Xp.
	'Xv' test	*Xv*	
	'Xg' test	*Xg*	

Abbreviations: Xe, *Xanthomonas euvesicatoria* pv. *euvesicatoria*; Xg, *X. hortorum* pv. *gardneri*; Xp, *X. euvesicatoria* pv. *perforans*; Xv, *X. vesicatoria*.

4.1.2 | DNA barcoding tests

DNA barcoding can be used for the identification of the three species causing bacterial spot of tomato and sweet pepper and *X. euvesicatoria* pathovars. A major advantage of DNA barcoding is that the obtained nucleotide sequences can be easily compared to reliable reference sequences. Protocols for routine barcoding using 16S rDNA, *gyrB* and *avrBs2* sequences are described in Appendix 2 of the EPPO Standard PM 7/129 (2) *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021). Reference sequences for those loci from strains are available at https://qbank.eppo.int/bacteria/. General procedures for sequencing are described in Appendices 7 and 8 of the EPPO Standard PM 7/129 (EPPO, 2021).

4.2 | Other tests

4.2.1 | Biochemical characteristics

Key phenotypic traits differentiating the four bacterial spot pathogens are described by Jones et al. (2004).

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Since then, however, taxonomy of these pathogens has changed (Constantin et al., 2016).

4.2.2 | Automated Biolog identification system

Differentiation of strains based on carbon utilization patterns using the Biolog GN Microplate system is possible (Jones et al., 2000).

4.2.3 | Hypersensitive reaction

In order to avoid performing identification tests on saprophytes, a hypersensitive reaction (HR) test may be performed on bean pods (Klement & Lovrekovich, 1961) by infiltrating aqueous suspensions containing approximately 10^8 cfu mL^{-1} of the putative xanthomonads. A positive HR strongly indicates the presence of a phytopathogenic *Xanthomonas* sp. Such a reaction will appear after 24 to 48 h.

4.2.4 | Pathogenicity test

For critical cases (see EPPO Standard PM 7/77(2)), a pathogenicity test may be performed if needed. The procedure for the pathogenicity test is described in Appendix 7.

5 | REFERENCE MATERIAL

Xanthomonas euvesicatoria* pv. *euvesicatoria Constantin et al. (2016).
Type strain: ATCC 11633; ICMP 109; ICMP 98; NCPPB 2968; LMG 27970.
Xanthomonas euvesicatoria* pv. *perforans Constantin et al. (2016).
Type strain: ATCC BAA-983; NCPPB 4321; ICMP 16690; LMG 28258.
Xanthomonas hortorum* pv. *gardneri Morinière et al. (2020).
Type strain: ATCC 19865; ICMP16689; NCPPB 881; LMG962.
Xanthomonas vesicatoria (*ex* Doidge 1920) Vauterin et al. (1995).
Type strain: ATCC 35937; ICMP 63; LMG 911; NCPPB 422; CFBP 2537.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CHARACTERISTICS

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

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Stefani E, Dept. Agricultural and Food Sciences, via Amendola 2, Pad. Besta, 42100 Reggio Emilia (IT). E-mail: emilio.stefani@unimore.it.

Aspin A, Fera Science Ltd, Sand Hutton, YO41 1LZ (United Kingdom). E-mail: andrew.aspin@fera.co.uk.

Koenraadt H, Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen, P.O. Box 40, 2370 AA Roelofarendsveen (NL) E-mail: h.koenraadt@naktuinbouw.nl.

Le Van A, Groupe d'Étude et de Contrôle des Variétés et des Semences (GEVES), 25 rue Georges Morel - CS 90024, 49071 Beaucouzé (FR) E-mail: amandine.levan@geves.fr.

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 diagnostic@eppo.int.

10 | PROTOCOL REVISION

A regular 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
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GB), M Rosello (Laboratorio de Diagnóstico Fitopatológico, Alicante, ES), E Stephani (Unimore, IT), S Loreti (CREA, IT), TK Baldwin (Geves, FR), H Koenraadt (Naktuinbouw, NL).

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APPENDIX 1 - EXTRACTION FROM SEEDS

Two methods are recommended to extract bacteria from a seed sample. The first involves soaking seeds; the second uses a Stomacher Laboratory Blender.

1. Soaking

Soak each subsample of seeds overnight (minimum 14 h) at 4–10°C in 3 mL g^{-1} seed of sterile 10 mM phosphate buffered saline (PBS, Appendix 2) or shake for 2 h at room temperature (18–24°C) and 115 rpm. Filter (e.g. through sterile gauze) and centrifuge at 10 000–12 000 *g* for 20 min at 10°C. Discard the supernatant and resuspend the pellet in 1–1.5 mL sterile distilled water or 10 mM PBS to obtain the final concentrate. Prepare 10-fold dilutions and perform direct isolation.

2. Stomacher procedure

Each subsample should be transferred into a strong sterile polythene bag, for example Stomacher type or Bioreba with an internal filter. Then 4 mL of sterile 50 mM phosphate buffer (PB-T, Appendix 2) is added per gram of seed and the subsamples are allowed to soak at 2–6°C overnight (minimum 14 h) before extraction, for example using a Bagmixer (Interscience) or Stomacher (Seward). The time and intensity of maceration is critical because internally located bacteria need to be released from the seeds: 4 min of maceration at maximum speed are recommended for the Interscience Bagmixer and 8 min for the Seward Stomacher. The seed extraction buffer becomes milky after maceration. The subsamples are allowed to settle for 5 min at ambient temperature and 20–50 mL of the seed extract is then concentrated by centrifugation at low speed (180 g for 5 min to 1000 g for 1 min) to clear the subsample from most of the debris. The supernatant is decanted in a sterile tube and centrifuged at 9000 g for 20 min at approximately 4°C[2]. The supernatant is discarded and the pellet resuspended by vortexing with 2–5 mL of 10 mM PBS to obtain the 10× concentrated seed extract. The resulting suspension is divided into 500-µL aliquots. Of these 1 × 500 µL is used for analysis and the second 1 × 500 µL for the spiked extract control in the isolation test. The remaining part is deep frozen for reference purposes.

[2]It should be noted that this additional centrifugation may not be possible for lots smaller than 2000 seeds.

APPENDIX 2 - BUFFERS AND MEDIA

1. Buffers

1.1 *Phosphate buffered saline (PBS) for extraction from symptomatic plants and seeds (soaking method)*

10 mM PBS, pH 7.2	g L^{-1}
$Na_2HPO_4{\cdot}12H_2O$	2.7 g
$NaH_2PO_4{\cdot}2H_2O$	0.4 g
NaCl	8.0 g
Distilled water	1 L
Autoclave 15 min at 121°C and cool to room temperature	

1.2 *Phosphate buffered Tween (PB-T) (pH 7.4) for the extraction of bacteria from seeds (Stomacher method)*

Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
$Na_2S_2O_3$ [a]	0.5 g
Distilled water	1 L
Autoclave 15 min at 121°C and cool to room temperature	
Add sterile Tween 20 (10% solution)	0.2 mL

[a]Recommended when seed have been treated with hypochlorite.

2. Media

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

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2.1 *CKTM agar medium* (Sijam et al., 1991, 1992)

Distilled water	900 mL
Soy peptone	2.0 g
Tryptone	2.0 g
Dextrose	1.0 g
L-glutamine	6.0 g
L-histidine	1.0 g
$(NH_4)_2HPO_4$	0.8 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.4 g
$CaCl_2$	0.25 g
Microbiological grade agar	12.0–15.0 g
Tween 80[a]	10 mL

[a]Autoclave separately.

Tween 80 and the following antibiotics are added after autoclaving the basal medium and cooling to approximately 45°C.

Bacitracin 2 mL (1.0 g in 20 mL distilled water);

Neomycin sulphate 0.5 mL (0.4 g in 20 mL distilled water);

100 mL distilled water containing: Cephalexin 65 mg, 5-fluorouracil 12 mg and Tobramycin 0.4 mg.

If needed, 35 mg L^{-1} nystatin or 100 mg L^{-1} cycloheximide can be added after autoclaving to inhibit fungal growth.

2.2 *Modified Tween Medium B agar (mTMB) (adapted* McGuire et al., 1986*)*

Distilled water	1 L
Bacto peptone	10.0 g
H_3BO_3	0.1 g
KBr	10.0 g
$CaCl_2$ (anhydrous)	0.25 g
Bacto agar	15.0 g
Tween 80[a]	10 mL

[a]Autoclave separately.

Tween 80 and the following antibiotics are added after autoclaving the basal medium and cooling to 45°C.

Cephalexin 65 mg;

5-fluorouracil 12 mg;

Tobramycin sulphate 0.2 mg.

If needed, 35 mg L^{-1} nystatin or 100 mg L^{-1} cycloheximide can be added after autoclaving to inhibit fungal growth.

2.3 *Yeast glucose calcium carbonate agar (YGCA)*

Distilled water	1 L
Yeast extract	5.0 g
Glucose	10.0 g
Calcium carbonate (light powder)	30.0 g
Microbiological grade agar	20.0 g

2.4 *Nutrient agar (NA)*

Use commercial preparations (such as Oxoid CM3 or Difco).

2.5 *Nutrient dextrose (ND) agar*

Add 1% (w/v) D-glucose to nutrient agar (such as Oxoid CM3 or Difco).

2.6 *Yeast extract–dextrose–calcium carbonate (YDC) agar*

Distilled water	1 L
Yeast extract	10.0 g
Dextrose (glucose)	20.0 g
Calcium carbonate ($CaCO_3$) (fine powder)[a]	20.0 g
Microbiological grade agar	15.0 g

[a]The autoclaved medium should be cooled to 50°C in a water bath and $CaCO_3$ suspended by swirling before pouring the plates.

2.7 *Nutrient broth yeast extract (NBY) agar*

Distilled water	1 L
Nutrient broth	8.0 g
Yeast extract	2.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
Glucose	2.5 g
Microbiological grade agar	15.0 g

2.8 *Yeast peptone glucose agar (YPGA)*

Distilled water	500 mL
Yeast extract	2.5 g
Bacto peptone	2.5 g
D(+) glucose	5.0 g
Difco bacto agar	7.5 g

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Prepare 500 mL medium in 1 L Erlenmeyer flasks. (If necessary, supplement with 50 mg L^{-1} adenine hemisulphate for faster growth)

If needed, 50 mg L^{-1} cycloheximide can be added after autoclaving to inhibit fungal growth.

2.9 *Wilbrink's medium (Koike, 1965)*

Distilled water	1 L
Peptone special	5.0 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Sucrose	10.0 g
Agar technical no. 3	18.0 g

Adjust pH to 7.0 before adding agar.

2.10 *Modified MXV medium (mMXV)* (*McGuire et al., 1986*; *Sijam et al., 1991*)

Distilled water	1 L
Yeast extract	0.5 g
KH_2PO_4	0.8 g
K_2HPO_4	0.8 g
Ammonium chloride	1.0 g
Lactose	10.0 g
Threhalose	4.0 g
Thiobarbituric acid	0.1 g
Microbiological grade agar	15.0 g
Tween 80[a]	10 mL

[a] Autoclave separately.

The pH is adjusted to 7.4.

Tween 80 and the following antibiotics are added after autoclaving and cooling the basal medium to approximately 45°C.

Cephalexine	32.5 mg
Bacitracine	100 mg
5-fluorouracil	6 mg
Neomycine sulphate	6750 µg
Tobramycine sulfat	0.2 mg
Cyclohemixide	100 mg

2.11 *Performance criteria available for the media*

Performance criteria for the isolation from symptomatic plants on Wilbrink's (NIVIP (NL))

Detection limit plating 20 µL on Wilbrink's medium was 3.8×10^3 cfu mL^{-1}

Performance criteria for isolation from seed extracts on the semi-selective media modified MXV and modified TMB (Naktuinbow (NL), 2012; the full validation report can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php))

Detection limits with simultaneous use of the semi-selective media mMXV and mTMB were found to be 53 cfu mL^{-1} (*X. euvesicatoria* pv. *euvesicatoria*), 24 cfu mL^{-1} (*X. vesicatoria*), 94 cfu mL^{-1} (*X. hortorum* pv. *gardneri*) and 334 cfu mL^{-1} (*X. euvesicatoria* pv. *perforans*). Lowest detectable concentration varied considerably between sample and semi-selective media. Particularly on the semi-selective medium mTMB, the lowest detectable concentration varied from 2 to 16 900 cfu mL^{-1} (*X. euvesicatoria* pv. *perforans*) between samples. Variation for the semi-selective medium mMXV was much lower, with the lowest detectable concentration ranging from 17 to 169 cfu mL^{-1} (*X. euvesicatoria* pv. *perforans*) between samples.

APPENDIX 3 - CONVENTIONAL DUPLEX PCR TESTS (KOENRAADT ET AL., 2009)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 The two conventional duplex-PCR tests are suitable for the identification of *X. euvesicatoria* pv. *euvesicatoria*, *X. vesicatoria*, *X. hortorum* pv. *gardneri* and *X. euvesicatoria* pv. *perforans*.

1.2 Using amplification fragment length polymorphism (AFLP) analysis, a specific marker has been identified previously for each of the four described species. Based on the sequence data of these markers, 4 primer combinations, Bs-XeF/Bs-XeR, Bs-XvF/Bs-XvR, Bs-XgF/Bs-XgR and Bs-XpF/Bs-XpR, have been developed, amplifying a specific fragment of each of the four species (Koenraadt et al., 2009). The amplification occurs in two separate reactions each including two primer combinations (duplex-PCR tests). Additionally, the primer combination BAC16-F/BAC16-R is also included in each amplification as an internal control, by which a fragment of 466 bp of the *16S rRNA* gene is amplified.

1.3 The test can be applied to bacterial colonies.

1.4 Oligonucleotides:

	Primer	Sequence	Amplicon size (including primer sequences)
Xanthomonas euvesicatoria pv. *euvesicatoria*			
Forward primer	Bs-XeF	5′-CAT GAA GAA CTC GGC GTA TCG-3′	173 bp
Reverse primer	Bs-XeR	5′-GTC GGA CAT AGT GGA CAC ATA C-3′	

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	Primer	Sequence	Amplicon size (including primer sequences)
Xanthomonas vesicatoria			
Forward primer	Bs-XvF	5′-GCA TGT GCC GTT GAA ATA CTT G-3′	138 bp
Reverse primer	Bs-XvR	5′-ACA AGA GAT GTT GCT ATG ATT TGC-3′	
Xanthomonas hortorum pv. *gardneri*			
Forward primer	Bs-XgF	5′-TCA GTG CTT AGT TCC TCA TTG TC-3′	154 bp
Reverse primer	Bs-XgR	5′-TGA CCG ATA AAG ACT GCG AAA G-3′	
Xanthomonas euvesicatoria pv. *perforans*			
Forward primer	Bs-XpF	5′-GTC GTG TTG ATG GAG CGT TC-3′	197 bp
Reverse primer	Bs-XpR	5′-GTG CGA GTC AAT TAT CAG AAT GTG G-3′	
16s rRNA internal control			
Forward primer	BAC16S-F	5′-TCC TAC GGG AGG CAG CAG T-3′	466 bp
Reverse primer	BAC16S-R	5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′	

1.5 PCR system: Cepheid SmartCycler (Cepheid, Sunnyvale, CA)

2. Methods

2.1 Nucleic acid extraction and purification:

Colony material from pure cultures is suspended in 100 μL molecular-grade water. DNA has been isolated from bacterial suspensions of approximately 1×10^8 cfu mL^{-1} according to the protocol of the High Pure PCR Template Preparation Kit (Roche, Switzerland).

2.2 Polymerase chain reaction (PCR).

Reagents (working concentration in brackets)	*X. euvesicatoria* pv. *euvesicatoria* *X. vesicatoria* Per reaction (μL)	Final concentration	*X. hortorum* pv. *gardneri* *X. euvesicatoria* pv. *perforans* Per reaction (μL)	Final concentration
Molecular-grade water	15.75		15.0	
PCR Rxn reaction buffer (10× Invitrogen)	2.5	1×	2.5	1×
$MgCl_2$ (50 mM, Invitrogen)	0.75	1.5 mM	1.5	3.0 mM
dNTPs (10 mM each)	0.5	0.2 mM	0.5	0.2 mM
BS-XeF (10 μM)	0.5	0.2 μM	–	
BS-XeR (10 μM)	0.5	0.2 μM	–	
BS-XvF (10 μM)	1.0	0.4 μM	–	
BS-XvR (10 μM)	1.0	0.4 μM	–	
BS-XgF (10 μM)	–		0.75	0.3 μM
BS-XgR (10 μM)	–		0.75	0.3 μM
Bs-XpF (10 μM)	–		0.75	0.3 μM
Bs-XpR (10 μM)	–		0.75	0.3 μM
BAC16S-F (10 μM)	0.15	0.06 μM	0.15	0.06 μM
BAC16S-R (10 μM)	0.15	0.06 μM	0.15	0.06 μM
Platinum Taq (5 U μL^{-1} Invitrogen)	0.2	1 U	0.2	1 U
Total	23.0		23.0	
Nucleic acid	2.0		2.0	
Total	25.0		25.0	

2.3 PCR cycling conditions: 2 min at 94°C, 40 cycles of 30 s at 95°C, 30 s at 64°C, 30 s at 72°C, 10 min at 72°C, and cooling at 20°C.

3. Essential procedural information

3.1 Controls:

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

- Negative isolation control to monitor contamination during nucleic acid extraction: for the NIC, 100 μL molecular-grade water is used.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated. For the PIC, a suspension of 10^9 cfu mL^{-1} *X. vesicatoria* (in the test performance, study isolate PD6003 was used).
- Negative amplification control (NAC) to rule out false positives due to contamination during preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification controls (PACs) to monitor the efficiency of the amplification of nucleic acid of each of the target organisms:
  PAC Xe: DNA of *X. euvesicatoria* pv. *euvesicatoria* 10^9 cfu mL^{-1} (e.g. PD3562)
  PAC Xv: DNA of *X. vesicatoria* 10^9 cfu mL^{-1} (e.g. PD5212)
  PAC Xp: DNA of *X. euvesicatoria* pv. *perforans* 10^9 cfu mL^{-1} (e.g. PD5515)

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PAC Xg: DNA of *X. hortorum* pv. *gardneri* 10^9 cfu mL^{-1} (e.g. PD5842)

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. For this reason, the primer combination BAC16-F/BAC16-R is also included in each amplification as an internal control (co-amplification of endogenous bacterial nucleic acid, *16s rRNA* internal control), by which a fragment of 466 bp of the *16S rRNA* gene is amplified.

3.2 Interpretation of results

Verification of controls

- NIC and NAC no band is visualized
- PIC and PAC
  X. euvesicatoria pv. *euvesicatoria* a band of 173 bp is visualized.
  X. vesicatoria a band of 138 bp is visualized.
  X. euvesicatoria pv. *perforans* a band of 197 bp is visualized.
  X. hortorum pv. *gardneri* a band of 154 bp is visualized.
- The IPC *16S rRNA* gene a band of 466 bp is visualized

When these conditions are met:

- A test will be considered positive if a band of the expected size is visualized (as for PIC and PAC).
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

The validation data reported below is from NVWA (NL). Further validation data was obtained by CREA with small adaptation of the test and is available in the EPPO database on diagnostic expertise.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1 Analytical sensitivity data

For *X. hortorum* pv. *gardneri* analytical sensitivity was 2.5×10^6 cfu mL^{-1}, for *X. euvesicatoria* pv. *perforans* 1.9×10^7 cfu mL^{-1}, for *X. euvesicatoria* pv. *euvesicatoria* 5.5×10^5 cfu mL^{-1} and for *X. vesicatoria* 1.6×10^6 cfu mL^{-1}.

4.2 Analytical specificity data

Fifty-three isolates of the former *X. campestris* pv. *vesicatoria* were analysed using sequence analysis of the *AvrBs2* gene (used on reference material on basis of the Quarantine Barcoding of Life (QBOL) protocol) and attributed the isolates to the following species: 27 *X. euvesicatoria* pv. *euvesicatoria*, three *X. hortorum* pv. *gardneri*, seven *X. euvesicatoria* pv. *perforans*, 12 *X. vesicatoria*, and four that could not be classified from their sequence analysis of the *AvrBs2* gene. PCR analysis attributed these 53 isolates to the following species: 27 *X. euvesicatoria* pv. *euvesicatoria*, five *X. hortorum* pv. *gardneri*, five *X. euvesicatoria* pv. *perforans*, 12 *X. vesicatoria*, and four that could not be classified. Two isolates identified using sequencing as *X. euvesicatoria* pv. *perforans* and one as *X. hortorum* pv. *gardneri* were not identified using this PCR test. Two isolates identified using PCR as *X. hortorum* pv. *gardneri* were not identified using sequencing. All other isolates were identified as belonging to the same species using both PCR and sequencing. Additionally, the analytical specificity was tested with 21 related bacterial isolates that can be present on tomato or pepper: *Clavibacter michiganensis* subsp. *michiganensis*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *Agrobacterium tumefaciens*, *P. corrugata*. Five (2 *P. syringae* pv. *tomato* isolates and 3 *P. corrugata* isolates) of the 21 related isolates gave weak (non-specific) amplicon(s) in the duplex PCR for *X. hortorum* pv. *gardneri/X. euvesicatoria* pv. *perforans* (very close to 154 and 197 bp) but not in the duplex PCR for *X. euvesicatoria* pv. *euvesicatoria /X. vesicatoria*. This underlines the risk of incorrect identification for *X. hortorum* pv. *gardneri* or *X. euvesicatoria* pv. *perforans*.

See also validation data from Appendix 6.

4.3 Data on repeatability: 93%.
4.4 Data on reproducibility: 100%.

APPENDIX 4 - MULTIPLEX REAL-TIME PCR TEST (STRAYER ET AL., 2016)

The test below differs from the one described in the original publication (see 1.2).

The test below is described as it was carried out to obtain the validation data generated by CREA provided in Section 4. Other equipment, kits or reagents may be used provided that a verification is carried out (see PM 7/98).

1. General information

1.1 This test is suitable for the detection of *Xanthomonas euvesicatoria* pv. *euvesicatoria*, *X. vesicatoria*, *X. euvesicatoria* pv. *perforans and X. hortorum* pv. *gardneri* in seeds. The test described in the original publication (Strayer et al., 2016), which uses slightly different master mixes and conditions, was also validated in plant material. The test can be run as a quadruplex real-time PCR or as two duplex real-time PCRs.

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Tests			Primer	Sequence	Amplicon size[a]
Duplex real-time PCR A	Quadruplex real-time PCR	Forward primer	FP1	5′-CGT CGA CGG CCT GGG CGA-3′	80 bp
		Reverse primer	RP1	5′-CCG GTG CCT GCG CCT GGA-3′	
		Probe	*X. euvesicatoria* pv. *perforans* probe	5′-FAM-CGG GCA AGG AGC CAT CGC CTG T-IABkFQ-3′	
		Probe	*X. euvesicatoria* pv. *euvesicatoria* probe	5′-TET-CGG GCA AGG CGC AAT CGC CTG T-BHQ_2-3′	
Duplex real-time PCR B		Forward primer	FP2	5′-AGG TCA GCC TGG GCG AGG T-3′	77 bp
		Reverse primer	RP2	5′-TGA AGC CCA CCA CCT CGG C-3′	
		Probe	*X. hortorum* pv. *gardneri* probe	5′-TexRd-XN-TGC GCC AGC GTG ACG GCA CGC-IAbRQSp-3′	
		Probe	*X. vesicatoria* probe	5′-Cy5-TGC GCC AGC GCG ATG GCA CGC-IAbRQSp-3′	

[a](including primer sequences)

1.2 This test was originally developed by Strayer et al. (2016) and further adapted by CREA (2017).
1.3 The real-time PCR test is targeting the *hrpB7* gene which belong to the hypersensitive response and pathogenicity gene cluster and is essential for type III protein secretion and pathogenicity.
1.4 Oligonucleotides:

1.5 Real-time PCR system: BIORAD CFX 96

2. Methods

2.1 Nucleic acid extraction and purification:

After centrifugation of 1.5 mL of seed extract (see Appendix 1 point 2), DNA from the pellet was extracted using DNeasy® Plant Mini Kit (Qiagen) following manufacturer's instructions. Note that a slightly different seed extraction protocol was used to produce the validation data described in Section 4.

2.2 Quadruplex real-time polymerase chain reaction

2.2.1 Master Mix

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		0.72[a]	
SsoAdvanced Universal Probes Supermix (Biorad)	2×	5.5	1×
Forward primers (×2)	10 µM	0.78 each[a]	0.71 µM each
Reverse primers (×2)	10 µM	0.78 each[a]	0.71 µM each
Probes (×4)	10 µM	0.165 each[a]	0.15 µM each
Subtotal		10.00	
DNA		1.00	
Total		11.00	

[a]If the test is run as two duplex PCR tests the volume of water and primers should be adjusted.

2.2.2 PCR cycling conditions: 30 s at 95°C, 40 cycles of 3 s at 95°C, 30 s at 69°C and 30 s at 72°C.

3. Essential procedural information

3.1 Controls

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

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is

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also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
– Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid. IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2 Interpretation of results

Verification of controls

- The PIC and PAC (as well as IPC if relevant) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

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

4. Performance characteristics available

Performance characteristics described below were obtained by CREA in an intralaboratory study (IS) and in a test performance study (TPS) organized in the framework of an Italian Project (ASPROPI) and involving 7 laboratories. Additional validation data are available in the original publication (Strayer et al., 2016) which uses a slightly different master mix and conditions. The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1 Analytical sensitivity data (IS)

The analytical sensitivity of the test for all four *Xanthomonas* spp. causing bacterial spot was 10^4 cfu$\,$mL^{-1} with DNA extracted from tomato seed extracts spiked with a mixture of bacterial suspensions of the four *Xanthomonas* species for the duplex real-time PCRs and the quadruplex real-time PCR and 100$\,$fg$\,\mu$L^{-1} with DNA extracted from pure culture (only evaluated for the quadruplex real-time PCR).

4.2 Analytical specificity data

Inclusivity was evaluated against 20 strains of *X. euvesicatoria* pv. *perforans*, 12 strains of *X. euvesicatoria* pv. *euvesicatoria*, 19 strains of *X. vesicatoria* and 15 strains of *X. hortorum* pv. *gardneri*. All 66 strains gave the expected results with the real-time TaqMan PCR test (Strayer et al., 2016).

Exclusivity evaluated against 30 non-target strains (CREA, DC), i.e. *Xanthomonas fragariae* (1), *Xanthomonas arboricola* pv. *juglandis* (1); *Xanthomonas arboricola* pv. *corylina* (1); *Xanthomonas arboricola* pv. *populi* (1); *Xanthomonas campestris* pv. *campestris* (2); *Xanthomonas hortorum* pv. *pelargonii* (1); *Xanthomonas axonopodis* pv. *poinsettiicola* (1); *Xanthomonas malvacearum* (1); *Xanthomonas campestris* pv. *begoniae* (1); *Pseudomonas viridiflava* (1); *Xanthomonas campestris* pv *raphani* (1); *Pseudomonas putida* (1); *Pseudomonas fluorescens* (1); *Xanthomonas arboricola* pv. *pruni* (1); *Xanthomonas citri* pv. *citri* (1); *Xanthomonas arboricola* pv. *celebensis* (1); *Ralstonia solanacearum* (1); *Clavibacter michiganensis* subsp. *michiganensis* (1); *Pseudomonas syringae* pv. *tomato* (1); saprophyte from tomato (6 isolates); *Pseudomonas corrugata* (1); *Pseudomonas mediterranea* (1); Cross-reactions were observed with:

Duplex real-time PCR	Quadruplex real-time PCR
Xanthomonas citri pv. *citri* (CREA-DC 1264), a saprophyte strain from tomato (CREA-DC 1495), *Xanthomonas axonopodis pv. poinsettiicola* (LMG 849)	*Xanthomonas campestris* pv. *campestris* (CREA-DC 1032), *Xanthomonas hortorum* pv. *pelargonii* (CREA-DC 1033), *Xanthomonas citri* pv. *citri* (CREA-DC 1264), *Xanthomonas arboricola* pv. *populi* (NCPPB 1832), *Xanthomonas campestris* pv. *raphani* (NCPPB 1946), a saprophyte strain from tomato (CREA-DC 1495), *Xanthomonas axonopodis pv. poinsettiicola* (LMG849).

4.3 Diagnostic sensitivity (DSE) and diagnostic specificity (DSP) (TPS)

	DSE	DSP
Duplex real-time PCR A	90%	82%
Duplex real-time PCR B	85%	85%
Quadruplex real-time PCR	92%	73%

4.4 Repeatability

IS: 100% on seed extract spiked with a 10^4 cfu$\,$mL^{-1} suspension of bacteria (3 biological replicates and 3 technical replicates)

Accordance based on TPS results:

	Accordance
Duplex real-time PCR A	80%
Duplex real-time PCR B	77%
Quadruplex real-time PCR	75%

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4.5 Reproducibility

Concordance based on TPS results:

	Concordance
Duplex real-time PCR A	76%
Duplex real-time PCR B	58%
Quadruplex real-time PCR	72%

APPENDIX 5 - TAQMAN REAL-TIME PCRS (ISF, 2017)

The tests below are described as they were carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 The tests described below are suitable for identification of isolates belonging to one of the following species/pathovars: *Xanthomonas euvesicatoria* pv. *euvesicatoria*, *X. vesicatoria*, *X. euvesicatoria* pv. *perforans and X. hortorum* pv. *gardneri*. However, when used as described below, the tests cannot be used to distinguish the isolates at the species/pathovar level. To identify each of the species/pathovars, the AFLP primers and probe sets can be used in simplex reactions.

1.2 The multiplex real-time PCR test described in 2.2 was developed in 2013 as described in Baldwin et al. (2023). This test was further adapted by ISF (2017). To include an internal positive control, the multiplex test was split in two separate tests (AFLP derived Taqman PCR and XopD Taqman PCR) as described in 2.3.

1.3 The AFLP derived Taqman PCR primers and probes are targeting the same loci as described in Appendix 3. The XopD Taqman PCR primers and probes target an effector gene common to the four *Xanthomonas* pathogens reported by Potnis et al. (2011).

1.4 Oligonucleotides:

1.4.1 AFLP derived Taqman PCR

	Primers and probes	Sequence	Amplicon size
Forward primer	XEF	5′-CTC GCT CAT CAA AGT GAT AAC GCC-3′	111 bp
Reverse primer	XER	5′-GGG CTT GGC AGG AAC GGC-3′	
Probe	XEFAM	5′-FAM-TCC GGC GAG GCA ATG CGC TAT AGC T-BHQ1-3′	
Forward primer	XVF	5′-GTG CCG TTG AAA TAC TTG CTA GCA G-3′	74 bp
Reverse primer	XVR	5′-CAC GCT ACG GGC CGC AA-3′	
Probe	XVFAM	5′-FAM-TTC GCA CCG CGG GCC CTG TTC T-BHQ1-3′	
Forward primer	XPF	5′-GTC GTG TTG ATG GAG CGT TCC C-3′	103 bp
Reverse primer	XPR	5′-CCG TCT GCT ACA CGA CTT CCG A-3′	
Probe	XPFAM	5′-FAM-TCT CCC ACA CCG CGA TAG GAT TGA CAG TAG A-BHQ1-3′	
Forward primer	XGF	5′-ACCTGCTCCACAAC GCGCTC-3′	97 bp
Reverse primer	XGR	5′-GCTTGAATCTG TTTTTTCAT TGGGATG-3′	
Probe	XGFAM	5′-FAM-TCC CAT CAA TAG TTG CTG CGC TAT AGC TTT TCT-BHQ1-3′	

1.4.2 XopD Taqman PCR

	Primers and probes	Sequence	Amplicon size
Forward primer	XDF	5′-TCG ACG GCA CCT TCG ACT ACG-3′	102 bp
Reverse primer	XDR	5′-CTG GAG CTT GCT CCG CTT GG-3′	
Probe	XDYY/ XDFAM[a]	5′-Yakima Yellow/FAM-CCT CAT CAG GGA TCG TCT TGC CCA AGC-BHQ1-3′	

[a]Yakima yellow was used in Baldwin et al. (2023). FAM is used in ISF (2017).

1.4.3 Internal amplification control

	Primers and probes	Sequence	Amplicon size
Forward primer	WuF	5′-CAA CGC GAA GAA CCT TAC C-3′	228 bp
Reverse primer	WuR	5′-ACG TCA TCC CCA CCT TCC-3′	
Probe	WuProbe1	5′-VIC[a]-ACG ACA ACC ATG CAC CAC CTG-QSY-3′	
Probe	WuProbe2	5′-VIC[a]-ACG ACA GCC ATG CAG CAC CT-QSY-3′	

[a]Different fluorophores were evaluated during validation: Yakima Yellow/BHQ1 and VIC/BHQ1.

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1.5 Real-time PCR system: Qiagen Rotor-Gene Q

2. Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 Prepare a slightly turbid cell suspension (with an OD_{600nm} of approximately 0.05) in sterile distilled water from each suspect colony on YDC medium. Cultures should not be older than 5 days after plating. Heat the suspensions for 10 min at 95–100°C.

2.1.2 DNA should preferably be stored at approximately −20°C.

2.2 Multiplex real-time PCR (Baldwin et al., 2023)

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	1.9	N.A.
Rotorgene Probe mix 2× (Qiagen)	2×	7.50	1×
Forward Primer (*XEF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XER*)	20 µM	0.30	0.4 µM
Forward Primer (*XVF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XVR*)	20 µM	0.30	0.4 µM
Forward Primer (*XPF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XPR*)	20 µM	0.30	0.4 µM
Forward Primer (*XGF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XGR*)	20 µM	0.30	0.4 µM
Probe (*XEFAM*)	5 µM	0.12	0.04 µM
Probe (*XVFAM*)	5 µM	0.12	0.04 µM
Probe (*XPFAM*)	5 µM	0.12	0.04 µM
Probe (*XGFAM*)	5 µM	0.12	0.04 µM
Forward Primer (*XDF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XDR*)	20 µM	0.30	0.4 µM
Probe (*XDYY*)	5 µM	0.12	0.04 µM
Subtotal		13	
Nucleic acid extract		2	
Total		15	

2.2.2 PCR conditions: 1 cycle of 94°C for 15 min; 40 cycles of 94°C for 15 s and 64°C for 30 s.

2.3 Real-time PCRs (ISF, 2017)

2.3.1 AFLP derived Taqman PCR

2.3.1.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	1.42	N.A.
Taqman Universal Mastermix II (Applied Biosystem)	2×	7.50	1×
Forward Primer (*XEF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XER*)	20 µM	0.30	0.4 µM
Forward Primer (*XVF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XVR*)	20 µM	0.30	0.4 µM
Forward Primer (*XPF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XPR*)	20 µM	0.30	0.4 µM
Forward Primer (*XGF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XGR*)	20 µM	0.30	0.4 µM
Probe (*XEFAM*)	5 µM	0.12	0.04 µM
Probe (*XVFAM*)	5 µM	0.12	0.04 µM
Probe (*XPFAM*)	5 µM	0.12	0.04 µM
Probe (*XGFAM*)	5 µM	0.12	0.04 µM
Forward Primer (*WuF*)	10 µM	0.30	0.2 µM
Reverse Primer (*WuR*)	10 µM	0.30	0.2 µM
Probe (*WuProbe1*)	10 µM	0.30	0.2 µM
Probe (*WuProbe2*)	10 µM	0.30	0.2 µM
Subtotal		13	
Nucleic acid extract		2	
Total		15	

2.3.1.2 PCR conditions: 1 cycle of 94°C for 15 min; 40 cycles of 94°C for 15 s and 64°C for 30 s.

2.3.2 XopD TaqMan PCR

2.3.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3.58	N.A.
Taqman Universal Mastermix II (Applied Biosystem)	2×	7.50	1×
Forward Primer (*XDF*)	20 µM	0.30	0.4 µM
Reverse Primer (*XDR*)	20 µM	0.30	0.4 µM
Probe (*XDFAM*)	5 µM	0.12	0.04 µM
Forward Primer (*WuF*)	10 µM	0.30	0.2 µM
Reverse Primer (*WuR*)	10 µM	0.30	0.2 µM
Probe (*WuProbe1*)	10 µM	0.30	0.2 µM
Probe (*WuProbe2*)	10 µM	0.30	0.2 µM
Subtotal		13	
Nucleic acid extract		2	
Total		15	

2.3.2.2 PCR conditions: 1 cycle of 94°C for 15 min; 40 cycles of 94°C for 15 s and 64°C for 30 s

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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: nucleic acid extraction and subsequent amplification of e.g. 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.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include specific amplification or co-amplification of nucleic acid control from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table described in point 2.2. Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

The ISF tests (see 2.3) include an internal positive control (adapted from Wu et al., 2008) to monitor each individual sample separately.

3.2 Interpretation of results: in order to assigning results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (and if relevant IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve for the AFLP derived Taqman probes (FAM fluorophore) or the XopD Taqman probe (FAM or Yakima Yellow fluorophore).
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential for the AFLP derived Taqman probes and the XopD Taqman probe.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data reported below for the test described in Section 2.2 are available from Baldwin et al. (2023). The ISF test (2017) as described in Section 2.3 was verified using 4 targets and 4 non-targets (T. Baldwin, *personal communication*).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist http://dc.eppo.int/validationlist.php).

4.1 Analytical sensitivity data

Not available.

4.2 Analytical specificity data

Inclusivity evaluated on 3 isolates of *Xanthomonas vesicatoria*, 4 isolates of *X. hortorum pv. gardneri*, 36 isolates of *Xanthomonas euvesicatoria* pv. *euvesicatoria* and 17 isolates of *X. euvesicatoria* pv. *perforans*:

Exclusivity evaluated using 35 look-alike isolates from seeds and 4 non-target *Xanthomonas* species: cross reactions observed with *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *vitians* with the *XopD* Taqman PCR test but not the AFLP-derived real time PCR test.

	Inclusivity	Exclusivity
AFLP-derived Taqman probes	77%	100%
XopD Taqman probe	97%	95%
Combined multiplex test	100%	95%

4.3 Data on Repeatability

Not available.

4.4 Data on Reproducibility

Not available.

4.5 Data on diagnostic sensitivity (DSE) and diagnostic specificity (DSP) data

	DSE	DSP	Accuracy
AFLP-derived Taqman probes	91.2%	95.6%	91.7%
XopD Taqman probe	92.6%	100%	93.4%

Detailed results

Results from a collection of *Xanthomonas* isolates shown to be pathogenic or non-pathogenic on pepper or tomato

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Number of isolates	Reference tests			Final assigned value	AFLP-derived Taqman probes result	XopD Taqman probe result
	First pathogenicity test result	AFLP-derived conventional PCR result	Second pathogenicity test result[a]			
172	Positive	Positive (1 Xv, 121 Xe, 48 Xp, 2 Xg)	Not evaluated a second time	Positive	Positive	Positive
15	Positive	Positive (15 Xv)	Not evaluated a second time	Positive	Positive	Negative
18	Positive	Negative	Positive	Positive	Negative	Positive
6	Positive	Negative	Negative	Negative	Negative	Negative
1	Negative	Negative	Negative	Negative	Positive	Negative
14	Negative	Negative	Not evaluated	Negative	Negvive	Negative
2	Negative	Not evaluated	Negative	Negative	Negative	Negative

Abbreviations: *Xe*, *Xanthomonas euvesicatoria* pv. *Euvesicatoria*; *Xg*, *X. hortorum* pv. *gardneri*; *Xp*, *X. euvesicatoria* pv. *perforans*; *Xv*, *X. vesicatoria*.

[a]When inconsistent test results were obtained, the pathogenicity test was repeated.

APPENDIX 6 - TAQMAN REAL-TIME PCRS (NAKTUINBOUW, RIJKZWAAN AND SYNGENTA)

The tests below are described as they were carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 The three tests described below are suitable for identification of isolates of *Xanthomonas vesicatoria* (Xv test), *X. hortorum* pv. *gardneri* (Xg test) or of isolates belonging to one of the following pathovars (Xep test): *Xanthomonas euvesicatoria* pv. *euvesicatoria* or *X. euvesicatoria* pv. *perforans*.

1.2 Those tests were developed by Naktuinbouw, RijkZwaan and Syngenta (Baldwin et al., 2023)

1.3 The Xep test is targeting *XopD* and *XopA* genes, the Xv test is targeting a locus encoding an hypothetical protein and a spacer and the Xg test is targeting two loci encoding an hypothetical protein and a type III effector.

1.4 Oligonucleotides:

1.4.1 Xep test

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	Xep1-F	5′-CAA ATT TGC TTC TTG AGA TTT CG-3′	102 bp
Reverse primer	Xep1-R	5′-CCA TTT CTT CCA GGT CTG CT-3′	
Probe	Xep1-P	5′-FAM-CCG CTG CTT GAA TCA TTG ATG CTG TAC G-BHQ1-3′	
Forward primer	Xep2-F	5′-GGC TGA GGC TAG TGT TGA GC-3′	76 bp
Reverse primer	Xep2-R	5′-GGA GAC ATT TTA CAG GCG AAG-3′	
Probe	Xep2-P	5′-HEX-CGG ACG CCT CGG TGG TTT ATC C-BHQ1-3′	

1.4.2 Xv test

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	Xv1-F	5′-ACA CAT TTA GCA CGC TAC GG-3′	111 bp
Reverse primer	Xv1-R	5′-ACC GTC CAT CTT CTT CGG TA-3′	
Probe	Xv1-P	5′-HEX-AAC AGA ACA GGG CCC GCG GT-BHQ1-3′	
Forward primer	Xv2-F	5′-TCC TAA CCA TTC GCA CGT C-3′	93 bp
Reverse primer	Xv2-R	5′-CCC ATC CCT GGT AGT CAG TCT-3′	
Probe	Xv2-P	5′-FAM-CAT GCG CAA GCA GAG CCT CAA C-BHQ1-3′	

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1.4.3 Xg test

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	Xg2-F	5′-CCT CTT CTG TGT AGA CGA CTT GGT T-3′	70 bp
Reverse primer	Xg2-R	5′-TTG CTC CAG CCA CTC GAT AA-3′	
Probe	Xg2-P	5′-HEX-TTC TCT GAC GCC GGT CCT CCG T-BHQ1-3′	
Forward primer	Xg5-F	5′-TAG GTG ACG CAG TTC CTG AG-3′	145 bp
Reverse primer	Xg5-R	5′-GTA TGC AAA ATA ACG GGT CAC TC-3′	
Probe	Xg5-P	5′-FAM-CAG TTG CGG TCG TAC TGC GC-BHQ1-3′	

1.4.4 Internal amplification control

	Primer	Sequence (5′→3′)	Amplicon size (including primer sequences)
Forward primer	WuF	5′-CAA CGC GAA GAA CCT TAC C-3′	228 bp
Reverse primer	WuR	5′-ACG TCA TCC CCA CCT TCC-3′	
Probe	WuProbe1	5′-TexRed-ACG ACA GCC ATG CAG CAC CT-BHQ2-3′	
Probe	WuProbe2	5′-TexRed-ACG ACA ACC ATG CAC CAC CTG-BHQ2-3′	

1.5 Enzyme: PerfeCTa qPCR toughmix (QuantaBio)
1.6 Real-time PCR system: BioRad CFX96

2. Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 Prepare a slightly turbid cell suspension (with an OD_{600nm} of approximately 0.05) in sterile distilled water from each suspect colony on YDC medium. Cultures should not be older than 5 days after plating. Heat the suspensions for 10 min at 95–100°C.

2.1.2 DNA should preferably be stored at approximately −20°C.

2.2 Real-time Polymerase Chain Reaction – real-time PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	3.0	N.A.
PerfeCTa qPCR toughmix (2×) (QuantaBio)	2×	12.5	1×
Forward Primer (*Xep1-F*[a])	20 µM	0.50	0.4 µM
Reverse Primer (*Xep1-R*[a])	20 µM	0.50	0.4 µM
Probe (*Xep1-P*[a])	10 µM	0.50	0.2 µM
Forward Primer (*Xep2-F*[a])	20 µM	0.50	0.4 µM
Reverse Primer (*Xep2-R*[a])	20 µM	0.50	0.4 µM
Probe (*Xep2-P*[a])	10 µM	0.50	0.2 µM
Forward Primer (*WuF*)	20 µM	0.25	0.2 µM
Reverse Primer (*WuR*)	20 µM	0.25	0.2 µM
Probe (*WuProbe1*)	10 µM	0.50	0.2 µM
Probe (*WuProbe2*)	10 µM	0.50	0.2 µM
Subtotal		20	
Nucleic acid extract		5	
Total		25	

[a]or primers from Xv or Xg tests.

2.2.2 PCR conditions: 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 62°C for 48 s. $5°C s^{-1}$ ramp rate

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: nucleic acid extraction and subsequent amplification of e.g. 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.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification

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procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

This test includes an internal positive control (adapted from Wu et al., 2008) to monitor each individual sample separately.

3.2 Interpretation of results: in order to assigning results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (and if relevant IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve for the two multiplexed target tests.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential for the two multiplexed target tests.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data are available from Baldwin et al. (2023).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist http://dc.eppo.int/validationlist.php).

4.1 Analytical sensitivity data (Syngenta validation data).

Evaluated on serial dilutions ($10–10^{-4}$) of 3 cell suspensions prepared from different colonies. For each test, all replicates of the 10 000 fold dilution were detected.

4.2 Analytical specificity data (Syngenta and Rijkzwaan validation data)

Inclusivity evaluated on 18 isolates of *Xanthomonas vesicatoria*, 13 isolates of *X. hortorum pv. gardneri*, 66 isolates of *Xanthomonas euvesicatoria* pv. *euvesicatoria* and 21 isolates of *X. euvesicatoria* pv. *perforans*: 100% for Xep, Xv and Xg tests.

Exclusivity evaluated on 100 non-target isolates: 100% for Xep and Xp tests. 97% for Xg test. The Xg test cross reacted with 3 isolates of *X. hortorum* pv. *vitians* from lettuce.

4.3 Data on Repeatability (Syngenta validation data)

See analytical sensitivity data. 100% for all tests at 10 000 fold dilution.

4.4 Data on Reproducibility (Syngenta validation data)

Evaluated by two different departments using the same samples comprising:

– cell suspensions of 3 colonies of *Xanthomonas vesicatoria*, *X. hortorum* pv. *gardneri*, *Xanthomonas euvesicatoria* pv. *euvesicatoria*, *X. euvesicatoria* pv. *perforans* and 4 non target isolates
– 3 ten-fold dilutions for each cell suspension

Cell suspensions were prepared by the two departments.

Reproducibility was 100% for the 3 tests.

APPENDIX 7 - PATHOGENICITY TEST

This test is carried out on young tomato or pepper plants with 4–5 true leaves. Susceptible cultivars (e.g. tomato cv. Moneymaker, cv. Bonny Best and pepper cv. Early Calwonder) should be used. Plants are grown at 23–35°C with normal watering and fertilization. Inoculum is prepared by suspending approximately 5×10^7 cfu mL^{-1} of a pure culture in PBS (Appendix 2). Leaves are inoculated before full expansion using a cotton swab saturated with inoculum with added carborundum for abrasion, or by infiltrating interveinal spaces. Inoculated plants should be covered with plastic bags to maintain high humidity for 48 h after inoculation. Positive (See Section 5) and negative controls should be included. Bacterial spot symptoms usually develop within 3 weeks at 25°C, appearing as lesions raised above the leaf surface with whitish haloes. Chlorosis and epinasty of tomato and pepper leaves also develop. Isolates of *X. vesicatoria* may incite sunken lesions without haloes. Re-isolation (see 3.2.2) and identification (see 4) of the isolates from symptomatic tissues should complete the positive pathogenicity test.

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