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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/110 (1)

Diagnostics
Diagnostic

PM 7/110 (1) *Xanthomonas* spp. (*Xanthomonas euvesicatoria, Xanthomonas gardneri, Xanthomonas 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*, *Xanthomonas gardneri*, *Xanthomonas perforans*, *Xanthomonas vesicatoria*)[1].

Specific approval and amendment

Approved in 2012–09.

Introduction

Bacterial spot of *Lycopersicon esculentum* 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 *Lycopersicon esculentum* and *Capsicum annuum* are cultivated.

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; Stall *et al.*, 1994; Vauterin *et al.*, 1995; Jones *et al.*, 2004). 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 (Vauterin *et al.*, 1995; Jones *et al.*, 2000) 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, maintained species status as *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*. The bacterial spot pathogens currently fall into four validly described species (*X. vesicatoria*, *X. euvesicatoria*, *X. perforans* and *X. gardneri*) and *X. axonopodis* pv. *vesicatoria* is no longer a valid name (Bull *et al.*, 2010). However, recent phylogenetic analyses based on DNA sequence similarity between single and multiple gene loci (Young *et al.*, 2008; Parkinson *et al.*, 2009; Hamza *et al.*, 2010) support three distinct species, showing *X. euvesicatoria* and *X. perforans* to be highly related to (if not synonymous with) each other and also with the recently described *X. alfalfa*. Furthermore, *X. gardneri* was found to be synonymous with the established *X. hortorum* and the more recently described *X. cynarae*. Although *X. vesicatoria* appears to be a well defined species, many xanthomonad pathovars are described for the *X. euvesicatoria/alfalfae/perforans* and *X. hortorum/cynarae/gardneri* species-level clades. Further investigation is needed to determine whether pathovar status for the tomato and pepper strains within these clades is deserved. Comparative studies of whole genome sequences from reference strains of *X. euvesicatoria*, *X. perforans*, *X. gardneri* and *X. vesicatoria* show considerable diversity between these pathogens and are identifying genes specific to pepper pathogens and other strain-specific genes, which may help to explain differences in virulence, aggressiveness and host preference (Potnis *et al.*, 2011).

The bacterial spot bacteria have been disseminated internationally in contaminated commercial seed lots, deposited

[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.

on the seed surface from infected pulp rather than as internal seed infections. 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 methods for infected or contaminated seeds as well as for diagnosis of bacterial spot in symptomatic tomato and pepper plants. In terms of identification, gene-sequencing methods (DNA barcoding) now offer a relatively simple, inexpensive and robust means to confirm identity and differentiate the *Xanthomonas* species causing bacterial spot. These methods are therefore preferred to the complex and expensive DNA:DNA homology analyses, which are not appropriate for use in routine diagnostics.

The diagnostic procedure for *Xanthomonas* spp. causing bacterial spot of tomato and sweet pepper is presented in Fig. 1.

Identity

Names: *Xanthomonas euvesicatoria* Jones *et al.* (2004).
Xanthomonas gardneri (e.g. Šutić 1957) Jones *et al.* (2004)
Xanthomonas perforans Jones *et al.* (2004).
Xanthomonas vesicatoria (e.g. Doidge 1920) Vauterin *et al.* (1995).
Synonyms: *Bacterium vesicatorium* Doidge 1920.
Bacterium exitiosum Gardner & Kendrick (1921).
Xanthomonas vesicatoria (Doidge 1920) Dowson 1939.
Pseudomonas gardneri Šutic (1957) = *Xanthomonas gardneri.*
Xanthomonas campestris pv. *vesicatoria* (Doidge 1920) Dye (1978).
Xanthomonas axonopodis pv. *vesicatoria* Vauterin *et al.* (1995).

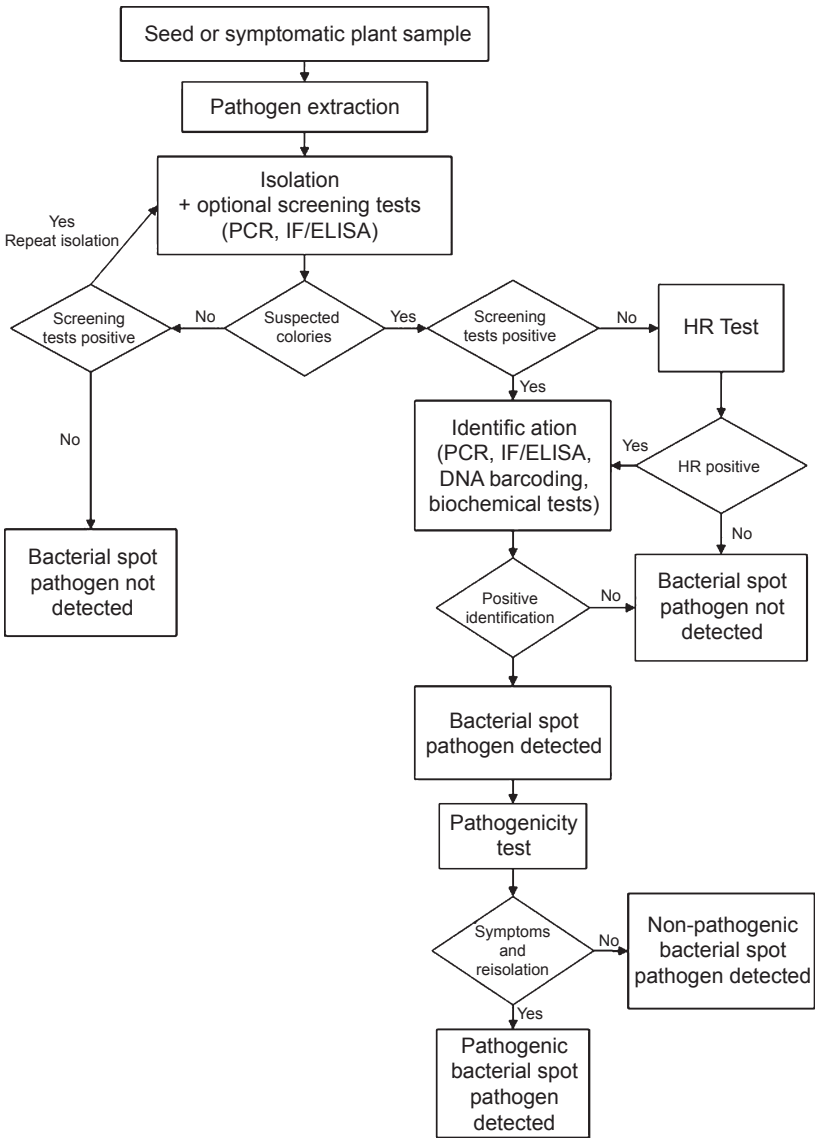

Fig. 1 Flow diagram for testing tomato and pepper seed and plant samples to detect *Xanthomonas* spp. causing bacterial spot.

(A type strains) = *Xanthomonas euvesicatoria.*
Xanthomonas axonopodis pv. *vesicatoria* Vauterin *et al.* (1995).
(C type strains) = *Xanthomonas perforans.*
Taxonomic position: Domain/empire: Bacteria; Division/phylum: Proteobacteria; Class: Gammaproteobacteria; Order: Xanthomonadales; Family: Xanthomonadaceae.
EPPO codes: XANTEU, XANTGA, XANTPF, XANTVE.
Phytosanitary categorization: All four species are EPPO A2 no. 157, EU Annex designation II/A2.

Detection

Symptoms

Lycopersicon esculentum
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. Foliar blight can occur when the lesions coalesce. Necrosis of the petioles and canker-like splits can be observed along the stem. On tomato leaves, bacterial speck lesions (*P. syringae* pv. *tomato*) look similar initially but are surrounded by a more distinct yellow halo.

Lesions on fruits begin as tiny, slightly raised blisters (Fig. 2A,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.

Capsicum annuum
Lesions on *Capsicum annuum* leaves are of irregular shape and necrotic, in some cases surrounded by a chlorotic halo (Figs 3A,B and 4A,B). When the infection is severe, foliar blight can occur and leaves may fall. On fruits, scab-like, raised, whitish lesions appear (Fig. 5A,B).

Detection in symptomatic plants

Extraction
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 one or two drops of sterile physiological saline (SPS = 0.85% NaCl in distilled water) or 1% peptone or 10 mM PBS.

Isolation
The resulting suspension or 100 µL aliquots of 10-fold dilutions in the same buffer can be streaked on a suitable non-selective medium, e.g. Wilbrink's (Koike, 1965), NSCAA (Schaad & Franken, 1996) or YGCA (see Appendix 2) and incubated at 25–28°C. Typical yellow colonies appear in 2–5 days. Suspect colonies may require further purification by re-streaking on the same medium before further identification.

Detection in seeds

A minimum sample size of 10 000 tomato or pepper seeds is recommended by the International Seed Federation (ISF) [http://www.worldseed.org/isf/ishi_vegetable.html] 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*. 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. For example, for protected cultivations or high-class hybrid seeds, the minimum number of seeds can be lowered to a single sample of 2000 seeds with 98% confidence of detecting down to 0.2% contamination. The tests described have not been validated on treated or pelleted seeds.

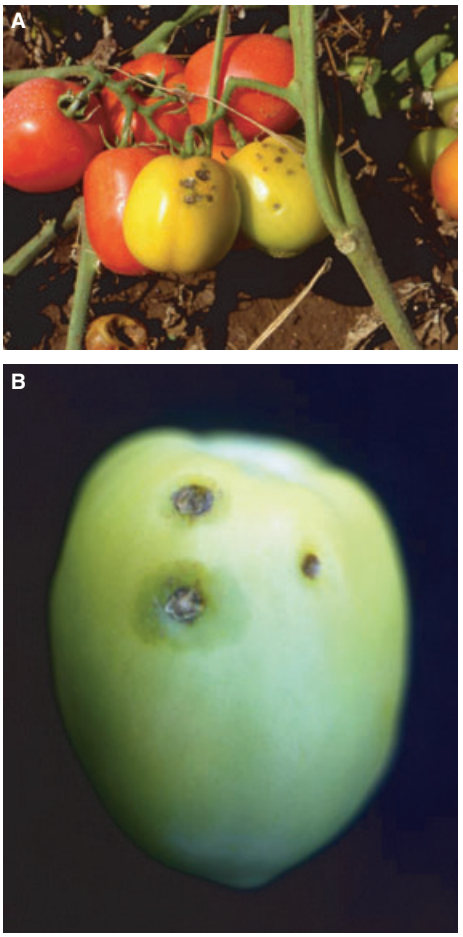

Fig. 2 (A,B) Typical bacterial spot lesions on the surface of tomato fruits.

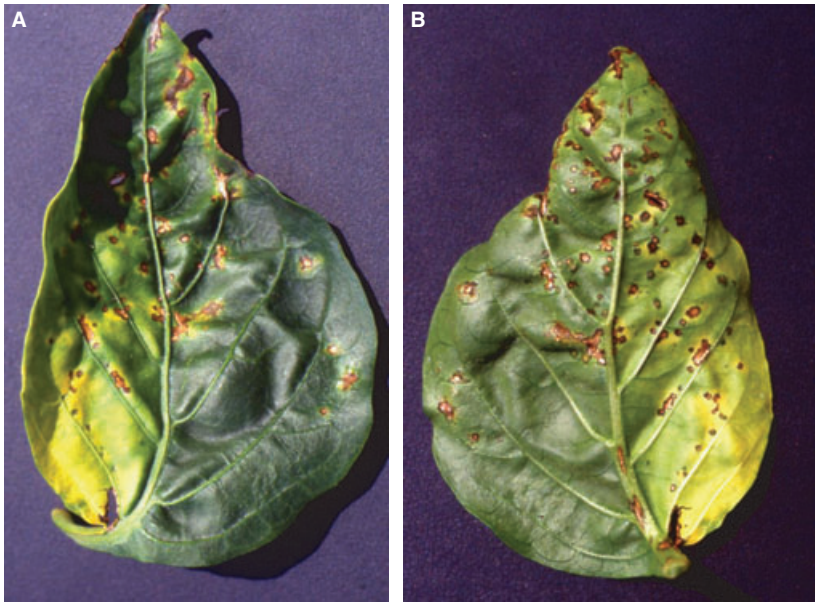

Fig. 3 (A,B) Bacterial spot lesions on pepper leaf (upper and lower surface).

Extraction

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 overnight at 4°C are not recommended, as saprophytes tend to multiply and outgrow the xanthomonads.

Isolation

For isolation, yeast–glucose–calcium carbonate agar (YGCA) is recommended, but other non-selective media 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 yeast peptone glucose agar (YPGA) may also be used (see Appendix 2). Semi-selective media such as CKTM agar (Sijam *et al.*, 1992), mMXV (Sijam *et al.*, 1991) or mTMB agar (McGuire *et al.*, 1986) may facilitate isolation in samples where high saprophyte populations prevent successful isolation of putative *Xanthomonas* colonies, although bacterial spot-forming *Xanthomonas* species may not all grow equally well on such media. When yellow-pigmented colonies are recovered after re-streaking on nutrient agar, tests for the identification of putative xanthomonads should be carried out.

Colony morphology

On YGCA, colonies are bright yellow, circular, with entire margin, wet and shining, mucoid and slightly raised. On other media, colonies appear pale or bright yellow, circular, mucoid and slightly raised. On CKTM medium, colonies appear circular, raised, yellow and surrounded by a white crystalline halo. 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. Use of Tween 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 nutrient agar on a non-selective medium. Identification tests of putative *Xanthomonas* spp. should then be carried out.

Possible screening tests

Serological methods

Antibodies are commercially available for use in immunofluorescence and ELISA (Jones *et al.*, 1997; Alvarez, 2004); however, published data on their specificity across *X. euvesicatoria*, *X. gardneri*, *X. perforans* and *X. vesicatoria* are not reported. Some monoclonal antibodies appear to be species-specific (Bouzar *et al.*, 1994a). The use of serological methods in screening seeds or plant extracts would therefore require prior validation of available antibodies against the full range of pathogen diversity to be detected. EPPO diagnostic protocols PM 7/97(1) and PM 7/101(1) describe indirect immunofluorescence tests and ELISA tests for use with validated antibodies (EPPO, 2009, 2010). Tests should be performed on seed or plant extracts and their 10-fold dilutions.

Polymerase chain reaction methods

Some published PCR assays are either species-specific (Moretti *et al.*, 2009) or of unknown specificity (Park *et al.*, 2009).

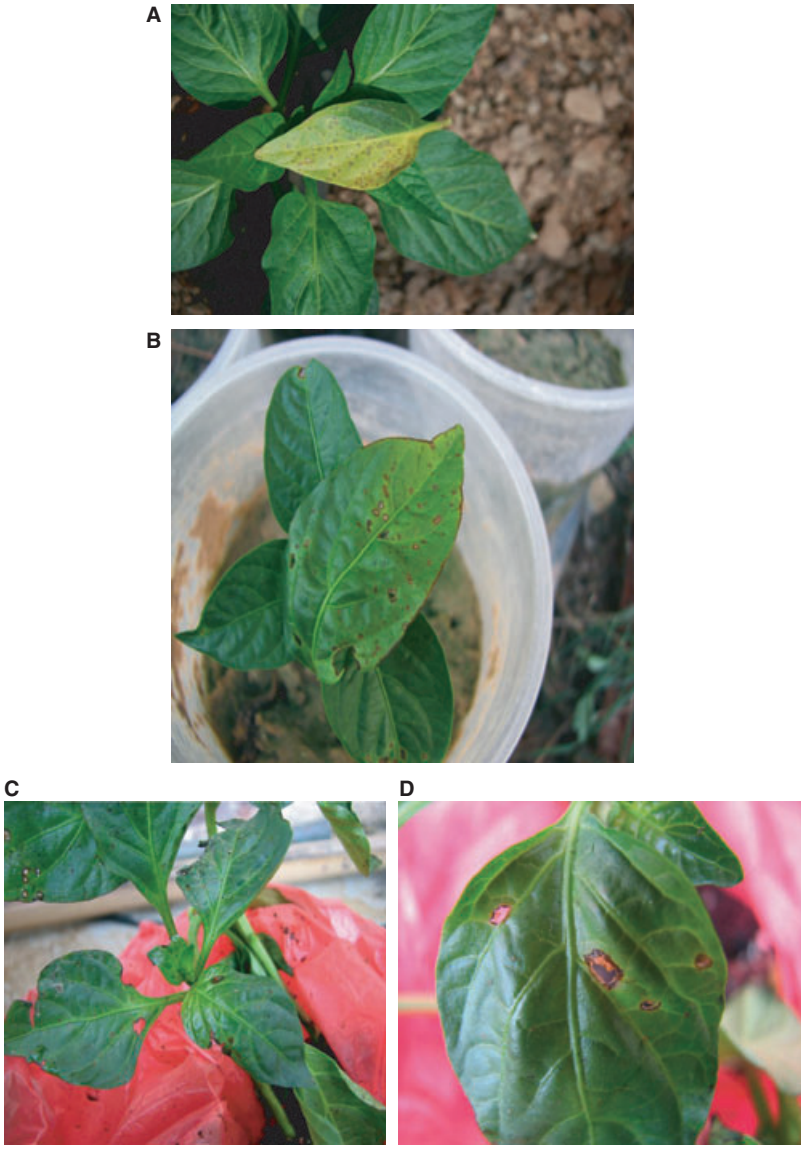

Fig. 4 (A,B,C,D) *Xanthomonas perforans* leaf spot symptoms on pepper. Figure 4A shows small necrotic leaf spots while figures 4B-4D show large necrotic leaf sports with perforated centres.

Identification

Purified isolates of the bacterial spot-causing *Xanthomonas* spp. can be identified using a combination of procedures described below. At least two tests based on different biological principles are recommended.

Hypersensitive reaction

In order to avoid performing identification tests on saprophytes, a hypersensitive reaction (HR) test can be performed on bean pods (Klement & Lovrekovich, 1961) by infiltrating tissues with aqueous suspensions containing approximately 10^7 cfu mL^{-1} of the putative xanthomonads. A positive HR strongly indicates the presence of a phytopathogenic *Xanthomonas* sp.

Biochemical characteristics

Xanthomonas spp. are easily differentiated from the other genera of aerobic, Gram-negative rods and other yellow-pigmented bacteria by the characters shown in Table 1 (Schaad & Stall, 1989; Schaad *et al.*, 2001). Key phenotypic traits differentiating the four *Xanthomonas* species that cause bacterial spot, as described by Jones *et al.* (2004), are shown in Table 2. *Xanthomonas euvesicatoria* is weakly amylolytic and weakly pectolytic (see below), has a distinct pattern of reaction to a panel of monoclonal antibodies (Bouzar *et al.*,

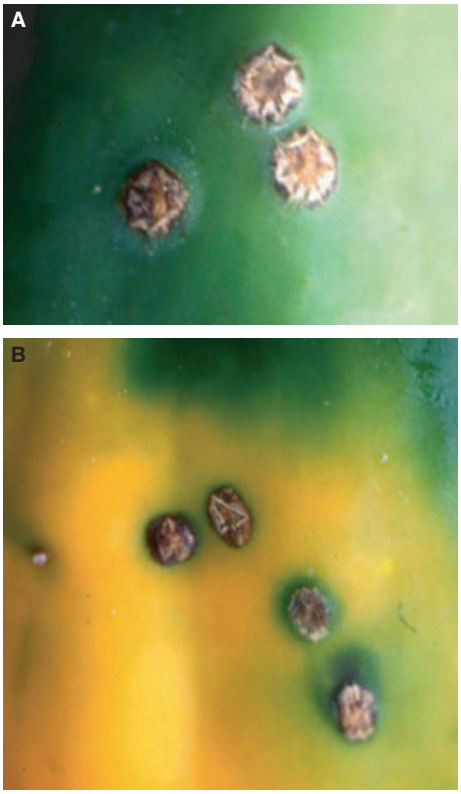

Fig. 5 (A,B) Typical bacterial spot lesions on the surface of pepper fruit.

1994a; Jones *et al.*, 2000), has a distinct SDS-PAGE profile (Bouzar *et al.*, 1994b), and utilizes *cis*-aconitic acid. *Xanthomonas vesicatoria* strains strongly digest starch and pectic substrates, react differently to the panel of monoclonal antibodies, do not utilize *cis*-aconitate, and have a specific 25- to 27-kDa protein in the SDS-PAGE profile. *Xanthomonas perforans* is strongly amylolytic and pectolytic, has a distinct pattern of reaction to a panel of monoclonal antibodies, and has a distinct SDS-PAGE profile. *Xanthomonas gardneri* is weakly amylolytic and pectolytic, and has a distinct pattern of reaction to a panel of monoclonal antibodies and a distinct SDS-PAGE profile.

Automated Biolog identification system

The new version (third generation) Biolog GENIII 96 microplate (Biolog, Omnilog, US) allows identification of isolated bacteria based on 94 phenotypic tests: 71 carbon-source utilization assays and 23 chemical sensitivity assays for biochemical and physiological properties including pH, salt, lactic acid tolerance and antibiotics. Every species tested creates a unique 'phenotypic fingerprint', which is automatically compared with a database created from 1200 aerobic species according to the manufacturer's instructions. *Xanthomonas gardneri*, unlike the other bacterial spot xanthomonads, does not utilize any of the carbon sources listed in Table 3.

Assays for amylolytic and pectolytic activity

The ability of isolates to degrade starch is tested on modified brilliant cresyl blue-starch medium (see Appendix 2) and on nutrient agar supplemented with 1% starch. The plates are incubated at 25–28°C for 2 days. Amylolytic isolates (Amy^+) grow profusely on mBS, whereas non-amylolytic isolates (Amy^-) do not. Both Amy^+ and Amy^- isolates grow on nutrient agar containing 1% starch. Starch hydrolysis is revealed by the production of an opaque zone surrounding bacterial growth. To confirm this hydrolysis, the plates can be flooded with Lugol's iodine (1% iodine, 2% potassium iodide) creating a clear, unstained zone where the starch has been hydrolysed. Pectolytic activity is tested on pectate medium (see Appendix 2). After incubation of the plates at 25–28°C for 1–2 days, a depression develops in the medium surrounding colonies of pectolytic isolates. Reference strains of each *Xanthomonas* species must be included as controls.

Fatty acid methyl ester analysis

Fatty acid methyl ester (FAME) analysis (as described by Weller *et al.*, 2000) will adequately identify *Xanthomonas* spp. isolated from tomato and distinguish them from other bacterial pathogens causing leaf spot symptoms.

Table 1 Phenotypic characteristics useful for differentiating *Xanthomonas* from *Pseudomonas* and other yellow-pigmented bacteria such as *Flavobacterium*

Test	*Xanthomonas*	*Pseudomonas*	*Flavobacterium*	*Pantoea*
Flagella	1, polar	>1, polar	None	Peritrichous
Xanthomonadin	+	–	–	–
Fluorescence	–	V	–	–
Levan from sucrose	+	V	–	–
H_2S from cysteine	+	–	–	–
Oxidase	–	V	+	–
Fermentation	–	–	–	+
Growth on 0.1% TTC	–	+	+	+

V, variable reaction.

Table 2 Main distinctive features of *Xanthomonas* spp. causing bacterial spot of tomato and pepper

Group	Name	Distribution	Reference strain	Specific protein size (kDa)	Amylolytic activity	Pectolytic activity
A	*Xanthomonas euvesicatoria*	Worldwide	ATCC11633^T (NCPPB2968)	32–35	–	–
B	*Xanthomonas vesicatoria*	Worldwide	ATCC35937^T (NCPPB422)	25–27	+	+
C	*Xanthomonas perforans*	Mexico, Thailand, US	ATCC BAA-983^T (NCPPB4321)	25–27	+	+
D	*Xanthomonas gardneri*	Brazil, Costa Rica, US	ATCC19865^T (NCPPB881)	25–27	–	–

Table 3 Differentiation of strains based on carbon utilization patterns using the Biolog GN Microplate system (Jones *et al.*, 2000)

Utilization of:	*X. euvesicatoria*	*X. vesicatoria*	*X. perforans*	*X. gardneri*
Dextrin	+	+	+	–
Glycogen	+	V	V	–
N-acetyl-D-glucosamine	+	V	+	–
D-galactose	+	V–	+	–
Gentibiose	+	V	+	–
α-D-lactose lactulose	V	V–	+	–
Acetic acid	V	–	+	–
Cis-aconitic acid	+	–	V	–
Malonic acid	+	V	+	–
Propionic acid	V–	V	+	–
D-alanine	V	V	+	–
Glycyl-L-aspartic acid	–	V–	+	–
L-threonine	V	V–	+	–

+ = positive reaction by all strains; V, 50% or more of strains utilized compound; V–, <50% of strains utilized compound; –, none of strains utilized compound.

Serological methods

Depending on the availability of validated antibodies, aqueous suspensions of presumptive isolates (containing approximately 10^6 cfu mL^{-1}) may be identified using immunofluorescence or ELISA tests according to EPPO diagnostic protocols PM 7/97 (1) *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009) and PM 7/101 (1) *ELISA tests for plant pathogenic bacteria* (EPPO, 2010), respectively.

Polymerase chain reaction methods

Aqueous suspensions of presumptive isolates (containing approximately 10^6 cfu mL^{-1}) can be identified using a series of conventional PCR tests (Appendix 3) as described by Koenraadt *et al.* (2009).

DNA fingerprinting methods

The comparison of genomic DNA between isolates and type strains by means of rep-PCR fingerprinting (Stern *et al.*, 1984; Sharples & Lloyd, 1990; Louws *et al.*, 1994; Koeuth *et al.*, 1995), can be a useful identification test (see EPPO Standard PM 7/100(1) *Rep-PCR tests for identification of bacteria* (EPPO, 2010)).

DNA barcoding methods

Allocation of the bacterial spot pathogens to the four *Xanthomonas* species has previously required analysis by complex DNA:DNA homology that is beyond the capability of most diagnostic laboratories. However, comparisons of commercially sequenced PCR products amplified from selected housekeeping gene loci now offer equivalent resolution with significantly reduced cost and effort. For example, single gene sequencing methods of Parkinson *et al.* (2009) and multilocus sequencing methods of Young *et al.* (2008) and Hamza *et al.* (2010) have been shown to adequately distinguish strains of *X. gardneri*, *X. vesicatoria*, *X. euvesicatoria* and *X. perforans.* Procedures are described in the EPPO Standard PM 7/XXX on *DNA barcoding as an identification tool for plant pests* (in preparation).

Pathogenicity test

Depending on the circumstances of use (see EPPO Standard PM 7/77(2)), a pathogenicity test may be performed if needed. This test may be conveniently carried out on young tomato or pepper plants with 4–5 true leaves. Susceptible cultivars (e.g. tomato cv. Moneymaker 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 phosphate buffer. Leaves are then 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 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 and identification of the isolates from symptomatic tissues should complete the positive pathogenicity test.

Reference material

Xanthomonas euvesicatoria Jones *et al.* (2004).
Type strain: ATCC 11633; ICMP 109; ICMP 98; NCPPB 2968.
Xanthomonas gardneri (e.g. Šutic, 1957) Jones *et al.* (2004)
Type strain: ATCC 19865; ICMP16689; NCPPB 881; LMG962.
Xanthomonas perforans Jones *et al.* (2004).
Type strain: ATCC BAA-983; NCPPB 4321; ICMP 16690.
Xanthomonas vesicatoria (e.g. Doidge 1920) Vauterin *et al.* (1995).
Type strain: ATCC 35937; ICMP 63; LMG 911; NCPPB 422; CFBP 2537.

Reporting and documentation

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

Performance criteria

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

Further information

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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

Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

Acknowledgements

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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.

Soaking

Soak each subsample of seeds for a minimum of 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.

Stomacher procedure (ISF recommended method; http://www.worldseed.org/isf/ishi_vegetable.html)

Transfer each subsample of seeds into a radiation sterile Stomacher bag (e.g. 105 × 50 mm) and add 3 mL sterile PBS-T per g seed (Appendix 2). Soak and refrigerate seeds overnight (minimum 14 h) under refrigeration. Crush the

seeds for 3 min in a Stomacher and allow the sediment to settle for 30 min at room temperature.

Filter the resulting homogenate through sterile gauze and centrifuge the filtrate at 10 000–12 000 *g* for 20 min at 10°C. Remove the supernatant and resuspend the pellet in 1.0–1.5 mL sterile water to obtain the final concentrate. Prepare 10-fold dilutions for direct isolation.

Aliquots of each seed extract can be frozen as a reference and used for any further testing.

Appendix 2 – Buffers and Media

Buffers

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	1000 mL
Autoclave 15 min at 121°C and cool to room temperature	

Phosphate buffered saline Tween (PBS-T) for the extraction of bacteria from seeds (Stomacher method).

50 mM PB, pH 7.4	g L^{-1}
$Na_2HPO_4 \times 12H_2O$	19.57 g
KH_2PO_4	1.65 g
$Na_2S_2O_3$*	0.5 g
Distilled water	1000 mL
Autoclave 15 min at 121°C and cool to room temperature	
Sterile Tween 20 (10% solution)	0.2 mL

*Recommended when seeds have been treated with hypochlorite.

Media

All media are sterilized by autoclaving at 121°C for 15 min.

CKTM agar medium (Sijam *et al.*, 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
Autoclave	
Tween 80*	10 mL

*Autoclave separately and add immediately after the combined media are autoclaved.

The following antibiotics are added after autoclaving 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.

Modified TMB agar (adapted McGuire *et al.*, 1986):

Distilled water to	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*	10 mL

*Autoclave separately and add immediately after the combined media are autoclaved.

The following antibiotics are added after autoclaving 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.

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

Nutrient agar (NA): Use commercial preparations such as Oxoid CM3 or Difco.

Nutrient dextrose (ND) agar: add 1% (w/v) D-glucose to nutrient agar (Oxoid CM3 or Difco).

Yeast dextrose chalk (YDC) agar:

Distilled water	1 L
Yeast extract	10.0 g
Dextrose (glucose)	20.0 g
Calcium carbonate (light powder)	20.0 g
Microbiological grade agar	15.0 g

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

Yeast peptone glucose agar (YPGA):

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

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

Modified brilliant cresyl blue-starch medium (mBS):

Distilled water	1 L
Potato starch	10.0 g
$K_2HPO_4 \cdot 3H_2O$	3.0 g
KH_2PO_4	5.0 g
L-methionin 0.25 g, nicotinic acid	0.25 g
L-glutamin	0.25 g
Agar technical	15.0 g

Adjust pH to 6.8–7.0.

Pectate medium (Paton, 1958).

Distilled water	1 L
Peptone	5.0 g
Lab-lemco	5.0 g
Calcium lactate	5.0 g
Oxoid agar No.3	12.0 g
L-glutamin	0.25 g
Agar technical	15.0 g

Adjust pH to 7.2 and autoclave.

Pectate overlayer ingredients

Distilled water	1 L
Sodium polypectate (e.g. Herbstreith and Fox, type CU 902, Neuenbürg, Germany)	2.0 g
Disodium ethylenediamine tetra-acetate (EDTA)	0.1 g
Bromothymol blue	Trace to colour

Gradually dissolve polypectate in hot water (80°C) with constant stirring. Add remaining ingredients; adjust pH to 7.2 and autoclave at 115°C for 4 min. Pour 5 mL overlayer per plate over set basal medium.

Wilbrink's medium: (Koike, 1965)

Distilled water	1 L
Peptone special (Oxoid/LP0072)	5.0 g
K_2HPO_4 (Merck/1.05101.)	0.5 g
$MgSO_4 \cdot 7H_2O$ (Merck/1.05886.)	0.25 g
Sucrose (Fluka/84100)	10.0 g
Agar technical no.3 (Oxoid/LP0013)	18.0 g

Adjust pH to 7.0 before adding agar.

Modified MXV medium (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*	10 mL

*Autoclave separately and add immediately after the combined media are autoclaved.

The pH is adjusted to 7.4.

The following antibiotics are added after autoclaving and cooling to approximately 45°C.

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

Nutrient starch cycloheximide antibiotic agar (NSCAA) (Schaad & Franken, 1996).

Distilled water	1 L
Potato Starch	15 g
Nutrient agar	23 g

The following antibiotics are added after autoclaving and cooling to 45°C.

Cycloheximide (Sigma/C-7698) 200 mg L^{-1} in methanol, nitrofurantion (Sigma/N-7878) 10 mg L^{-1} in dimethylformamide and vancomycin (Sigma/V-2002) 0.5 mg L^{-1} in distilled water.

Performance criteria available for the media

Performance criteria for the isolation from symptomatic plants on Wilbrink's and NSCAA

Detection limit on NSCAA was 1.06×10^3 cfu mL^{-1} and on Wilbrink's 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

Detection limits on the semi-selective media mMXV and mTMB were found to be 53 cfu mL^{-1} (*X. euvesicatoria*), 24 cfu mL^{-1} (*X. vesicatoria*), 94 cfu mL^{-1} (*X. gardneri*) and 34 cfu mL^{-1} (*X. perforans*). Lowest detectable concentration varied considerably between sample and semi-selective medium. Particularly on the semi-selective medium mTMB, the lowest detectable concentration varied from 1.7 to 16900 cfu mL^{-1} (*X. perforans*) between samples. Variation for the semi-selective medium mMXV was much lower, with the lowest detectable concentration ranging from 16.9 to 169 cfu mL^{-1} (*X. perforans*) between samples.

Appendix 3 – Conventional PCR tests according to Koenraadt *et al.* (2009)

1. General information

1.1 Two conventional duplex-PCR tests are available for identification purposes to distinguish the four described species: *X. euvesicatoria*, *X. vesicatoria*, *X. gardneri* and *X. perforans.* 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.

1.2 The test can be applied to bacterial colonies.

1.3 Oligonucleotides:

Xanthomonas euvesicatoria
Bs-XeF (5′-CAT GAA GAA CTC GGC GTA TCG-3′)
Bs-XeR (5′-GTC GGA CAT AGT GGA CAC ATA C-3′)
Xanthomonas vesicatoria
Bs-XvF: 5′-CCA TGT GCC GTT GAA ATA CTT G -3′
Bs-XvR: 5′-ACA AGA GAT GTT GCT ATG ATT TGC-3′
Xanthomonas gardneri
Bs-XgF: 5′-TCA GTG CTT AGT TCC TCA TTG TC-3′
Bs-XgR: 5′-TGA CCG ATA AAG ACT GCG AAA G-3′
Xanthomonas perforans
Bs-XpF: 5′-GTC GTG TTG ATG GAG CGT TC-3′
Bs-XpR: 5′-GTG CGA GTC AAT TAT CAG AAT GTG G-3′
16s rRNA internal control
BAC16S-F: 5′-TCC TAC GGG AGG CAG CAG T-3′
BAC16S-R (5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′)

1.4 Amplicon sizes in base pairs: Bs-XeF/R primers 173 bp; Bs-XvF/R primers 138 bp; Bs-XpF/R primers 197 bp; Bs-XgF/R primers 154bp; BAC16S-F/R (internal control) primers 466 bp.

1.5 Enzyme: platinum Taq polymerase (Invitrogen/Life technologies, US).

2. Methods

Nucleic acid extraction and purification: colony material from pure cultures is suspended in 100 µL molecular-grade water. For the negative isolation control (NIC), 100 µL molecular-grade water is used. 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).

2.1 Polymerase chain reaction (PCR).

Reagents (working concentration in brackets)	*X. euvesicatoria* *X. vesicatoria* Per reaction (µL)	Final concentration	*X. gardneri* *X. perforans* Per reaction (µL)	Final concentration
Molecular-grade water*	15.75		15.0	
PCR Rxn reaction buffer (10× Invitrogen)	2.5	1x	2.5	1x
$MgCl_2$ (50 mM, Invitrogen)	0.75	1.5 mM	1.5	3.0 mM
dNTPs (10 mv 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	

2.2 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* 10^9 cfu mL^{-1} (e.g. PD 3562)
  PAC Xv: DNA of *X. vesicatoria* 10^9 cfu mL^{-1} (e.g. PD5212)
  PAC Xp: DNA of *X. perforans* 10^9 cfu mL^{-1} (e.g. PD5515)
  PAC Xg: DNA of *X. gardneri* 10^9 cfu mL^{-1} (e.g. PD5842)

In addition to the external positive controls (PIC and PAC), use of an internal positive control (IPC) contributes by monitoring 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 should produce no amplicons
- PIC and PAC should produce amplicons of the relevant size

X. euvesicatoria should give a product of 173 bp
X. vesicatoria should give a product of 138 bp
X. perforans should give a product of 197 bp
X. gardneri should give a product of 154 bp

- The IPC *16S rRNA* gene should give a product of 466 bp

When these conditions are met:

- A test will be considered positive if amplicons of bp as indicated above for PIC and PAC are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

For *X. gardneri* analytical sensitivity was 2.5×10^6 cfu mL^{-1}, for *X. perforans* 1.9×10^7 cfu mL^{-1}, for *X. 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 *Xanthomonas 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 *Xanthomonas euvesicatoria*, three *Xanthomonas gardneri*, seven *Xanthomonas perforans*, 12 *Xanthomonas 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 *Xanthomonas euvesicatoria*, five *Xanthomonas gardneri*, five *Xanthomonas perforans*, *12 Xanthomonas vesicatoria*, and four that could not be classified. Two isolates identified using sequencing as *Xanthomonas perforans* and one as *Xanthomonas gardneri* were not identified using PCR. Two isolates identified using PCR as *Xanthomonas 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*, *Pseudomonas syringae* pv. *syringae*, *Agrobacterium tumefaciens*, *Pseudomonas corrugata*. Five (two *P. syringae* pv. *tomato* isolates and three *Pseudomonas corrugata* isolates) of the 21 related isolates gave weak (non-specific) amplicon(s) in the duplex PCR for *X. gardneri/X. perforans* (very close to 154 and 197 bp) but not in the duplex PCR for *Xanthomonas euvesicatoria/Xanthomonas vesicatoria*. This underlines the risk of wrong identifications for *X. gardneri* or *X. perforans*.

4.3 Data on repeatability:

93%.

4.4 Data on reproducibility:

100%.