Bulletin OEPP/EPPO Bulletin (2016) **46** (3), 429–443 ISSN 0250-8052. DOI: 10.1111/epp.12329

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/128 (1)

Diagnostics
Diagnostic

PM 7/128 (1) *Xanthomonas axonopodis* pv. *allii*

Specific scope

This Standard describes a diagnostic protocol for *Xanthomonas axonopodis* pv. *allii*.[1]

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

Specific approval and amendment

First approved in 2016-09.

1. Introduction

Xanthomonas axonopodis pv. *allii* (bacterial blight of onion) is a pest of *Allium* species. Symptoms typical of bacterial blight of *Allium cepa* L. (onion) were initially observed in Barbados in 1971 (Paulraj & O'Garro, 1993). The first identification of the causal agent as a xanthomonad was made in Hawaii, where the disease appeared in 1975 (Alvarez *et al.*, 1978). A disease inducing similar symptoms was reported in the mainland USA in the 1940s and ascribed to the non-valid '*Xanthomonas striaformans*' (Thomas & Weinhold, 1953), whose biochemical characteristics do not match those of the genus (Swings *et al.*, 1993). The causal agent of BBO has since then been characterized as *Xanthomonas axonopodis* pv. *allii* (Roumagnac *et al.*, 2004a) and this bacterium has subsequently been reported in several countries: Brazil (Neto *et al.*, 1987), Cuba (Roumagnac *et al.*, 2004a), Japan (Kadota *et al.*, 2000), Mauritius and Réunion Island (Picard *et al.*, 2008), South Africa (Serfontein, 2001), several continental states in the USA (Isakeit *et al.*, 2000; Schwartz & Otto, 2000; Nunez *et al.*, 2002; Sanders *et al.*, 2003) and Venezuela (Trujillo & Hernandez, 1999). Although primarily associated with outbreaks on *A. cepa* L. worldwide, *X. axonopodis* pv. *allii* was found to infect *Allium fistulosum* (Welsh onion), *Allium sativum* (garlic), *Allium porrum* (leek), *Allium schoenoprasum* (chive) and *A. cepa* var. *ascalonicum* (shallot) after artificial inoculation (Bowen *et al.*, 1998; Kadota *et al.*, 2000; Roumagnac *et al.*, 2004a). The three former hosts were also reported to be susceptible in the field in addition to *A. cepa* L. (Kadota *et al.*, 2000; Picard *et al.*, 2008). Non-*Allium* plant species (primarily citrus and legume species) have been reported as hosts under artificial inoculation (O'Garro & Paulraj, 1997; Gent *et al.*, 2005) but no symptoms caused by *X. axonopodis* pv. *allii* have ever been reported from these species under field conditions, making their host status uncertain. Yield losses vary depending on the timing of infection, weather conditions and cultivar susceptibility, but yield reductions ranging from 20 to 50% have been recorded under conditions conducive to efficient development of disease (Schwartz & Otto, 2000; Nunez *et al.*, 2002).

Xanthomonas axonopodis pv. *allii* is seed borne and seed transmitted (Roumagnac *et al.*, 2004b; Humeau *et al.*, 2006). Further information can be found in the EPPO data sheet on *X. axonopodis* pv. *allii* (EPPO, 2016a). For geographical distribution see the EPPO Global Database (EPPO, 2016b). A flow diagram for the detection and identification of *X. axonopodis* pv. *allii* is given in Figure 1.

2. Identity

Name: *Xanthomonas axonopodis* pv. *allii* (Kadota *et al.*, 2000; Roumagnac *et al.*, 2004a).

Synonyms: *Xanthomonas campestris* pv. *allii* (Kadota *et al.*, 2000).

Taxonomic position: Proteobacteria: Gammapropteobacteria: *Xanthomonadales*: *Xanthomonadaceae*.

The analyses based on rep-PCR, amplified fragment length polymorphism (AFLP) and partial sequencing of housekeeping genes (Roumagnac *et al.*, 2004a; Gent *et al.*, 2005; Bui Thi Ngoc *et al.*, 2010) have placed *X. axonopodis* pv. *allii* into the 9.2 genetic cluster of *X. axonopodis* (Rademaker *et al.*, 2000).

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

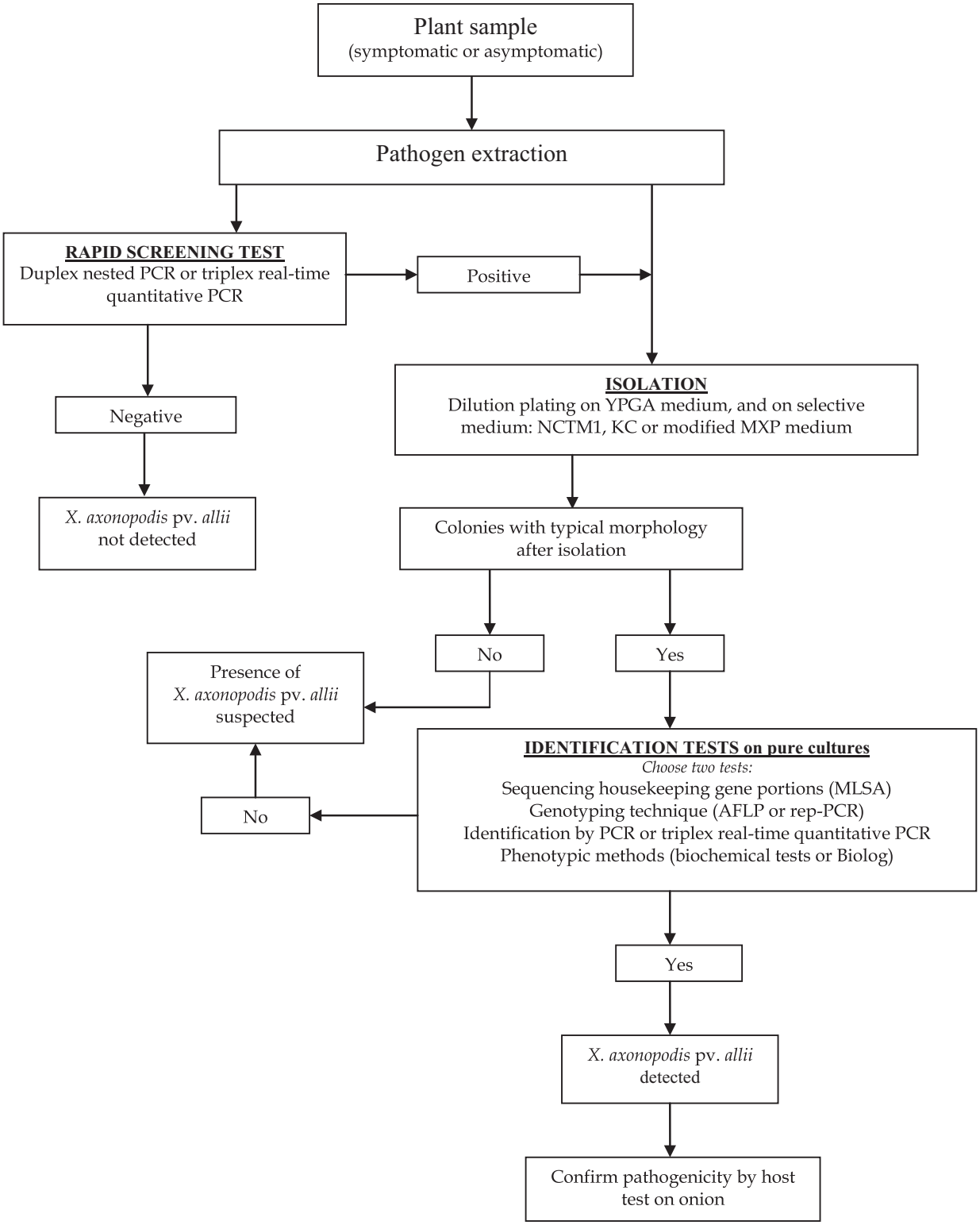

Fig. 1 Flow diagram for the detection and identification of *Xanthomonas axonopodis* pv. *allii* in samples of symptomatic or asymptomatic *Allium* or other putative host species.

EPPO code: XANTAA.
Phytosanitary categorization: EPPO A1 List, no. 353.

3. Detection

3.1. Disease symptoms

Xanthomonas axonopodis pv. *allii* causes symptoms at any stage of crop development on short-day *A. cepa* L. cultivars, but symptoms mostly develop during or after bulb initiation on long-day cultivars. Symptoms develop on leaves and flowering stems but not on roots and bulbs. Lesions first appear as small lenticular-shaped chlorotic lesions with water-soaked margins. Lesions quickly enlarge, become tan to brown in colour, and cause extensive water-soaking. Chlorotic streaks may run down the entire length of leaves. When weather conditions become hot and dry, infected tissues or lesions dry out and become brittle but retain their characteristic tan to brown colour.

As the disease progresses, lesions coalesce and cause tip dieback, and extensive blighting of outer older leaves occurs. The loss of leaf area results in stunted plants and undersized bulbs. In severe infections, all leaves may become completely blighted, resulting in premature death of the plant. Symptoms are similar on *A. schoenoprasum*, *A. sativum*, *A. porrum*, *A. cepa* var. *ascalonicum* and *A. fistulosum*, but tend to be most severe on *A. cepa* L. Pictures of symptoms are presented in Figs 2–4.

Fig. 2 Leaf lesions in onion bacterial blight caused by *Xanthomonas axonopodis* pv. *allii*.

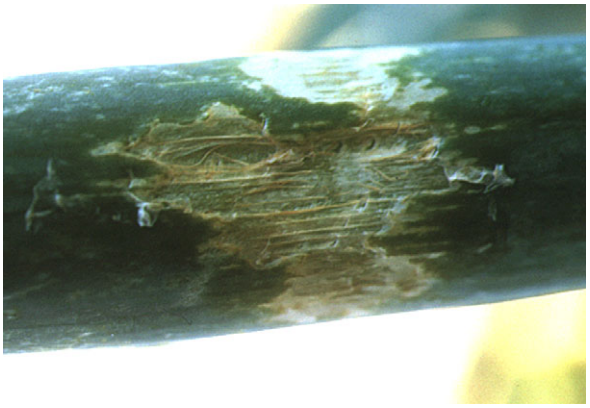

Fig. 3 Scape lesion in onion bacterial blight caused by *Xanthomonas axonopodis* pv. *allii*.

Fig. 4 Onion bacterial blight lesions caused by *Xanthomonas axonopodis* pv. *allii* on a garlic leaf.

3.2. Screening tests

Direct isolation from *A. cepa* L. seeds or from plant material, PCR (duplex nested-PCR) and a triplex real-time quantitative PCR test can be used as screening tests (Robène-Soustrade *et al.*, 2010; Robene *et al.*, 2015). The conventional PCR and real-time PCR tests are described in Appendices 2 and 3, respectively. Using nested-PCR protocols gives an increased risk for false positives due to contamination with amplicons produced in the first round of amplification. A commercial all-inclusive diagnostic kit based on this duplex nested-PCR is also available (Qualiplante). No specific serological test is currently available for *X. axonopodis* pv. *allii*.

3.3. Extraction

3.3.1. Extraction from plant material

Infected leaves or stems are quickly surface disinfected using 70% ethanol. Pieces of leaf or stem tissues including the spots or necrosis are removed with a disinfected scalpel

blade and transferred to approximately 2 mL of 10 mM sterile Sigma 7–9 buffer (pH 7.2). Tissue parts are cut aseptically into small pieces or crushed with a sterile pestle. The preparation is then left for 5–10 min at room temperature for diffusion of bacteria. Extraction can also be performed using a homogenizer grinder (e.g. a Homex grinder from Bioreba) and extraction bags (0.25 g of tissue in 5 mL of 10 mM sterile Sigma 7–9 buffer, pH 7.2). This is particularly appropriate for rapid screening tests (duplex nested-PCR and triplex real-time PCR).

Plant extracts (up to 2 mL) should be centrifuged for 10 min at 20 000 *g* and the supernatant discarded.

The extraction has not been evaluated for asymptomatic plant material.

3.4. Extraction from seeds

Sampling

Tests are performed on subsamples each containing 10 g of onion seeds. The number of subsamples to be tested is determined based on the hypergeometric distribution.

The minimal recommended sample size is 30 000 seeds (with a maximum subsample size of 2500 seeds), providing a 95% probability of detecting a 0.01% incidence of contamination in the seed lot.

Seed samples (10 g each) are soaked in 50 mL of 10 mM sterile Sigma 7–9 buffer (pH 7.2) for 48 h at approximately 4°C and homogenized (30 s at low speed) using a Stomacher (Stomacher®400 from Seward Medical) or equivalent.

3.5. Isolation

Plant extracts: 50 µL of plant extract are plated onto a non-selective rich medium such as YPGA and/or on a semi-selective medium such as NCTM1, modified MXP or KC (see Appendix 1). Plates are incubated for 2–3 days at approximately 28°C.

Seed macerates: are streaked on a semi-selective medium such as NCTM1, modified MXP or KC (see Appendix 1). Plates are incubated at approximately 28°C for up to 96 h.

3.6. Colony morphology

Xanthomonas axonopodis pv. *allii* colonies on YPGA or semi-selective media listed in Appendix 1 are circular, convex, mucoid and yellow. Comparison with a reference strain on the same medium is recommended.

Because of an important bacterial microflora naturally associated with onion seeds and plants, the detection of the pathogen from seed should be preferentially achieved using the PCR procedure described in Appendix 2 or the real-time PCR described in Appendix 3. Additional plating on semi-selective media allows one to check for viability of the target and recover cultures that can be precisely genotyped (see below).

4. Identification

The identification of *X. axonopodis* pv. *allii* should be performed using two or more tests and including at least identification PCR. Checking for pathogenicity is also recommended. Relevant tests are described below.

4.1. Biochemical and physiological tests

Xanthomonas axonopodis pv. *allii* strains are aerobic rods with one polar flagellum. They produce yellow xanthomonadin pigment.

The biochemical and physiological characteristics to be tested for *X. axonopodis* pv. *allii* strains are given in Table 1.

Table 1. Biochemical characteristics of *Xanthomonas axonopodis* pv. *allii* (Roumagnac *et al.*, 2004a)

Characteristics	Results
Gram's reaction	−
Cytochrome *c* oxidase reaction	−
Aerobic metabolism of glucose	+
Anaerobic metabolism of glucose	−
Nitrate reduction	−
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Tween 80 hydrolysis	+
Milk proteolysis	+
H_2S production from cysteine	+
Urease test	−
Indole production	−
Fluorescent pigment production (on Kings Medium B)	−
Tolerance to 3% NaCl	+
Pectolytic activity	+

4.2. Automated Biolog identification system

Cultures to be identified can also be submitted to Biolog tests for Gram-negative bacteria. The metabolic fingerprint of *X. axonopodis* pv. *allii* is provided in Table 2. The new version (third generation) Biolog GENIII 96 microplate allows rapid identification of isolated bacteria (both Gram-negative and Gram-positive) using the same microplate. The identification system is based on 94 phenotypic tests: 71 carbon source utilization tests and 23 chemical sensitivity tests including biochemical and physiological properties such as pH, salt, lactic acid tolerance and antibiotics. Every species tested creates a unique 'phenotypic fingerprint' which is automatically compared with a database of 1200 aerobic species.

The microplate and the program are commercially available (Biolog, Omnilog, US).

The manufacturer's instructions should be followed for automatic identification of suspected strains.

Table 2. Percentages of metabolic activities of 33 *Xanthomonas axonopodis* pv. *allii* strains with 95 carbon sources of the Biolog GN microplate

	1*	2	3	4	5	6	7	8	9	10	11	12
A	Water	0	100	100	100	70	0	100	0	6	0	100
		(−)	(+)	(+)	(+)	(v)	(−)	(+)	(−)	(−)	(−)	(+)
B	0	100	70	100	97	100	0	9	100	100	0	100
	(−)	(+)	(v)	(+)	(+)	(+)	(−)	(−)	(+)	(+)	(−)	(+)
C	73	0	94	39	0	0	100	100	33	0	100	100
	(v)	(−)	(+)	(v)	(−)	(−)	(+)	(+)	(v)	(−)	(+)	(+)
D	85	100	70	3	0	0	0	0	0	58	21	0
	(v)	(+)	(v)	(−)	(−)	(−)	(−)	(−)	(−)	(v)	(v)	(−)
E	0	0	100	100	15	18	91	79	0	0	0	100
	(−)	(−)	(+)	(+)	(v)	(v)	(+)	(v)	(−)	(−)	(−)	(+)
F	100	76	0	100	97	100	100	18	58	100	67	100
	(+)	(v)	(−)	(+)	(+)	(+)	(+)	(v)	(v)	(+)	(v)	(+)
G	6	42	64	3	0	94	0	0	100	91	0	0
	(−)	(v)	(v)	(−)	(−)	(+)	(−)	(−)	(+)	(+)	(−)	(−)
H	0	3	3	0	0	0	0	0	88	12	18	3
	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(v)	(v)	(v)	(−)

*The names of the carbon sources used, as indicated by letters (rows) and numbers (columns), are those of the Biolog GN microplate assay.(−), 0–10% of strains positive; (+), 90–100% of strains positive; (v), 11–89% of strains positive.

4.3. Molecular tests

4.3.1. PCR

A suspension containing approximately 10^8 cfu mL^{-1} in molecular-grade sterile water is prepared from a 24-h growing culture on YPGA. No DNA extraction is required when pure cultures are used but cell lysis by boiling, as described in Appendix 2.1.1, should be performed. Appropriate PCR[2] procedures are applied to generate specific amplicons targeting *PilW*/*PilX* and *avrRxv* avirulence genes in *X. axonopodis* pv. *allii* as described in Appendix 2 (Robène-Soustrade *et al.*, 2010) and Appendix 3 (Robene *et al.*, 2015). In the conventional PCR test, the first PCR round of the duplex nested-PCR is performed. A few strains mostly belonging to *X. axonopodis* genetic group 9.2 *sensu* Rademaker produce amplicons of the same size. The probability of finding these pathovars on *A. cepa* L. plants or seeds is negligible because the capacity to induce symptoms on onion plants is specific to *X. axonopodis* pv. *allii* strains. Nevertheless, the presence of signature sequences for most of the strains can be used to differentiate the other pathovars from *X. axonopodis* pv. *allii* if a false-positive signal is suspected (Robène-Soustrade *et al.*, 2010; Appendix 2).

4.3.2. DNA sequencing

Comparisons of sequenced PCR products amplified from selected housekeeping genes allow identification of *X. axonopodis* pv. *allii* (and distinction from other *Xanthomonas* pathogens). For example, all isolates of *X. axonopodis* pv. *allii* tested to date were differentiated from other taxa according to partial *gyrB* gene sequencing using the method of Parkinson *et al.* (2009). Other genes used are *atpD*, *dnaK* and *efp* (Bui Thi Ngoc *et al.*, 2010).

4.3.3. Genotyping techniques

Although less easy to implement than sequencing, rep-PCR (Louws *et al.*, 1994; Gent *et al.*, 2004, 2005) and AFLP (Vos *et al.*, 1995; Roumagnac *et al.*, 2004a; Bui Thi Ngoc *et al.*, 2010) have been shown to be highly reliable for the identification of xanthomonad strains at the species and infraspecies level (e.g. genetic clusters of *X. axonopodis*), including comprehensive collections of *X. axonopodis* pv. *allii*. For details on the procedure see EPPO PM 7/100 *rep-PCR tests for identification of bacteria*.

4.4. Pathogenicity tests

Methods for inoculation are presented in Appendix 4.

5. Reference material

Pathotype strain CFBP 6107 (=ICMP 17031 = MAFF 311173).

A draft genome sequence of strain CFBP 6369 is publicly available (Gagnevin *et al.*, 2014).

[2]The bacterial blight onion pathogen primers and their use in diagnostic tests are protected by CIRAD's French patent: publication no. FR 2 951 459, date 22-04-2011. This patent was extended by PCT (Europe and USA) under the number PCT/IB2010/054774, date 21-10-2010. The patent was published in Europe under the no. EP 2491136, date 29-08-2012, and in the USA under the no. US 2013/0130239. No express or implied rights for their use are provided under any patent applications, trade secrets or other proprietary rights via this publication. Those desiring to use or license the patent rights are asked to contact the CIRAD to request permission to do so at the following address: Office of Technology Transfer and Development, TA 40/PS1 Boulevard de la Lironde 34398 Montpellier Cedex 5, France (or e-mail: valobios@cirad.fr).

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 criteria

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

8. Further information

Further information on this organism can be obtained from:

CIRAD, UMR PVBMT, Pôle de Protection des Plantes, 7 chemin de l'IRAT, 97410 Saint Pierre, Réunion Island (FR). Fax: +262 262 499 293, email: isabelle.robene@cirad.fr or olivier.pruvost@cirad.fr

Anses, National Plant Health Laboratory, Unit for Tropical Pests and Diseases, Pôle de Protection des Plantes, 7 chemin de l'IRAT, Ligne Paradis, 97410 Saint Pierre, Reunion Island (FR). E-mail: saint-pierre.lsv@anses.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 diagnostics@eppo.int

10. Protocol revision

An annual review process is in place to identify the need for revision of Diagnostic Protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press this will also be marked on the website.

Acknowledgements

This protocol was originally drafted by I. Robène-Soustrade and O. Pruvost, CIRAD, UMR PVBMT (FR) and A. Chabirand, Anses (FR). It was reviewed by the Panel on Diagnostics in Bacteriology.

References

Alvarez AM, Buddenhagen IW, Buddenhagen ES & Domen HY (1978) Bacterial blight of onion, a new disease caused by *Xanthomonas* sp. *Phytopathology* **68**, 1132–1136.

Audy P, Braat CE, Saindon G, Huang HC & Laroche A (1996) A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blights in bean seed. *Phytopathology* **86**, 361–366.

Bowen P, Gibbs HAA & O'Garro LW (1998) Garlic, chives, challot and leek are alternative hosts to *Xanthomonas campestris*, the pathogen of leaf blight of onion *Allium cepa* L. In *7th Int. Cong. Plant Pathol.*, pp. 6–96. ISPP, Edinburgh (GB).

Bui Thi Ngoc L, Vernière C, Jouen E, Ah-You N, Lefeuvre P, Chiroleu F *et al.* (2010) Amplified fragment length polymorphism and multilocus sequence analysis-based genotypic relatedness among pathogenic variants of *Xanthomonas citri* pv. *citri* and *Xanthomonas campestris* pv. *bilvae*. *International Journal of Systematic and Evolutionary Microbiology* **60**, 515–525.

Chandelier A, Planchon V & Oger R (2010) Determination of cycle cut off in real-time PCR for the detection of regulated plant pathogens. *EPPO Bulletin* **40**, 52–58.

EPPO (2014) PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. *EPPO Bulletin* **44**, 117–147.

EPPO (2016a) *Xanthomonas axonopodis* pv. *allii*. *EPPO Bulletin* **46**, 4–7.

EPPO (2016b) EPPO Global Database available at https://gd.eppo.int/ (last accessed 1 June 2016).

Gagnevin L, Bolot S, Gordon JL, Pruvost O, Vernière C, Robène I *et al.* (2014) Draft genome sequence of *Xanthomonas axonopodis* pv. *allii* strain CFBP 6369. *Genome Announcements* **2**, e00727-14.

Gent DH, Schwartz HF, Ishimaru CA, Louws FJ, Cramer RA & Lawrence CB (2004) Polyphasic characterization of *Xanthomonas* strains from onion. *Phytopathology* **94**, 184–195.

Gent DH, Al-Saadi A, Gabriel DW, Louws FJ, Ishimaru CA & Schwartz HF (2005) Pathogenic and genetic relatedness among *Xanthomonas axonopodis* pv. *allii* and other pathovars of *X. axonopodis*. *Phytopathology* **95**, 918–925.

Gent DH & Schwartz HF (2005) Effect of nitrogen fertilization and seed contamination on epiphytic populations of *Xanthomonas axonopodis* pv. *allii* and developement of *Xanthomonas* leaf blight of onion. *Plant Health Progress*. doi:10.1094/PHP-2005-0331-01-RS.

Humeau L, Roumagnac P, Picard Y, Robène-Soustrade I, Chiroleu F, Gagnevin L *et al.* (2006) Quantitative and molecular epidemiology of bacterial blight of onion in seed production fields. *Phytopathology* **96**, 1345–1354.

Isakeit T, Miller ME, Barnes LW, Dickstein ER & Jones JB (2000) First report of leaf blight of onion caused by *Xanthomonas campestris* in the Continental United States. *Plant Disease* **84**, 201.

Kadota I, Uehara K, Shinohara H & Nishiyama K (2000) Bacterial blight of Welsh onion: a new disease caused by *Xanthomonas campestris* pv. *allii* pv. nov. *Journal of General Plant Pathology* **66**, 310–315.

Louws FJ, Fulbright DW, Stephens CT & De Bruijn FJ (1994) Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environment Microbiology* **60**, 2286–2295.

Neto JR, Malavolta VA Jr, Cardelli MA & Sinigaglia C (1987) Ocorrencia de uma nova doença bacteriana em cebola, no estado de Sao Paulo. *Summa Phytopathologica* **13**, 10.

Nunez JJ, Gilbertson RL, Meng X & Davis RM (2002) First report of *Xanthomonas* leaf blight of onion in California. *Plant Disease* **86**, 330.

O'Garro LW & Paulraj LP (1997) Onion leaf blight caused by *Xanthomonas campestris*: Alternative hosts and resistant onion genotypes. *Plant Disease* **81**, 978–982.

Parkinson N, Cowie C, Heeney J & Stead D (2009) Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology* **59**, 264–274.

Paulraj L & O'Garro LW (1993) Leaf blight of onions in Barbados caused by *Xanthomonas campestris*. *Plant Disease* **77**, 198–201.

Picard Y, Roumagnac P, Legrand D, Humeau L, Robène-Soustrade I, Chiroleu F *et al.* (2008) Polyphasic characterization of *Xanthomonas axonopodis* pv. *allii* associated with outbreaks of bacterial blight on three *Allium* species in the Mascarene archipelago. *Phytopathology* **98**, 919–925.

Pruvost O, Roumagnac P, Gaube C, Chiroleu F & Gagnevin L (2005) New media for the semi-selective isolation and enumeration of *Xanthomonas campestris* pv. *mangiferaeindicae*, the causal agent of mango bacterial black spot. *Journal of Applied Microbiology* **99**, 803–815.

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L *et al.* (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* **50**, 665–677.

Rademaker JLW, Louws FJ, Schultz MH, Rossbach U, Vauterin L, Swings J *et al.* (2005) A comprehensive species to strain taxonomic framework for *Xanthomonas*. *Phytopathology* **95**, 1098–1111.

Robene I, Perret M, Jouen E, Escalon A, Maillot MV, Chabirand A *et al.* (2015) Development and validation of a real-time quantitative PCR assay to detect *Xanthomonas axonopodis* pv. *allii* from onion seed. *Journal of Microbiological Methods* **114**, 78–86.

Robène-Soustrade I, Legrand D, Gagnevin L, Chiroleu F, Laurent A & Pruvost O (2010) Multiplex nested PCR for detection of *Xanthomonas axonopodis* pv. *allii* from onion seeds. *Applied and Environmental Microbiology* **76**, 2697–2703.

Roumagnac P, Gagnevin L & Pruvost O (2000) Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium. *European Journal of Plant Pathology* **106**, 867–877.

Roumagnac P, Gagnevin L, Gardan L, Sutra L, Manceau C, Dickstein ER *et al.* (2004a) Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. *International Journal of Systematic and Evolutionary Microbiology* **54**, 15–24.

Roumagnac P, Pruvost O, Chiroleu F & Hughes G (2004b) Spatial and temporal analyses of bacterial blight of onion caused by *Xanthomonas axonopodis* pv. *allii*. *Phytopathology* **94**, 138–146.

Sanders FH, Langston DB Jr, Brock JH, Gitaitis RD, Curry DE & Torrance RL (2003) First report of a leaf blight of onion caused by *Xanthomonas* spp. in Georgia. *Plant Disease* **87**, 749.

Schwartz HF & Otto K (2000) First report of leaf blight of onion caused by *Xanthomonas campestris* in Colorado. *Plant Disease* **84**, 922.

Serfontein JJ (2001) *Xanthomonas* blight of onion in South Africa. *Plant Disease* **85**, 442.

Swings J, Vauterin L & Kersters K (1993) The bacterium *Xanthomonas*. In *Xanthomonas* (Ed. Swings J & Civerolo EL), pp. 121–156. Chapman & Hall, London, Glasgow, New York, Tokyo, Melbourne, Madras.

Thomas WDJ & Weinhold AR (1953) *Xanthomonas striaformans*, a new bacterial pathogen of onion. *Journal of the Colorado-Wyoming Academy of Science* **4**, 23.

Trujillo G & Hernandez Y (1999) Identificacion de bacterias fitopatogenas en semillas de plantas cultivadas. In *XVI Congreso Venezolano de Fitopatologia*. Sociedad Venezolano de Fitopatologia, Barquisimeto (VE).

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407–4414.

Appendix 1 – Preparation of media

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

All prepared culture media should ideally be plated a few days before the tests are performed. Media may be stored away from light in a dry place at 4°C for a maximum of 1 month. Plates should be stored upside down to avoid the presence of water on agar and allowed to dry in a sterile laminar flow hood before being used.

1. Media

Yeast peptone glucose agar (YPGA).

Yeast extract	7.0 g
Bactopeptone	7.0 g
Glucose	7.0 g
Microbiological-grade agar	18.0 g
Distilled water	1.0 L

pH is adjusted to 7.2.

NCTM1 medium (Roumagnac *et al.*, 2000).

Yeast extract	7.0 g
Bactopeptone	7.0 g
Glucose	7.0 g
Microbiological-grade agar	18.0 g
Distilled water	1.0 L

The following antibiotics and other components are added after autoclaving.

Pivmecillinam	100.0 mg (to dissolve in water)
Cephalexin	30.0 mg (to dissolve in NaOH 1N)
Trimethoprim	3.0 mg (to dissolve in 95% ethanol)
Neomycin	10.0 mg (to dissolve in water)
Propiconazole	20.0 mg (to dissolve in water)

The pH is adjusted to 7.2.

Some discrepancies in the plating efficiency of NCTM1 have been reported (Roumagnac *et al.*, 2000; Gent & Schwartz, 2005) suggesting the importance of testing the efficiency of media prior to experiments. Pivmecillinam can be difficult to find in some countries. It can be obtained from Sigma, for example. Alternative semi-selective media (modified MXP and KC) have also been used for isolating *Xanthomonas axonopodis* pv. *allii* from diseased plant material but these were not evaluated for detecting the pathogen from seed.

Modified MXP medium (Gent & Schwartz, 2005).

K_2HPO_4	0.8 g
KH_2PO_4	0.6 g
Yeast extract	0.7 g
Soluble potato starch	8.0 g
KBr	10.0 g
Glucose	1.0 g
Microbiological-grade agar	15.0 g

Distilled water 1.0 L.

The following antibiotics and other components are added after autoclaving (amounts for 1 L).

Cycloheximide	50.0 mg (to dissolve in 95% ethanol)
Cephalexin	50.0 mg (to dissolve in NaOH 1N)
Kasugamycin	50.0 mg (to dissolve in water)
Gentamycin	2.0 mg (to dissolve in water)
Methyl violet 2B	30.0 µL of a 1% solution in 20% ethanol
Methyl green	60.0 µL of a 1% solution in water

KC medium (Pruvost *et al.*, 2005).

Yeast extract	7.0 g
Bactopeptone	7.0 g
Glucose	7.0 g
Microbiological grade agar	18.0 g

Distilled water 1.0 L.

The following antibiotics and other components are added after autoclaving the YPGA medium (amounts for 1 L).

Kasugamycin	20.0 mg (to dissolve in water)
Cephalexin	40.0 mg (to dissolve in NaOH 1N)
Propiconazole	20.0 mg

The pH is adjusted to 7.2.

Appendix 2 – Duplex nested-PCR (Robène-Soustrade *et al.*, 2010).

1. General information

1.1 This test is described in Robène-Soustrade *et al.* (2010). The duplex nested-PCR protocol is suitable for detecting and/or identifying *Xanthomonas axonopodis* pv. *allii* strains pathogenic to *Allium* species and has been developed particularly to detect the pathogen in onion seeds.

1.2 The PCR primers are designed to target two sequences showing sequence similarity with bacterial genes encoding the PilW/PilX proteins (PIL marker) and the *avrRxv* avirulence gene (AVR marker).

1.3 Amplicon sizes are 995 bp (AVR marker) and 697 bp (PIL marker) for the first-round PCR, and 401 bp (AVR marker) and 447 bp (PIL marker) for the nested step.

Note: for the PIL marker a 3-bp deletion was observed for some strains (size: 694 bp and 444 bp for the first step and nested amplicons, respectively).

1.4 Identification of *X. axonopodis* pv. *allii* strains pathogenic to *Allium* species is achieved with the first PCR round of the duplex nested-PCR (followed if necessary by enzymatic restriction).

The primers used in the first round are:

Pxaa1U (5′-GGCTCTAATACGACGTTGACGAT-3′)
Pxaa1L (5′-AAATTCATGCGCGTTTTCAATAG-3′)
Pxaa2U (5′-CTCAAGCAGCAGTCGTTTTCA-3′)
Pxaa2L (5′-ATGCTTCGATTGACATGCTGT-3′).

For the detection of *X. axonopodis* pv. *allii* from asymptomatic plant material, the nested-PCR protocol is required because the second PCR round greatly increases sensitivity.

The primers used in the second round are:

Nxaa1U (5′-TTACGTCGCAAACAATCCAGATA-3′)
Nxaa1L (5′-GGGCACCATTGACATTATCAGTT-3′)
Nxaa2U (5′-ATGCCTGGTTTCGTGAA-3′)
Nxaa2L (5′-CTACGGCTCAGCGACTC-3′).

1.5 Among various Taq polymerases tested, GoTaq® G2 Flexi DNA Polymerase (Promega) was particularly successful. Another enzyme, Red Diamond Taq®, has not been validated with this PCR protocol. For a mix containing the four nucleotides, 10 mM each (New England Biolabs, Ozyme), 1.25 units of GoTaq® Flexi DNA Polymerase was used. The 5X Green GoTaq® Flexi Buffer concentrated buffer and 25 mM $MgCl_2$ supplied with the Flexi DNA Polymerase were used. The 9600, 9700 and Veriti™ thermal cyclers systems (Applied Biosystems) were successfully used. All reactions were performed with HPLC grade water. The use of nested-PCR protocols gives an increased risk for false positives due to the risk of contamination with amplicons produced in the first round of amplification.

A commercial all-inclusive diagnostic kit based on this duplex nested-PCR is also available (Qualiplante, Clapiers, FR) and has been validated in an inter-laboratory comparison (see Section 4.4).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Pure cultures

Suspend a single colony of a fresh pure culture in 1 mL of sterile deionized water, boil for 2 min and immediately chill on ice for 1 min, and vortex vigorously. Samples can be stored at −18°C or below.

2.1.2 Seeds

Seed samples (10 g each) are soaked in 50 mL of 0.01 M sterile Sigma 7–9 buffer (pH 7.2) for 48 h at approximately 4°C and homogenized (for 30 s at low speed) using a stomacher (Stomacher®400 from Seward Medical) or equivalent.

Nucleic acid extraction is performed on the macerates.

Two DNA extraction protocols can be used.

(i) A quick alkaline DNA extraction method adapted from Audy *et al.* (1996). Four millilitres of macerate are centrifuged at 10 000 *g* for 30 min and pellets are dissolved in

100 µL of 0.5 N NaOH with 0.5% polyvinylpyrrolidone (PVP). The lysate (5 µL) is transferred to 1.5-mL microfuge tubes containing 495 µL of 20 mM Tris–HCL, pH 8.0.

(ii) DNA extraction with a commercial kit (DNeasy®Plant Mini Kit, Qiagen). Follow the protocol provided by the manufacturer, starting by adding 400 µL of buffer AP1 to the homogenate. Elute twice with 50 µL of buffer AE. DNA thus extracted can be stored at −18°C or below.

2.1.3 Detection from infected tissues

DNA extraction is then performed using a commercial kit (DNeasy®Plant Mini Kit, Qiagen) following the manufacturer's instructions.

2.2. Conventional PCR

2.2.1 First PCR round

Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	to make up to 25 µL	NA
PCR buffer (Green GoTaq® Flexi Buffer, Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	1.5	1.5 mM
dNTP (NED)	10 mM	0.25	100 µM
Pxaa 1 U	5 µM	1	0.2 µM
Pxaa 1 L	5 µM	1	0.2 µM
Pxaa 2 U	5 µM	1	0.2 µM
Pxaa 2 L	5 µM	1	0.2 µM
Polymerase (GoTaq® G2 Flexi DNA Polymerase, Promega)	5 U μL^{-1}	0.2	1 U
Genomic DNA extract		See note†	
Total		25	

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

†For identification purposes the template DNA is 1 µL of bacterial suspension. For seed extracts obtained with the alkaline procedure (Audy *et al.*, 1996), one aliquot of 5 µL is used as the template. For seed or plant extracts obtained with the commercial kit (DNeasy®Plant Mini Kit, Qiagen), one aliquot of 2 µL is used as the template.

PCR conditions

The amplification program includes denaturation at 94°C for 5 min, 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 2 min, and a final extension step at 72°C for 5 min.

PCR amplification products are detected by electrophoresis in 1% agarose (Seakem LE agarose, FMC Bioproducts) and are stained with ethidium bromide.

For the next step, first-round amplicons are diluted 1/100 in deionized water prior to nested-PCR amplification for symptomatic plant material (detection) or bacterial suspensions (identification), in order to prevent inhibition of the PCR due to high concentrations of DNA in the template.

2.2.2 Nested-PCR

Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	13.05	NA
PCR buffer (Green GoTaq® Flexi Buffer, Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	1.5	1.5 mM
dNTP (NED)	10 mM	0.25	100 µM
Nxaa 1 U	5 µM	1	0.2 µM
Nxaa 1 L	5 µM	1	0.2 µM
Nxaa 2 U	5 µM	1	0.2 µM
Nxaa 2 L	5 µM	1	0.2 µM
Polymerase (GoTaq® G2 Flexi DNA Polymerase, Promega)	5 U μL^{-1}	0.2	1 U
Subtotal		24	
First-round DNA amplicon		1	
Total		25	

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

PCR tubes containing the first reaction amplicons must be opened with extreme care to avoid creation of aerosols which would cause contamination with amplification products.

PCR conditions

Use the following PCR cycling parameters: denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 40 s, and a final extension at 72°C for 5 min.

PCR amplification products are detected by electrophoresis in 3% agarose (Seakem LE agarose, FMC Bioproducts) and are stained with ethidium bromide.

2.3. Restriction fragment length polymorphism (RFLP) reaction

2.3.1 Preparation of DNA solution

RFLP analyses are performed on DNA amplicons obtained after the first or the second nested-PCR step (DNA concentration around 20 ng μL^{-1}). These DNA solutions can be stored at less than −18°C.

2.3.2 Enzymatic digestion

*Cfr*I (*Eae*I)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10	N.A.
RFLP buffer (Ozyme)	10×	1.5	1×
*Cfr*I (*Eae*I) (Ozyme)	3 U µL^{-1}	1	3 U
(purified) PCR product	20 ng µL^{-1}	2.5	
Total		15	

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

Incubation temperature: 37°C overnight.

Denaturation time and temperature: none.

*Nhe*I

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	7	N.A.
RFLP buffer (Ozyme)	10×	1	1×
*Nhe*I (Ozyme)	1 U µL^{-1}	1	1 U
Subtotal		9	
(purified) PCR product	20 ng µL^{-1}	1	
Total		10	

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

Incubation temperature 37°C overnight.

Denaturation time and temperature: none.

2.3.3 Interpretation of band patterns

RFLP analysis can be performed on the first-round amplicons in conditions to distinguish *X. axonopodis* pv. *allii* strains from *X. axonopodis* pv. *vesicatoria* and *X. axonopodis* pv. *begoniae* strains.

*Cfr*I does not cleave the AVR marker amplification product generated from strains of *X. axonopodis* pv. *allii* (995 bp), while two fragments (882 bp and 113 bp) are obtained for *X. axonopodis* pv. *begoniae* strains.

*Nhe*I generates two DNA fragments from the PIL marker amplicon for *X. axonopodis* pv. *vesicatoria* strains (590 bp and 107 bp), while no cleavage occurs for *X. axonopodis* pv. *allii* strains (697 bp).

RFLP analysis can be performed also on the nested product to distinguish *X. axonopodis* pv. *allii* strains from *X. axonopodis* pv. *vesicatoria* and *X. axonopodis* pv. *begoniae* strains.

*Cfr*I does not cleave the AVR marker amplification product generated from strains of *X. axonopodis* pv. *allii* (401 bp), while two fragments (346 bp and 55 bp) are obtained for *X. axonopodis* pv. *begoniae* strains.

*Nhe*I generates two DNA fragments from the PIL marker amplicon for *X. axonopodis* pv. *vesicatoria* strains (363 bp and 84 bp), while no cleavage occurs for *X. axonopodis* pv. *allii* strains (447 bp).

3. Essential information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of target nucleic acid extraction and amplification.

3.1.1 Identification

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR 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, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

The PAC should preferably be near to the limit of detection.

A pure culture of the *X. axonopodis* pv. *allii* reference strain, extracted with the thermal lysis method, can be used as positive control (CFBP 6107).

3.1.2 Detection

Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or 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. infected plant material or synthetic control prepared by adding pure culture of the *X. axonopodis* pv. *allii* reference strain to a macerate of healthy seeds).

3.2. Interpretation of results

Verification of the controls

NIC and NAC should produce no amplicons.

PIC and PAC should produce amplicons of the expected sizes: first step 995 bp (AVR) or 694–697 bp (PIL), or both amplicons observed; nested step 401 bp (AVR) or 444–447 bp (PIL) or both amplicons observed.

When these conditions are met

The PCR test (identification or detection) is negative if the expected amplicons are not observed or if the observed amplicons are not of the expected sizes.

The PCR identification test is positive if one amplicon of the expected size is observed, 995-bp amplicon (AVR) or 694–697 bp (PIL), or both amplicons are observed.

The PCR detection test is positive if one amplicon of the expected size, 401-bp amplicon (AVR) or 444- to 447-bp amplicon (PIL), or both amplicons are observed.

No cleavage is observed when the RFLP analysis is performed on the PIL marker amplicon using *Nhe*I (first round 697 bp, nested round 447 bp) or on the AVR marker amplification product using *Cfr*I (first round 995 bp, second round 401 bp).

Tests should be repeated if any contradictory or unclear results are obtained and should be coupled with other tests based on different biological properties.

4. Performance criteria available

Validation experiments were performed according to both the protocol of PM 7/98 (2) (EPPO, 2014) and European Standard EN ISO 16140:2003 (Robene *et al.*, 2015). Anses (FR) and CIRAD (FR) also conducted an inter-laboratory comparison (December, 2014).

4.1. Analytical sensitivity data

Analytical sensitivity was determined using healthy onion seeds artificially contaminated with serially 10-fold diluted suspensions of *X. axonopodis* pv. *allii* strains CFBP 6385, CFBP 6366 or CFBP 6367 and CFPB 6107 (1×10^2 to 1×10^7 cfu $mL^{-1,}$ corresponding to 5×10^2 to 5×10^7 cfu g^{-1}). Two independent dilution series were performed for each strain and six tests of duplex nested-PCR were performed on each series, with two replicates for each dilution. 100% of samples were detected as positive at a concentration of 1×10^3 cfu mL^{-1} (detection level 100%). At 1×10^2 cfu mL^{-1}, 81% samples tested positive.

4.2. Analytical specificity data

Inclusivity was evaluated by testing 79 strains of *X. axonopodis* pv. *allii* pathogenic to onion and/or other *Allium* species isolated in different regions of the world (suspensions adjusted to 1×10^4 cfu mL^{-1}). See the EPPO validation database http://dc.eppo.int/validationlist.php for the list of strains used for the validation. The internal primers used in the duplex nested-PCR test directed amplification for all 79 *X. axonopodis* pv. *allii* strains tested, resulting in a 401-bp amplicon (AVR marker), a 444- to 447-bp amplicon (PIL marker) or both amplicons, depending on the strain, giving 100% inclusivity.

Exclusivity was tested on a collection of 135 non-target strains (suspensions adjusted to 1×10^6 cfu mL^{-1}). See the EPPO validation database http://dc.eppo.int/validationlist.php for the list of strains used for the validation resulting in 82.2% exclusivity (89% exclusivity when considering the taxa). No amplification was obtained for any unrelated phytopathogenic bacteria or for any saprophytic bacteria commonly isolated from onion leaves and seeds. Most *Xanthomonas* strains also did not produce amplicons, except for a few strains classified in *X. axonopodis* genetic subgroup 9.1 or 9.2 (Rademaker *et al.*, 2005): *X. axonopodis* pv. *begoniae, X. axonopodis* pv. *vesicatoria*, *X. axonopodis* pv. *citrumelo*, *X. axonopodis* pv. *cassavae, X. axonopodis* pv. *desmodii*, *X. axonopodis* pv. *desmodiiganetici*, *X. axonopodis* pv. *phyllanthi*, *X. axonopodis* pv. *tamarindi* and *X. axonopodis* pv. *lespedezae*, and also two strains of *X. vasicola* pv. *musacearum*. Depending on the pathovar, one or both markers were observed. The probability of finding these pathovars on onion plants or seeds is negligible because the capacity to induce symptoms on onion plants is specific to *X. axonopodis* pv. *allii* strains. Nevertheless, the presence of signature sequences for most of the strains can be used to differentiate the other pathovars from *X. axonopodis* pv. *allii* (Robène-Soustrade *et al.*, 2010). Interestingly, enzymatic digestion with *Nhe*I of the first-round or nested amplicons from the PIL marker allows one to distinguish *X. axonopodis* pv. *allii* from *X. axonopodis* pv. *vesicatoria.* Also, enzymatic digestion of the first- or second-round amplicons from the AVR marker with *Cfr*I allows differentiation of *X. axonopodis* pv. *allii* from *X. axonopodis* pv. *begoniae.*

4.3. Data on repeatability

Repeatability was calculated using data on determination of analytical sensitivity (Section 4.1) for concentrations ranging from 1×10^2 to 1×10^4 cfu mL^{-1} (96 replicates for each concentration, regardless of the strain). We calculated the accordance (the percentage chance of finding the same result, i.e. both negative or both positive, from two identical test portions analysed in the same laboratory, under repeatability conditions) using the following formula: $(pr/t)^2 + (nr/t)^2$, where pr and nr are the number of positive and negative responses, respectively, and t is the total number of responses. From 1×10^4 to 1×10^3 cfu mL^{-1} accordance was 100%. At 1×10^2 cfu mL^{-1} accordance was 70%.

4.4 Data on reproducibility

A collaborative study involving five laboratories has been conducted in order to test the performance of the nested-PCR test. The 10 samples tested were DNA extracts from pure culture of target and non-target strains or DNA extracts from healthy seed spiked with different concentrations of bacteria (1×10^3 to 1×10^7 cfu mL^{-1}) and controls. The main reagents were sent to laboratories but the equipment and operators were different. Despite these differences, 100% concordance was obtained for this evaluation (the concordance is the percentage of all pairings giving the same results on all possible pairings of data).

The commercial diagnostic kit based on the duplex nested-PCR was tested in the same collaborative study and the same results were obtained in terms of specificity,

sensitivity and reproducibility as for the duplex nested-PCR test.

4.5 Detection on naturally infected seed

Determination of the detection threshold according to the contamination rate was determined by repeatedly 'diluting' naturally infected seed with healthy seed: theoretical contamination rates of 1/10 000, 1/21 000, 1/30 000 and 1/138 000 in Robène-Soustrade *et al.* (2010) and theoretical contamination rates of 1.75/10 000, 1/30 000, 1/60 000 and 1/120 000 in Robene *et al.* (2015). At least two independent analyses of 35 seed lots were performed for each contamination rate. Seed contamination rate (cr) derived from duplex nested-PCR analyses was calculated according to the formula $cr = 1 - (A/B)^{1/n}$, where A is the number of samples in which the pathogen was not detected, B is the total number of samples analysed and n is the number of seeds per replicate sample ($n = 245$; Robène-Soustrade *et al.*, 2010). The sensitivity threshold of the duplex nested test was found to be 1 infected seed in 27 340 seeds (Robène-Soustrade *et al.*, 2010) and 1 infected seed in 1/27,260 (Robene *et al.*, 2015).

Appendix 3 – Triplex real-time quantitative PCR (Robene *et al.*, 2015)

1. General information

1.1. This test, described in Robene *et al.* (2015), is a 5′ nuclease real-time PCR protocol (Taqman®) developed to screen onion seed lots for the detection and identification of *Xanthomonas axonopodis* pv. *allii*. This real-time PCR test is an improvement on the conventional PCR test (Robène-Soustrade *et al.*, 2010) and includes an internal control (amplifying plant DNA) to give a more rapid method adapted to high-throughput screening.

1.2. The real-time PCR test targets three DNA sequences: (i) the two *X. axonopodis* pv. *allii* genes *avrRxv* (AVR) and *pilW* (PIL); (ii) the 5.8S rDNA sequence that is highly conserved in plants, and particularly in different *Allium* species including *Allium cepa*.

1.3. The amplicon sizes are 74 bp (AVR), 56 bp (PIL) and 69 bp (5.8S).

1.4. Oligonucleotides

Target: *X. axonopodis* pv. *allii*
Xaa-pil MGB (Vic®) 5′-TGGTGGCCTCAGGAG-3′
Xaa-pilF 5′-CACGACCACTGCTGGAACA-3′
Xaa-pilR 5′-CATATCGACCGGCAAGGTTT-3′
Xaa-avr MGB (Fam™) 5′-TGCTGAGTCAGCCTC-3′
Xaa-avrF 5′-TCGAGCAGCAGTCGTTTTCA-3′
Xaa-avrR 5′-GGAGGCGTAGACGCCTTACT-3′
Target: *Allium* sp.
Xaa-5.8S MGB (Ned™) 5′-ATCCCGTGAACCATCG-3′
Xaa-5.8SF 5′-GCGAAATGCGACACTTGGTGTGA-3′
Xaa-5.8SR 5′-GCGCAACTTGCATTCAAAGA-3′

1.5. Real-time PCR reactions were performed using the StepOnePlus system (Applied Biosystems, Courtaboeuf, FR) with reagents recommended by the supplier (Master Mix, primers and probes Taqman®, Applied Biosystems), but detection of *X. axonopodis* pv. *allii* was also successfully achieved using other real-time PCR equipment (see Section 4.4).

1.6. The cycle cut-off (i.e. the number of PCR cycles above which any sample response is considered to be a false positive) was estimated statistically at 36.3 considering a risk of false positives of 1% (according to Chandelier *et al.*, 2010).

2. Methods

2.1 Nucleic acid extraction and purification

Use the same procedure as in Appendix 2, except that nucleic acid extraction is performed only using a commercial kit (DNeasy®Plant Mini Kit, Qiagen).

2.2 Real-time PCR reaction

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	1.91	N.A.
Taqman Universal PCR Master Mix (Applied Biosystems)	2×	10	1×
Xaa-avrF	20 µM	1.2	1.2 µM
Xaa-avrR	20 µM	1.2	1.2 µM
Xaa-avr MGB probe (Fam™)	10 µM	0.1	0.05 µM
Xaa-pilF	20 µM	1.6	1.6 µM
Xaa-pilR	20 µM	1.6	1.6 µM
Xaa-pil MGB probe (Vic®)	10 µM	0.15	0.075 µM
Xaa-5.8SF	20 µM	0.07	0.07 µM
Xaa-5.8SR	20 µM	0.07	0.07 µM
Xaa-5.8S MGB probe (Ned™)	10 µM	0.1	0.05 µM
Subtotal		18	
DNA		2	
Total		20	

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

2.2.2. PCR conditions

The real-time PCR cycling conditions included a step at 50°C for 2 min, an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation and

annealing/elongation for 15 s at 95°C and 1 min at 60°C, respectively. Automatic analyses were performed using the StepOnePlus software version 2.2.2.

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.

3.1.1 Identification

A negative amplification control (NAC) containing no target nucleic acid 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.

A positive amplification control (PAC) containing nucleic acid to monitor the efficiency of the amplification.

A pure culture, extracted using the thermal lysis method, of the *X. axonopodis* pv. *allii* reference strain can be used as positive control (CFBP 6107).

3.1.2 Detection

A negative isolation control (NIC) to monitor contamination during nucleic acid extraction and subsequent amplification. This consists of performing a nucleic acid extraction using a known 'blank' sample that does not include target nucleic acid (e.g. uninfected plant material or clean extraction buffer).

A positive isolation control (PIC). This consists of performing a nucleic acid extraction using a known sample that includes target nucleic acid (e.g. infected plant material or synthetic control prepared by adding pure culture of *X. axonopodis* pv. *allii* reference strain to a macerate of healthy seeds).

In addition to the positive extraction controls, the co-amplification of an internal positive control (5.8S) is used here to monitor each individual sample separately.

3.2 Interpretation of results

The cycle cut-off value is set at 36.3 according to statistical analyses and was obtained using the equipment/materials and chemistry used as described in this Appendix.

The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

The PIC, PAC and 5.8S amplification curves should be exponential.

PIC and PAC should have a Ct value below the cut-off value (36.3).

For each sample the 5.8S value should be as expected (the 5.8S Ct value is usually between 15 and 22).

NAC and NIC should be negative (Ct > cut-off).

When these conditions are met

A sample will be considered positive if it produces an exponential amplification curve and a Ct value < 36.3 for at least one of the bacterial markers (PIL or AVR).

A sample will be considered negative if a Ct value ≥ 36.3 is obtained for both bacterial markers.

Tests should be repeated if any contradictory or unclear results are obtained and should be coupled with other tests based on different biological properties.

4. Performance criteria available

Validation experiments were performed according to both PM 7/98 (2) (EPPO, 2014) and European Standard EN ISO 16140:2003 (Robene *et al.*, 2015). Anses (FR) and CIRAD (FR) also conducted an inter-laboratory comparison (December, 2014).

4.1 Analytical sensitivity data

Analytical sensitivity was determined using healthy onion seeds artificially contaminated with serially 10-fold diluted suspensions of *X. axonopodis* pv. *allii* strains CFBP 6385, CFBP 6366 or CFBP 6367 and CFPB 6107 (1×10^2 to 1×10^7 cfu $mL^{-1,}$ corresponding to 5×10^2 to 5×10^7 cfu g^{-1}). Three independent dilution series were performed for each strain and five tests of duplex nested-PCR were performed on each series, with three replicates for each dilution. DL 100% was obtained at a concentration of 1×10^4 cfu mL^{-1}. At 1×10^3 cfu mL^{-1}, 95% of samples tested positive.

4.2 Analytical specificity data

Inclusivity was evaluated by testing 79 strains of *X. axonopodis* pv. *allii* pathogenic to onion and/or other *Allium* species isolated in different regions of the world (suspensions adjusted to 1×10^4 cfu mL^{-1}). See the EPPO validation database http://dc.eppo.int/validationlist.php for the list of strains used for the validation. All *X. axonopodis* pv. *allii* strains were detected as positive by real-time PCR, resulting in 100% inclusivity.

Means (standard deviation) of Ct values were 31.15 (0.74) and 31.99 (0.63) for AVR and PIL markers, respectively.

Exclusivity was tested on a collection of 135 non-target strains (suspensions adjusted to 1×10^6 cfu mL^{-1}). See the EPPO validation database http://dc.eppo.int/validationlist.php for the list of strains used for the validation. This resulted in 82.2% exclusivity (89% exclusivity considering the taxa). In particular, no amplification product was observed when the real-time PCR test was performed for saprophytic strains isolated from onion or for other bacteria pathogenic to onion. Most *Xanthomonas* strains tested negative, except for 15 strains belonging to *X. axonopodis* genetic group 9.2

sensu Rademaker *et al.* (2005) for which a clear positive signal was observed: *X. euvesicatoria*, *Xanthomonas axonopodis* pv. *citrumelo*, *X. axonopodis* pv. *cassavae*, *Xanthomonas axonopodis* pv. *desmodii*, *X. axonopodis* pv. *desmodiigangetici*, *X. axonopodis* pv. *lespedezae*, *X. axonopodis* pv. *phyllanthi* and *X. axonopodis* pv. *tamarindi*. Depending on the pathovar, one or both markers were amplified. Amplification was also observed for the AVR marker for three strains of *Xanthomonas axonopodis* pv. *begoniae* (genetic group 9.1 *sensu* Rademaker *et al.* 2005) and two strains of *X. vasicola* pv. *musacearum*. These data are entirely in agreement with the *in silico* analysis of sequences available on NCBI databases.

4.3 Data on repeatability

Repeatability was calculated using data on determination of analytical sensitivity (Section 4.1) for concentrations ranging from 1×10^3 to 1×10^4 cfu mL^{-1}.

The accordance (the percentage chance of finding the same result, i.e. both negative or both positive, from two identical test portions analysed in the same laboratory, under repeatability conditions) was calculated according to the following formula: $(pr/t)^2 + (nr/t)^2$, where pr and nr are the number of positive and negative responses, respectively, and t is the total number of responses. The accordance was 100% for bacterial concentrations ranging from 1×10^4 to 1×10^7 cfu mL^{-1}. At a concentration of 1×10^3 cfu mL^{-1} the accordance was 99%.

For quantitative results, intra-test coefficients of variation (Cv) based on Ct mean values ($Cv = \sigma/\mu$), were computed with all triplicates of the same set of DNA samples tested in the same run with concentrations ranging from 1×10^3 to 1×10^7 cfu mL^{-1} (450 sets of triplicates). Inter-test Cv values were calculated from the five runs performed independently for each series (90 sets of five replicates, each replicate being the mean of the DNA sample triplicates). Inter-test Cv values were also calculated among the three independent dilution series (30 sets of 15 replicates, each replicate being the mean of the DNA sample triplicates). Intra-test Cv values ranged from 0.01 to 4.37% with a median of 0.09%. Inter-test Cv values computed on the five independent series ranged from 0.13 to 2.87% with a median of 1.11%. Inter-test Cv values calculated with the three independent series ranged between 1.09 and 5.10% with a median of 2.24%.

4.4 Data on reproducibility

A collaborative study involving five laboratories was conducted in order to test the performance of the triplex qPCR test. The 10 samples tested were DNA extracts from pure cultures of target and non-target strains or DNA extracts from healthy seed spiked with different concentrations of bacteria (1×10^3 to 1×10^7 cfu mL^{-1}). The main reagents were sent to laboratories, but equipment and operators were different and some laboratories also performed comparative experiments using the master mix usually used in their laboratories.

Four different qPCR equipments were used: StepOnePlus™ Real-Time PCR System (Applied Biosystems), ABI PRISM® 7000 or 7500 (Applied Biosystems) or Rotor-Gene Q (Qiagen). For the Rotor-Gene, the fluorophore CY5 replaced NED in the triplex real-time PCR amplification. Taqman® Universal Master Mix (Applied Biosystems) was mainly used. Alternatively, some tests were performed with the QuantiFast multiplex PCR + Rox (Qiagen).

100% concordance was obtained whatever the qPCR equipment used when the Taqman® Universal Master Mix (Applied Biosystems) was used. Some optimization has to be performed when using the QuantiFast multiplex PCR + Rox (Qiagen) (80% concordance). (The concordance is the percentage of all pairings giving the same results on all possible pairings of data.)

4.5 Detection on naturally infected seed

Determination of the detection threshold according to the contamination rate was determined by repeatedly 'diluting' naturally infected seed with healthy seed (theoretical contaminations rates of 1.75/10 000, 1/30 000, 1/60 000 and 1/120 000) (Robene *et al.*, 2015). At least two independent analyses of 35 seed lots were performed for each contamination rate. Seed contamination rates (cr) derived from triplex qPCR analyses were calculated according to the formula $cr = 1 - (A/B)^{1/n}$, where A is the number of samples in which the pathogen was not detected, B is the total number of analysed samples and n is the number of seeds per replicate sample ($n = 245$; Robène-Soustrade *et al.*, 2010). The sensitivity threshold of the triplex real-time PCR test was found to be 1 infected seed in 1/32 790 seeds.

Appendix 4 – Pathogenicity tests

Plant growth stage

Allium cepa L. (onion) plants approximately 20 cm high are inoculated.

Numbers of plants tested and controls

Ten plants are inoculated for each bacterial strain.

For the detached leaf test, eight leaf fragments are tested for each bacterial strain.

Pathogenicity tests should always contain positive (an authenticated strain of *Xanthomonas axonopodis* pv. *allii*) and negative (sterile water or buffer) controls.

Methods of inoculation

Detached leaf test

Allium cepa L. (onion) leaves preferably of a sensitive cultivar (i.e. cv. Red Creole) should be surface disinfested with 1% NaOCl and cut into fragments about 5 cm long. Leaf fragments are placed on the surface of water agar (10 g L^{-1}) supplemented with 50 mg mL^{-1} benlate. Bacterial suspensions prepared in sterile distilled water or Sigma 7–9 buffer (Appendix 2) and containing approximately 1×10^4 cfu mL^{-1} are obtained from serial dilutions of suspensions adjusted spectrophotometrically to contain approximately 1×10^8 cfu mL^{-1}. For inoculation, a 10 µL droplet of inoculum is deposited on a single wound made in the centre of each fragment using a sterile needle. Plates are sealed by wrapping with Parafilm™, and incubated for 6 days at 28 ± 1°C with a 12-h photoperiod with a light intensity of approximately 40 000 lm.

Plant inoculation

Another method of inoculation is by atomizing bacterial suspensions containing $\approx 1 \times 10^7$ cfu mL^{-1} onto leaf surfaces of *A. cepa* L. plants and maintaining them at saturating humidity for 16–24 h (e.g. by placing a clear polyethylene bag over the spray-inoculated plants).

Method of incubation

Plates are incubated at approximately 28°C under conditions of high relative humidity (approximately 80%).

Symptoms

Bacterial blight lesions typically develop within 5–10 days (depending on the growth stage of the leaves).

Koch's postulates should be fulfilled by re-isolating dense *Xanthomonas*-like cultures from the produced lesions and identifying them as *X. axonopodis* pv. *allii* using one of the above-proposed identification techniques (preferably molecular).