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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/127 (1)

Diagnostics
Diagnostic

PM 7/127 (1) *Acidovorax citrulli*

Specific scope

This Standard describes a diagnostic protocol for *Acidovorax citrulli*.[1]

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

Specific approval and amendment

Approved in 2016-09.

1. Introduction

Acidovorax citrulli is the causal agent of bacterial fruit blotch and seedling blight of cucurbits (Webb & Goth, 1965; Schaad *et al.*, 1978). This disease was sporadic until the late 1980s (Wall & Santos, 1988), but recurrent epidemics have been reported in the last 20 years (Zhang & Rhodes, 1990; Somodi *et al.*, 1991; Latin & Hopkins, 1995; Demir, 1996; Assis *et al.*, 1999; Langston *et al.*, 1999; O'Brien & Martin, 1999; Burdman *et al.*, 2005; Harighi, 2007; Holeva *et al.*, 2010; Popović & Ivanović, 2015). The disease is particularly severe on watermelon (*Citrullus lanatus*) and on several genotypes of melon (*Cucumis melo*). Other cucurbits might be affected, although to a lesser extent: cucumber (*Cucumis sativus*), squash and gourd varieties (*Cucurbita pepo*) and winter squash (*Cucurbita moschata*). Citron melon (*Citrullus lanatus* var. *citroides*), a cucurbitaceous weed, is a potential latent host for *A. citrulli* (Dutta *et al.*, 2014). Other *Citrullus* spp., such as *Citrullus colocynthis*, present in several areas around the Mediterranean Basin, are not known to be hosts of *A. citrulli*. Genetically and pathogenically, *A. citrulli* is divided into two groups[2]: Group I isolates, including the American Type Culture Collection (ATCC 29625) type strain, were mainly isolated from non-watermelon, cucurbit hosts such as cantaloupe melon (*Cucumis melo* var. *cantalupensis*), cucumber (*Cucumis sativus*), honeydew melon (*Cucumis melo* var. *indorus*), squash and pumpkin (*Cucurbita pepo, Cucurbita maxima* and *Cucurbita moschata*) whereas Group II isolates were mainly recovered from watermelon (Walcott *et al.*, 2000, 2004; Burdman *et al.*, 2005). While Group I isolates were moderately aggressive on a range of cucurbit hosts, Group II isolates were highly aggressive on watermelon but moderately aggressive on non-watermelon hosts (Walcott *et al.*, 2000, 2004). *Acidovorax citrulli* is seedborne, present on both the outside and inside of the seed (Webb & Goth, 1965; Sowell & Schaad, 1979; Rane & Latin, 1992; Hopkins & Thompson, 2002), and from seeds it is easily transmitted to seedlings (Kucharek *et al.*, 1993). Current seed disinfection methods do not eliminate the pathogen (Hopkins *et al.*, 1996, 2003; Fessehaie & Walcott, 2005; Walcott, 2008).

Flow diagrams describing the diagnostic procedure for *Acidovorax citrulli* are presented in Figs 1 and 2.

2. Identity

Name: *Acidovorax citrulli* (Schaad *et al.*, 1978, 2008).
Synonyms: *Acidovorax avenae* subsp. *citrulli*, *Pseudomonas pseudoalcaligenes* subsp. *citrulli, Pseudomonas pseudoalcaligenes* subsp. *citrulli*.
Common names: (bacterial) fruit blotch, seedling blight.
Taxonomic position: Bacteria: Proteobacteria: Betaproteobacteria: *Burkholderiales*: *Comamonadaceae*.
EPPO code: PSDMAC.
Phytosanitary categorization: EPPO A1 List no. 379.

[1]The use of brand names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable.

[2]The Panel on Diagnostics in Bacteriology is aware that a third group (which also reacts with the tests included in this protocol) has recently been identified based on multilocus sequence typing but no publication is available yet.

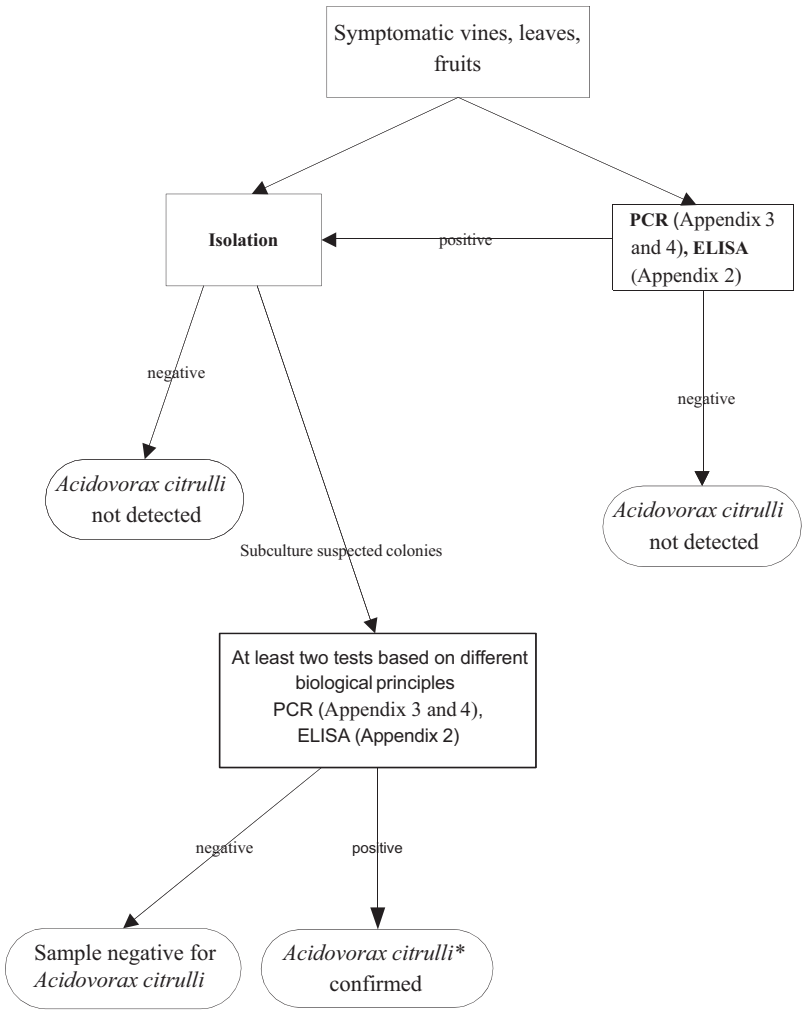


Fig. 1 Diagnostic procedure for *Acidovorax citrulli* in symptomatic plant material (except sweat box procedure).

3. Detection

Acidovorax citrulli can be present on plants and plant parts such as seeds, seedlings, fruits, leaves and vines (Schaad *et al.*, 1978; Walcott, 2005; Feng *et al.*, 2013).

Detection is possible from symptomatic plant material and from seeds.

3.1. Detection in plants

Detection in asymptomatic plants is not recommended as an appropriate sample size has not been determined.

3.1.1. Symptoms

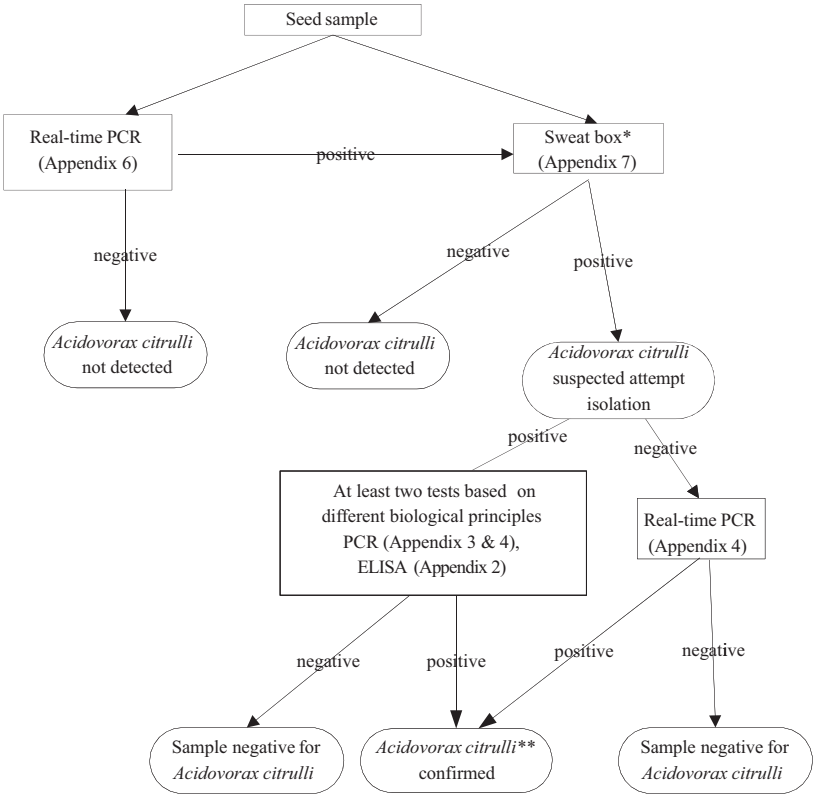
Symptoms of the disease can be observed on leaves, vines and fruits.

On leaves, small dark brown, somewhat angular and water-soaked lesions can appear, but they are generally inconspicuous (Fig. 3).

On seedlings, water-soaked areas on the underside of cotyledons can be seen and lesions on the hypocotyl cause collapse of the emerging plants.

On vines, brown necrotic streaks may appear, later cracking to form a longitudinal canker (Fig. 4). Bacterial ooze may emerge from cankers.

On watermelon fruits, symptoms appear as a dark olive green stain (or blotch) on the upper side of the fruit. Lesions usually become apparent shortly before fruit ripening. The epidermis of the rind can then rupture and bacterial ooze is frequently produced. On susceptible watermelon cultivars, lesions may develop as large cracking and rotting areas (Figs 5 and 6). On melon fruits, symptoms



*Experience with performing the sweat box test on cucurbit seeds other than watermelon (*Citrullus lanatus*), melon (*Cucumis melo*) and cucumber (*Cucumis sativa*) is limited (see section 3.2.2)
**in critical cases confirmation with a pathogenicity test may be required (Appendix 8)

Fig. 2 Diagnostic procedure for *Acidovorax citrulli* in seeds.

Fig. 3 Necrotic spots and lesions caused by *Acidovorax citrulli* on a watermelon leaf. Courtesy D. Giovanardi, University of Modena and Reggio Emilia (IT).

Fig. 4 Necrotic lesions on a watermelon vine caused by *Acidovorax citrulli*. Courtesy D. Giovanardi, University of Modena and Reggio Emilia (IT).

Fig. 5 Initial symptoms of bacterial blotch on watermelon: initial small cracks are visible, with some water soaking. Courtesy A. Obradović, University of Belgrade (RS).

Fig. 6 Advanced symptoms of bacterial blotch on watermelon: cracks are clearly visible on fruit, together with an extensive water soaked and rotting area all around. Courtesy A. Obradović, University of Belgrade (RS).

appear as small sunken lesions (sometimes very many) which do not expand on the fruit surface. When numerous, such lesions may coalesce and cause large rotting areas and long cracks on fruits Fig. 7. Symptoms on leaves are presented in Fig. 8.

3.1.2. Sample preparation

Samples from symptomatic plants should be preferably processed within 24–48 h to allow maximum recovery of the pathogen and to avoid heavy multiplication of saprophytes. After reception in the laboratory, they should be kept at 4–8°C until analysis. Freshly prepared sample extracts are necessary for successful detection and isolation of the pathogen. The remaining plant samples can be cold-stored at 4–8°C for up to 1 week to be processed for additional verification, if needed.

Symptomatic vine segments (8–12 cm), leaves or fruits with necrotic lesions are thoroughly rinsed with sterile

Fig. 7 Symptoms of bacterial blotch on melon. Courtesy SALIM (GP).

Fig. 8 Necrotic spots and lesions caused by *Acidovorax citrulli* on a melon leaf. Courtesy SALIM (GP).

water and blotted dried and briefly disinfected in ethanol (70%). From the leaf or fruit lesions 8–10 small pieces (approximately 1 cm) of infected tissue are aseptically removed, put into a tube with 2–5 mL of sterile saline solution or sterile water, and left to soak for a few minutes. For vine segments, 3 to 5 pieces of approximately 1 cm of infected tissue (margins of a vine canker) are processed in bulk as described for leaf lesions or preferably individually for isolation. Alternatively, fragments of infected tissue can be comminuted (cut into approximately 1 cm pieces) in 2–5 mL of sterile saline solution (or sterile water) and allowed to soak for 2–3 min. These washing suspensions or plant extracts are commonly used for direct isolation, but they can also be used for PCR and serological tests.

3.1.3. Screening tests

For symptomatic material, direct isolation, PCR and serological tests can be performed as screening tests.

3.1.3.1. Isolation. Isolation of *A. citrulli* from symptomatic samples may be inhibited by the presence of saprophytic

microflora which may overgrow the medium in 36–48 h after plating. Therefore, NSA-250 (Giovanardi *et al.*, 2015) or King's B medium (supplemented) should preferably be used (Appendix 1). Colonies grow more slowly on media supplemented with antibiotics compared with the same media without antibiotics.

Isolation from plant extracts is done by plating of 20–50 µL of the plant extract or plant washing solution obtained as above and its 10-, 100- and 1000-fold dilutions onto agar plates with a sterile Drigalski stick. Plates are incubated for 3–6 days at approximately 27°C. As a control, a suspension of an *A. citrulli* control strain (see reference material) at a concentration of about 10^4–10^5 cfu mL^{-1} is plated onto the same medium. Plates should be observed between the 3rd and 6th days after plating for the presence of whitish pinhead-sized colonies. Suspect colonies require a further purification step by streaking on NSA or King's B medium. Incubation is done at approximately 27°C before proceeding to further identification.

Colony morphology—After 4 days of incubation on NSA-250 medium at 27°C colonies are 1.0–1.5 mm in diameter, convex, slightly spreading with clear margins and whitish-grey in colour (Fig. 9).

After 3 days of incubation on King's B medium colonies are non-fluorescent white, flat and with entire margins. Colony size is affected by the presence of adjacent colonies of other bacteria (Fig. 10).

3.1.3.2. Serological tests. Serological tests (ELISA and immuno strips) are commercially available (see Appendix 2).

Instructions to perform an ELISA test are provided in EPPO Standard PM 7/101 (EPPO, 2010b) *ELISA tests for plant pathogenic bacteria*. It should be noted that ELISA is not recommended on symptomatic seedlings from the sweat box test due to the high number of false negative and false positive results obtained.

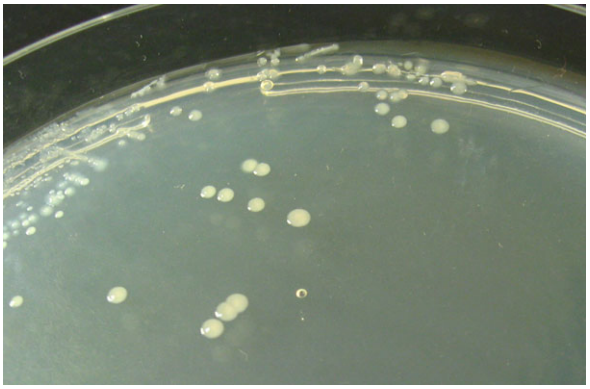


Fig. 9 *Acidovorax citrulli* on NSA250, showing 4-day-old colonies. Courtesy of R Çetinkaya Yildiz, Biological Control Research Institute, Ankara (TR).

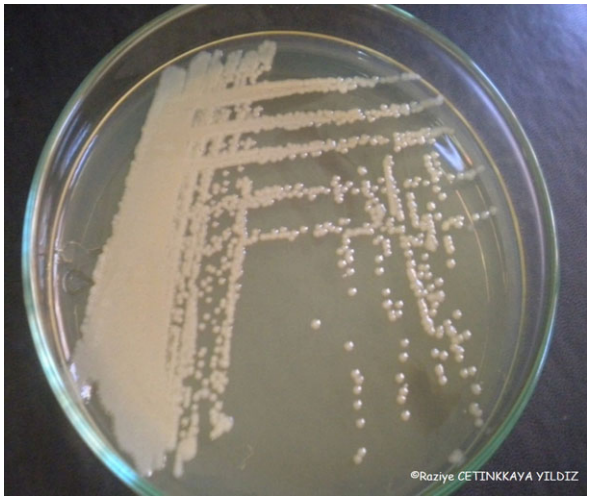


Fig. 10 *Acidovorax citrulli* on King's B medium. Courtesy Davide Giovanardi, Dept. Universita degli Studi, UNIMORE (IT).

3.1.3.3. Molecular tests. Conventional and real-time PCR are described in Appendices 3 and 4, respectively. Another PCR test (Bahar *et al.*, 2008) has been developed for the detection of *A. citrulli*. Validation studies are in progress in Anses (FR) and will be made available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int) when completed.

3.2. Detection in seeds

3.2.1. Sampling

The International Seed Federation (ISHI-Veg, ISF) recommends a minimum sample size of 10 000 seeds per seed lot.

3.2.2. Screening tests

For seeds, screening tests involve real-time PCR of direct seed washes; alternatively, a sweat box test (followed by a confirmation) can be done. These tests are described in Appendices 6 and 7.

Experience with performing the sweat box with cucurbit seeds other than melon and watermelon is limited. Using larger and higher boxes and/or more than 12 boxes with a smaller number of seeds per box may be considered due to the size of such seeds.

3.2.3. Extraction for testing by real-time PCR

The maximum number of seeds that can be processed in one test is 5000 and a minimum of two subsamples are tested per seed lot. Cucurbit seeds are of varying sizes and weights, depending on species and variety: therefore, the weight of 5000 seeds may vary greatly (from 120 g for cucumber to 1000 g for *Cucurbita maxima*) (Grubben & Denton, 2004). Pathogen extraction is based on soaking the seeds in a soaking buffer (Appendix 1) followed by a concentration step to obtain a final concentrate. The full procedure is described in Appendix 4.

It is recommended that the extract is divided into two parts: one used to perform the test recommended in this Diagnostic Protocol and the second intended for storage (by adding 15–20% of sterile glycerol with storage at −80°C for possible further confirmation).

Spiked controls should be included. Spiking with, for example, *Acidovorax cattleyae* spike (NBC430 or NCPPB961) is performed before centrifugation.

4. Identification

The identification of *A. citrulli* should be performed using two or more of the following tests based on different biological principles and including at least one PCR test.

4.1. Serological tests

ELISA as described in Section 3.1.3.2 can also be performed on pure cultures. Instructions for performing an ELISA test are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010b).

4.2. Molecular tests

4.2.1. PCR

Conventional and real-time PCR are described in Appendices 3 and 4. These can be performed for confirmation on a suspension containing approximately 10^6 cfu mL^{-1} in molecular-grade sterile water, prepared from a 24-h growing culture on NSA.

Another PCR test (Bahar *et al.*, 2008) has been developed for the detection of *A. citrulli*. Validation studies are in progress in Anses (FR) and will be made available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int) when completed.

4.2.2. DNA fingerprinting methods

Repetitive PCR (rep-PCR) has been shown to be highly reliable for identifying strains of pseudomonads, xanthomonads and other bacteria at the species and infraspecies level (Louws *et al.*, 1994; Rademaker & de Bruijn, 1997) by means of amplification of interspersed repetitive DNA sequences present in bacterial genomes. Guidance on how to perform rep-PCR tests for the identification of bacterial isolates is given in EPPO PM 7/100 *Rep-PCR tests for identification of bacteria* (EPPO, 2010a).

4.3. Other tests that can provide information

The following tests may be useful in excluding non-pathogenic bacteria and can provide indication on the identity of *A. citrulli* but are not sufficient for a positive identification.

4.3.1. Hypersensitive reaction

In order to avoid performing identification tests on saprophytes, a hypersensitive reaction (HR) test can be performed on tobacco leaves (Klement, 1963; Lelliott & Stead, 1987) by infiltrating tissues with aqueous suspensions of the putative *Acidovorax*. A positive HR strongly indicates the presence of a phytopathogenic bacterium.

4.3.2. Biochemical and physiological tests (Rane & Latin, 1992; modified, Hugh & Leifson, 1953)

Acidovorax citrulli strains give the following results:

Tests	Results
Gram's reaction	−
Cytochrome *c* oxidase reaction	+
Oxidative	+
Fluorescent pigment on King's B medium	−
Tobacco hypersensitivity	+
Gelatin liquefaction	−
Growth at 41°C	+
Motility	+
Levan	−
Arginine dihydrolase	−
Lipase	+
Utilization of:	
β-alanine	+
L-arabinose	+
Fructose	+
Glycerol	+
Glucose	+
Galactose	+
L-leucine	+
Trehalose	+
Sucrose	−
Lactose	−
Sorbitol	−
Mannitol	−
Rhamnose	−
Cellobiose	−

4. 3.3. Pathogenicity tests

A pathogenicity test may be required for the completion of the diagnostic procedure (in particular for critical cases, see EPPO PM 7/76 *Use of EPPO diagnostic protocols*). The test is described in Appendix 8.

5. Reference material

Acidovorax citrulli (Schaad *et al.*, 1978, 2008)

Pathotype strain: NCPPB 3679 (ex ATCC 29625). This pathotype strain is also available as CFBP 4459 or LMG 5376.

The following collections can provide different *A. citrulli* reference strains:

1 National Collection of Plant Pathogenic Bacteria (NCPPB), FERA, York (GB) (http://www.nppb.-comhttp://www.nppb.com).
2 Collection Française de Bactéries associées aux Plantes (CIRM-CFBP), INRA, IRHS, 42 rue Georges Morel, BP 60057, 49071 Beaucouzé Cedex (France) (http://

www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria; http://www-intranet.angers.inra.fr/cfbp/catalogue).

3 Laboratory of Microbiology Gent (BCCM/LMG), Gent (BE) (http://lmg.ugent.be).

Authenticity of the strains can only be guaranteed if they are obtained directly from the culture collections.

Acidovorax cattleyae spike

1 NBC430: Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen (The Netherlands)
2 NCPPB961: National Collection of Plant Pathogenic Bacteria (NCPPB), FERA, York (GB) (http://www.nppb.com).

6. Reporting and documentation

Guidelines on reporting and documentation are 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 are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports).

8. Further information

Further information on this organism can be obtained from:

E. Stefani, Dept. of Life Sciences, University of Modena and Reggio Emilia, via Amendola 2, 42122 Reggio Emilia (Italy) (stefani.emilio@unimore.it)

H.M.S. Koenraadt, Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen (The Netherlands) (H.Koenraadt@Naktuinbouw.nl)

M. Bruinsma, Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen (The Netherlands) (m.bruinsma@naktuinbouw.nl)

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 send it to 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 prepared in the framework of the European Union-funded project Testa for the section on seed testing. It was originally drafted by M. Bruinsma and H. M. S. Koenraadt, Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen (The Netherlands) and E. Stefani, Dept. of Life Sciences, University of Modena and Reggio Emilia, via Amendola 2, 42122 Reggio Emilia (Italy). This protocol was reviewed by the Panel on Diagnostics in Bacteriology.

References

Assis SMP, Mariano RLR, Silva-Hanlin DMW & Duarte V (1999) Bacterial fruit blotch caused by Acidovorax avenae subsp. citrulli in melon in the state of Rio Grande do Norte, Brazil. *Fitopatologia Brasileira* **24**, 191.

Bahar O, Efrat M, Hadar E, Dutta B, Walcottand RR & Burdwan S (2008) New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. *Plant Pathology* **57**, 754–763.

Burdman S, Kots N, Kritzman G & Kopelowitz J (2005) Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp. *citrulli* isolates from watermelon and melon in Israel. *Plant Disease* **89**, 1339–1347.

Crosse JE (1959) Bacterial canker of stone fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. *Annals of Applied Biology* **47**, 306–317.

Demir G (1996) A new bacterial disease of watermelon in Turkey: bacterial fruit blotch of watermelon (*Acidovorax avenae* subsp. *citrulli* (Schaad *et al.*) Willems *et al.*). *Journal of Turkish Phytopathology* **25**, 43–49.

Dutta B, Sanders H, Langston DB, Booth C, Smith S & Gitaitis RD (2014) Long-term survival of *Acidovorax citrulli* in citron melon (*Citrullus lanatus* var. *citroides*) seeds. *Plant Pathology* **63**, 1130–1137.

EPPO (2006) PM7/77 *Documentation and reporting on a diagnosis*. *EPPO Bulletin* **36**, 459–460.

EPPO (2010a) PM 7/100 *Rep-PCR tests for identification of bacteria*. *EPPO Bulletin* **40**, 365–368.

EPPO (2010b) PM 7/101 *ELISA tests for plant pathogenic bacteria*. *EPPO Bulletin* **40**, 369–372.

Feng JJ, Li JQ, Walcott RR, Zhang GM, Luo LX & Kang L (2013) Advances in detection of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch of cucurbits. *Seed Science Technology* **41**, 1–15.

Fessehaie A & Walcott RR (2005) Biological control to protect watermelon blossoms and seed from infection by *Acidovorax avenae* subsp. *citrulli*. *Phytopathology* **95**, 413–419.

Frei M (2011) Centrifugation basics. *BioFiles* **6**, 3–13.

Gilbert P, Andrews J, Collier PJ & Brown MRW (1995) Methods of harvesting of cultured bacteria. In *Microbiological Quality Assurance: A Guide Towards Relevance and Reproducibility of Inocula*. (ed. Brown MRW & Gilbert P), pp. 122–127.

Giovanardi D, Ferrari M & Stefani E (2015) Seed transmission of *Acidovorax citrulli*: implementation of detection in watermelon seeds and development of disinfection methods. In: Proceedings of the VII Congress on Plant Protection, Zlatibor, Serbia, November 24-28, 2014 (In press).

Grubben GJH & Denton OA (2004) *Plant Resources for Tropical Africa 2. Vegetables*, p. 668. PROTA Foundation, Backhuys Publishers, CTA, Wageningen, Leiden, the Netherlands.

Harighi B (2007) Bacterial leaf spot of Christ's thorn, a new disease caused by *Acidovorax avenae* subsp. *citrulli* in Iran. *Journal Plant Pathology* **89**, 283–285.

Holeva MC, Karafla CD, Glynos PE & Alivizatos AS (2010) *Acidovorax avenae* subsp. *citrulli* newly reported to cause bacterial fruit blotch of watermelon in Greece. *Plant Pathology* **59**, 797.

Hopkins DL, Cucuzza JD & Watterson JC (1996) Wet seed treatment for the control of bacterial fruit blotch of watermelon. *Plant Disease* **80**, 529–532.

Hopkins DL & Thompson CM (2002) Seed transmission of *Acidovorax avenae* subsp. *citrulli* in cucurbits. *HortScience* **37**, 924–926.

Hopkins DL, Thomson CM, Hilgren J & Lovic B (2003) Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. *Plant Disease* **87**, 1495–1499.

Hugh R & Leifson E (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *Journal of Bacteriology* **66**, 24–26.

King EO, Ward MK & Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* **44**, 301–307.

Klement Z (1963) Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature* **199**, 299–300.

Koenraadt HMS, Borst RC, vanVliet A, Hoekstra M, van Schie J & Buimer M(2005) Detection of *Acidovorax avenae* subsp. *citrulli* with the sweatbox method. In: Mededelingenblad van de Koninklijke Nederlandse Plantenziektekundige Vereniging. Supplement Gewasbescherming 36: 86S.

Kucharek T, Perez Y & Hodge C (1993) Transmission of the watermelon blotch bacterium from infested seed to seedlings. *Phytopathology* **83**, 467.

Langston DB Jr, Walcott RR, Gitaitis RD & Sanders FH Jr (1999) First report of a fruit rot of pumpkin caused by *Acidovorax avenae* subsp. *citrulli* in Georgia. *Plant Disease* **83**, 199.

Latin RX & Hopkins DL (1995) Bacterial fruit blotch of watermelon. The hypothetical exam question becomes reality. *Plant Disease* **79**, 761–765.

Lelliott RA & Stead DE (1987) Methods for the diagnosis of bacterial diseases of plants. In *Methods in Plant Pathology*, Vol. **2**, (ed. Preece TF), pp. 44–56. Blackwell Scientific Publications, Oxford, UK.

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 Environmental Microbiology* **60**, 2286–2295.

O'Brien RG & Martin HL (1999) Bacterial blotch of melons caused by strains of Acidovorax avenae subsp. citrulli. *Australian Journal of Experimental Agriculture* **39**, 479–485.

Popović T & Ivanović Ž (2015) Occurrence of *Acidovorax citrulli* causing Bacterial fruit blotch of watermelonin Serbia. *Plant Disease* **33**, 886.

Rademaker JLW & de Bruijn FJ (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. In *DNA Markers: Protocols, Applications and Overviews* (eds Caetano-Anolles G & Gresshoff PM), pp. 151–171. John Wiley, New York.

Rane KK & Latin RX (1992) Bacterial fruit blotch of watermelon: association of the pathogen with seed. *Plant Disease* **76**, 509–512.

Schaad NW, Sowell G Jr, Goth RW, Colwell RR & Webb RE (1978) *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology* **28**, 117–125.

Schaad NW, Song WY & Hatziloukas E (2000) PCR primers for detection of plant pathogenic species and subspecies of *Acidovorax*. US Patent 6,146,834.

Schaad NW, Postnikova E, Sechler A, Claflin LE, Vidaver AK, Jones JB *et al.* (2008) Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino 1911) comb. nov., *A. citrulli* (Schaad *et al.*, 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Systematic and Applied Microbiology* **31**, 434–446.

Somodi GC, Jones JB, Hopkins DL, Stall RE, Kucharek TA, Hodge NC *et al.* (1991) Occurrence of a bacterial watermelon fruit blotch in Florida. *Plant Disease* **75**, 1053–1056.

Sowell G Jr & Schaad NW (1979) Pseudomonas pseudoalcaligenes subsp. citrulli on watermelon: seed transmission and resistance of plant introductions. *Plant Disease Reporter* **63**, 437–441.

Walcott RR (2005) Bacterial fruit blotch of cucurbits. *The Plant Health Instructor*. doi:10.1094/PHI-I-2005-1025-02.

Walcott RR (2008) Integrated pest management of bacterial fruit blotch of cucurbits. In *Integrated Management of Plant Pests and Diseases* (Eds Ciancio A & Mukerji KG), pp. 191–209. Springer, Dordrecht, the Netherlands.

Walcott RR, Fessehaie A & Castro AC (2004) Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit hosts. *Journal of Phytopathology* **152**, 277–285.

Walcott RR, Langston DB Jr, Sanders FH Jr & Gitaitis RD (2000) Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. *Phytopathology* **90**, 191–196.

Wall GC & Santos VM (1988) A new bacterial disease of watermelon in the Mariana Islands. *Phytopathology* **78**, 1605.

Webb RE & Goth RW (1965) A seedborne bacterium isolated from watermelon. *Plant Disease Reporter* **49**, 818–821.

Woudt B, Beerepoot AJ & Carter LL. (2009a) A new PCR method for detection of *Acidovorax citrulli* on seed. Poster APS.

Woudt B, Koenraadt HMS, Oosterhof J & vanBetteray B (2009b) Development of specific primers for the molecular detection of *Acidovorax avenae* subsp. *citrulli*. Proceedings of the EPPO Conference on diagnostics. http://archives.eppo.int/MEETINGS/2009_conferences/diagnostic/Diag_York2009_brochure.pdf

Zhang X & Rhodes BB (1990) A new devastating disease of watermelon: bacterial fruit blotch. *China Watermelon Muskmelon* **2**, 44–45.

Appendix 1 – Media and buffers

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

Media

NSA (nutrient sucrose agar) (Crosse, 1959)

Nutrient broth	8.0 g
Sucrose	50.0 g
Bacteriological agar	18.0 g
Distilled water	1.0 L

The pH is adjusted to 7.2.

NSA-250 (Giovanardi *et al*., 2015)

Nutrient broth	8.0 g
Sucrose	50.0 g
Bacteriological agar	18.0 g
Distilled water	1.0 L

The pH is adjusted to 7.2.

After autoclaving, cool to about 50°C and then add 10 mL of a 25 mg mL^{-1} solution of cycloheximide in 70% ethanol and 10 mL of a 20 mg mL^{-1} aqueous solution of ampicillin.

With antibiotics store for up to 1 month refrigerated in the dark.

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

Proteose peptone no. 3	20.0 g
Bacterial glycerol	10.0 mL
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Microbiological-grade agar	15.0 g
Distilled water	to 1 L

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

Buffers

Soaking buffer for seeds

Tween 20 (Tween 10% solution)	5 mL
Nystatin (>4400 U mg^{-1}) stock solution	450 µL
PBS buffer	up to 1000 mL

PBS

Sodium chloride (NaCl)	8.0 g
Di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	3.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride (KCl)	0.2 g
Distilled water to adjust pH to 7.2–7.4	1 L

Store the prepared buffer at room temperature. The shelf life of stock is 6 months.

Appendix 2 – Commercially available serological tests and associated validation data

Data as provided by Agdia and Loewe (for more information contact the companies).

Agdia monoclonal antibody for DAS-ELISA on symptomatic plants and bacterial cultures

Analytical sensitivity

The range 1.6×10^5 to 6.2×10^7 cells mL^{-1} (cfu mL^{-1}) gave a positive result with four pure cultures.

Analytical specificity

No cross-reactions were seen with the following plant pathogens: *Acidovorax avenae* pv. *avenae*, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium vitis*, *Burkholderia glumae*, *Clavibacter michiganensis* subsp. *insidiosus*, *Clavibacter michiganensis* subsp. *michiganensis*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Clavibacter michiganensis* subsp. *sepedonicus*, *Clavibacter michiganensis* subsp. *tessellarius*, *Curtobacterium flaccumfaciens* subsp. *poinsettiae*, *Dickeya chrysanthemi*, *Erwinia amylovora*, *Erwinia tracheiphila*, *Pantoea agglomerans*, *Pantoea stewartii*, *Pectobacterium atroseptica*, *Pectobacterium carotovora* subsp. *carotovora*, *Pseudomonas fuscovaginae*, *Pseudomonas savastanoi* pv. *glycinea*, *Pseudomonas savastanoi* pv. *phaseolicola*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *tomato*, *Ralstonia solanacearum*, *Spiroplasma citri*, *Stenotrophomonas maltophili*, *Xanthomonas albilineans*, *Xanthomonas arboricola* pv. *celebensis*, *Xanthomonas arboricola* pv. *pruni*, *Xanthomonas axonopodis* sp., *Xanthomonas axonopodis* pv. *begoniae*, *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *dieffenbachiae*, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas campestris* pv. *armoraciae*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas campestris* pv. *zinnae*, *Xanthomonas citri* pv. *aurantifolii*, *Xanthomonas citromelo*, *Xanthomonas fragariae*, *Xanthomonas hortorum* pv. *pelargonii*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas translucens* pv. *translucens*, *Xanthomonas vesicatoria*, *Xylella fastidiosa*.

No cross-reactions were seen with following non-plant pathogens: *Acinetobacter calcoaceticus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus faecalis*.

Agdia ImmunoStrips on symptomatic plants and bacterial cultures

Analytical sensitivity

The lowest detectable concentration of the target organism was 4×10^5 cfu mL^{-1}.

Analytical specificity

No cross-reactions were seen with the following plant pathogens: *Clavibacter michiganensis* subsp. *insidiosus*, *Clavibacter michiganensis* subsp. *michiganensis*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Clavibacter michiganensis* subsp. *sepedonicus*, *Clavibacter michiganensis* subsp. *tessellarius*, *Curtobacterium flaccumfaciens* subsp. *poinsettia*, *Erwinia carotovora*, *Pseudomonas syringae* subsp. *syringae*, *Pseudomonas syringae* subsp. *tomato*, *Ralstonia solanacearum*, *Xanthomonas campestris* subsp. *armoraciae*, *Xanthomonas axonopodis* pv. *dieffenbacchiae*, *Xanthomonas vesicatoria*.

Bioreba polyclonal antibody for for DAS-ELISA on symptomatic plants and bacterial cultures

Analytical sensitivity

10^4 cfu mL^{-1} (bacteria from pure cultures diluted in buffer). This detection limit is nearly reached when bacteria are diluted in healthy watermelon leaves.

Analytical specificity

Cross-reaction is possible with *Acidovorax cattleyae* or *Acidovorax avenae*.

Loewe: polyclonal antibody for DAS-ELISA on symptomatic plants and bacterial cultures

Analytical sensitivity

10^2 cfu mL^{-1}

Analytical specificity

Cross-reactions were observed with *Acidovorax avenae*, *Acidovorax anthurii*, various isolates of *Acidovorax valerianella*, various isolates of *Acidovorax cattleyae* and *Acidovorax konjaci.*

No cross-reactions were observed with *Pseudomonas syringae* pv. *lachrymans*, *Erwinia carotovora* subsp. *carotovora*, *Xanthomonas cucurbitae* or *Erwinia tracheiphila*

Appendix 3 – PCR on plant extracts from symptomatic material or pure cultures (Schaad *et al.*, 2000)

1. General information

1.1 The test can be performed on plant extracts in the case of symptomatic material, or as one of the identification test to be done on pure cultures of putative *Acidovorax citrulli*.

1.2 Primers for the detection of plant pathogenic species and subspecies of *Acidovorax* were patented and published in 2000 (Schaad *et al.*, 2000).

1.3 Primers were designed on 625- and 617-bp fragments of the inner spacer region of 16S–23S rDNA of a set of pathogenic isolates belonging to the species *Acidovorax avenae* (including the former subspecies *citrulli*), obtained from several host plants, including watermelon.

1.4 Oligonucleotides:SEQ ID no. 3: 5′-GGA AGA ATT CGG TGC TAC CC-3′SEQ ID no. 4: 5′-TCG TCA TTA CTG AAT TTC AAC A-3′

1.5 Amplicon size: 450 bp

1.6 This method has been successfully performed on an Applied Biosystems 2720 Thermal Cycler.

2. Methods

2.1. Nucleic acid extraction

The plant extract obtained either from soaking or from comminuting the symptomatic plant tissue (cutting into small pieces) in sterile PBS buffer is centrifuged (10 000 × *g* for 15 min) and the pellet is suspended with 2 mL of PBS buffer to obtain the final concentrate. 1 mL of it is used for DNA extraction and the remaining part is mixed with sterile glycerol 20% and stored at −20°C.

Plant material: nucleic acid extraction is done using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions.

Pure cultures: nucleic acid extraction is done as follows. A single colony of a fresh pure culture is suspended in 0.9 mL of PCR-grade water, supplemented with 0.1 mL of a 0.5 M NaOH solution. The suspension is then heated for 4 min at approximately 95°C and immediately chilled on ice for 10 min.

2.2. PCR

The reaction is prepared for a total volume of 25 µL in each reaction tube.

For plant extracts, in addition to the undiluted DNA extract it is recommended that 10- and 100-fold dilutions are also used to overcome possible inhibition problems. The master mix is prepared as explained below. The DNA polymerase should always be a 'hot start' polymerase; the master mix can be conveniently prepared also using GoTaq® Hot Start Polymerase.

Master mix prepared according to Giovanardi *et al.* (2015), adapted from Schaad *et al.* (2000):

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	5.00 (adjust the quantity to make up to 25 µL depending on DNA input; see below)	N.A.
Polymerase buffer	5×		1×
$MgCl_2$	25 mM	1.50	1.5 mM
dNTPs	10 mM	0.5	0.2 mM
Primer SEQ ID no. 3	20 µM	0.25	0.2 µM
Primer SEQ ID no. 4	20 µM	0.25	0.2 µM
Taq DNA polymerase (i.e. Go Taq® G2 Flexi DNA polymerase)	5 U μL^{-1}	0.20	0.04 U μL^{-1}
DNA obtained from bacterial suspensions		2–5	1–60 ng μL^{-1}
or			
DNA obtained from plant extract		2–5	
Total		25.00	

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

PCR cycling conditions (Giovanardi *et al.*, 2015; amended from Schaad *et al.*, 2000):

	Temp.(°C)	Time
Hold	94	10 min
	94	30 s
	56	45 s
35 cycles	72	60 s
	72	7 min
Hold	4	∞

3. Essential procedural information

(a) Controls

For a reliable PCR test, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative internal control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected cucurbit plant matrix or, if not available, clean extraction buffer.
- Positive internal 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 cucurbit plant matrix sample containing the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

(b) Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the expected size.

When these conditions are met

- A test will be considered positive if amplicons of 450 bp 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 specificity extracted from the patent

Specificity was evaluated with 2 isolates of *A. citrulli,* 2 isolates of *A. cattleyae*; 2 isolates of *Burkholdera glumae* and 2 isolates of *A. avenae*.

No cross-reactions were observed.

Appendix 4 – Real-time PCR on symptomatic plants and pure cultures

1. General information

1.1 Two primer and probe sets were designed by Monsanto based on the amplified fragment length polymorphism sequence. The sequences are located at *A. citrulli* Contig 21, ATPase and Contig 22, a non-coding region.

1.2 The following real-time TaqMan PCR test is performed for the detection and identification of *Acidovorax citrulli* in symptomatic plants of watermelon (*Citrullus lanatus*), melon (*Cucumis melo*) and other cucurbits (Cucurbitaceae) and seedlings under suspicion after a sweat box test. It can also be performed on presumptive cultures.

Acit F Contig 21: 5′-ACCGAACAGAGAGTAATTCTCAAAGAC-3′ Acit R Contig 21: 5′-GAGCGTGATGGCCAATGC-3′ Acit Contig 21 probe: 5′-FAM-CATCGCTTGAGCAGCAA-MGB NFQ-3′ Acit F Contig 22: 5′-GAAAGTGGTTGTTCTGGTGATCAA-3′ Acit R2 Contig 22: 5′-TTCGGAGGACTCGGGATTT-3′ Acit probe Contig 22: 5′-VIC-ATGGTCTGCGAGCCAG-MGB NFQ-3′

1.3 An internal control is included. *Acidovorax cattleyae* is a related bacterium added in a known quantity to check extraction and PCR efficiency (see Appendix 5, point 1.2). The primer set was originally developed by Syngenta and modified by Naktuinbouw.

Acat 2-F: 5′-TGTAGCGATCCTTCACAAG-3′ Acat 2-R: 5′-TGTCGATAGATGCTCACAAT-3′ Acat 1-probe: 5′-Texas Red-CTTGCTCTGCTTCTCTATCACG-BHQ2-3′

1.4 The method has been validated using PerfeCTa Multiplex qPCR ToughMix (Quanta Biosciences) on a Bio-Rad QPCR CFX100.

2. Methods

2.1 Nucleic acid extraction

Plant material (except seedlings; see below)

The plant extract, obtained either from soaking or from comminuting the symptomatic plant tissue (cutting it into small pieces) in sterile PBS buffer, is centrifuged (10 000 × *g*, 15 min at 4°C) and added with 2 mL of the same buffer to obtain the final concentration: half of it is used for DNA extraction and half is added with 20% sterile glycerol and stored at −20°C,

DNA is extracted using different extraction kits. Add an *A. cattleyae* spike (for spike preparation see Appendix 5, point 1.2) before DNA extraction. The following kits have been validated: Sbeadex Maxi Kit for *A. citrulli* by LGC on a KingFisher Work Station by Thermo Scientific and the DNeasy Plant Mini Kit by Qiagen. Kits are used following the instructions in Appendix 6 or the manufacturer's instructions, respectively (for the Sbeadex Maxi Kit a manual extraction without the KingFisher Work Station is also possible).

- Seedlings from the sweat box test: symptomatic seedlings from the sweatbox test (Appendix 7) can be ground in 1 mL of NaPBS buffer per cotyledon.
- Pure culture: for cultures, prepare a cell suspension from each putative *A. citrulli* isolate. Heat at 95°C for 15 min and cool on ice. Centrifuge for 1 min at 10 000 × *g*.

Prepare 10× and 100× dilutions for cultures and seedlings and add an *A. cattleyae* spike (for spike preparation see Appendix 5, point 1.2) to monitor PCR performance.

2.2 TaqMan PCR

2.2.1 Master mix for triplex PCR.

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	11.25	N.A.
PerfeCTa Multiplex qPCR Tough Mix	5×	5.0	1×
Acit F Contig 21	20 µM	0.5	0.4 µM
Acit R Contig 21	20 µM	0.5	0.4 µM
Acit F Contig 22	20 µM	0.5	0.4 µM
Acit R2 Contig 22	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acit Contig 21-probe FAM	20 µM	0.25	0.2 µM
Acit Contig 22 probe VIC	20 µM	0.25	0.2 µM
Acat 1-probe Texas Red	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

2.2.2 Master mix for two duplex PCR tests. TaqMan qPCR mix multiplex Contig 21 and Acat

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.5	N.A.
5× PerfeCTa multiplex qPCR Tough Mix	5×	5.0	1×
Acit F Contig 21	20 µM	0.5	0.4 µM
Acit R Contig 21	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acit Contig 21 probe FAM	20 µM	0.25	0.2 µM
Acat 1-probe Texas Red	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

TaqMan qPCR mix multiplex Contig 22 and Acat

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.5	N.A.
5× PerfeCTa multiplex qPCR Tough Mix	5×	5.0	1×
Acit F Contig 22	20 µM	0.5	0.4 µM
Acit R2 Contig 22	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acit Contig 22 probe VIC	20 µM	0.25	0.2 µM
Acat 1-probe Texas Red	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

2.2.3 PCR conditions

Incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 48 s. The ramp speed should be 5°C s^{-1}.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction

and subsequent amplification preferably of a sample of uninfected melon or watermelon tissue or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of naturally infected melon or watermelon tissue or tissue spiked with *A. citrulli*. In addition to the external positive controls (PIC), internal positive controls (IPC) are used to monitor each individual sample separately. The samples are spiked with another bacterium, *A. cattleyae,* to monitor isolation and amplification.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of *A. citrulli*.

3.2. Interpretation of results

The Ct value given below is as established in Naktuinbouw. As a Ct cut-off value is dependent on equipment, material and chemistry and needs to be verified in each laboratory when implementing the test.

Verification of the controls
- The PIC, PAC and IPC amplification curves should be exponential.
- NIC and NAC should be negative.
- PIC and PAC should have a Ct value below 35.
- For each negative sample the *A. cattleyae* value (IPC) should be as expected.

When these conditions are met
- A sample will be considered positive if it produces an exponential amplification curve and a Ct value below 35.
- A sample will be considered negative if it produces no exponential amplification curve and a Ct value equal or above 35 and the *A. cattleyae* value IS between 28 and 30 as expected.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data for the specificity of the primer sets Contig 21 and Contig 22 were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. The DNA extraction has been validated for seeds (see Appendix 5).

4.1. Analytical specificity

A total of 222 isolates were tested, 168 *A. citrulli* and 54 non-targets (lookalikes). Both primer sets detected all *A. citrulli* isolates. Contig 21 cross-reacted with 4 non targets (lookalike) isolates and Contig 22 with 1 non-target (lookalike) isolate (see the validation report in the EPPO Database on Diagnostic Expertise).

Appendix 5 – Preparation of the seed extracts for testing with real-time PCR and spike preparation

Pathogen extraction from seeds is done by soaking (for the soaking buffer see Appendix 1). The soaking fluid is then collected and centrifuged to prepare the final concentrate.

1. Methods

1.1 Soaking

1.1.1 Determine the thousand grain weight (TGW) of the seeds and weigh 2 (10 000 seeds) subsamples of 5000 seeds into suitable containers.

1.1.2 Add sterile soaking buffer in a certain ratio to the weight of the seed, according to Table 1.

1.1.3 Close containers properly and shake at 90 r.p.m. at room temperature for 20–24 h. Make a small hole in the container lids in case fermentation occurs during soaking.

Table 1 Amount of soaking buffer for different Cucurbitaceae seeds

Seed	mL soaking buffer per gram of seed
Melon	2
Watermelon	2
Cucumber	2
Zucchini	3.5
Rootstock	3.5

1.2 Preparation of spike

1.2.1 Grow a single colony of *Acidovorax cattleyae* on a nutrient medium (described in Section 3.1.3.1) and incubate at 28°C for 24–48 h.

1.2.2 Transfer 1 inoculation loop (±1 µL) to 4 mL of Tryptic Soy Broth (TSB) medium and incubate at 28°C and 250 r.p.m.

1.2.3 Centrifuge for 10 min at 3000 × *g*. Gently remove the supernatant.

1.2.4 Add sterile 25% glycerol in 0.05M PBS to an optical density (OD) of 0.8.

1.2.5 Make a dilution series to determine the appropriate dilution to have a Ct-value between 28 and 30 in the sample.

1.2.6 Store the tubes in a freezer at −80°C.

1.3 Concentration of bacteria

1.3.1 For each subsample pipette 10 mL of seed extract into a 15-mL centrifuge tube and add an *A. cattleyae* spike. Centrifuge for 5 min at 1200 × *g*. Pour as much supernatant as

possible gently into a clean 15-mL tube (avoid getting parts of the pellet in the tube).

1.3.2 Centrifuge the supernatant for 20 min at 3400 × *g*.[3]

1.3.3 Remove the tubes from the centrifuge immediately and carefully remove the entire supernatant and use the pellet for DNA extraction (Appendix 6).

Appendix 6 – Real-time PCR on seed extracts

1. General information

1.1 The IS1002 TaqMan was developed on the AAC00-1 genomic sequence (GenBank Accession no. NC_008752). It targets the IS1002 element which is present in 19 nearly identical copies in the AAC00-1 genome (Woudt *et al.*, 2009a,b). Primer set Contig 22 was developed on the basis of results of an amplified fragment length polymorphism project by Keygene on Contig 22, a single-copy non-coding region.

1.2 The following real-time TaqMan PCR test is performed for the detection and identification of *Acidovorax citrulli* in seeds of watermelon (*Citrullus lanatus*), melon (*Cucumis melo*) and other cucurbits (Cucurbitaceae). It uses specific primers and probes developed by Syngenta (Primer set IS1002; Woudt *et al.*, 2009a,b) and by Monsanto (Primer set Contig 22; see Table 1). Validation was performed within the European Union-funded project TESTA.

1.3 The protocol was developed by Syngenta and modified by Naktuinbouw.

1.4 IS1002 TaqMan:Acit 1-F IS1002: 5′-GAGTCTC ACGAGGTTGTT-3′Acit 1-R IS1002: 5′-GACCC TACGAAAGCTCAG-3′Acit 1-probe IS1002: 5′-6FAM-TGCAGCCCTTCATTGACGG-BHQ1-3′

1.5 Contig 22 TaqMan:Acit F Contig 22: 5′-GAAAG TGGTTGTTCTGGTGATCAA-3′Acit R2 Contig 22: 5′-TTCGGAGGACTCGGGATTT-3′Acit probe Contig 22: 5′-VIC-ATGGTCTGCGAGCCAG-M GB NFQ-3′

1.6 An internal control is included. *Acidovorax cattleyae* is a related bacterium added in a known quantity to check the efficiency of extraction and PCR. The primer set was originally developed by Syngenta and modified by Naktuinbouw.
Acat 2-F: 5′-TGTAGCGATCCTTCACAAG-3′
Acat 2-R: 5′-TGTCGATAGATGCTCACAAT-3′
Acat 1-probe: 5′-Texas Red-CTTGCTCTGCTT CTCTATCACG-BHQ2-3′

1.7 The method has been validated with the PerfeCTa multiplex qPCR Tough Mix (Quanta Biosciences) on a Bio-Rad QPCR CFX100.

1.8 All samples should be tested in duplicate.

2. Methods

2.1 Nucleic acid extraction

2.1.1 DNA is extracted using different extraction kits. The following kits have been validated: Sbeadex Maxi Kit for *A. citrulli* by LGC on the KingFisher Work Station by Thermo Scientific and the DNeasy Plant Mini Kit by Qiagen. Kits are used following the instructions in Section 2.1.2 or the manufacturer's instructions, respectively. The Sbeadex Maxi Kit for *A. citrulli* can also use a manual protocol (without the KingFisher Work Station).

2.1.2 Resuspend the pellet in 230 µL of fresh lysis mix (200 µL lysis buffer PVP, 22 µL protease K and 8 µL DTT per subsample). Incubate the samples for 1 h at 55°C in a thermo shaker (850 r.p.m.) and then put them on ice. Fill the KingFisher plates according to Table 1. Centrifuge the samples for 5 min at 16 000 × *g* and add all of the lysate to the sample plate. Start the KingFisher program (see below).

Table 2. KingFisher mL plates with reagents. The volume is shown per sample

Name plate	Type	Reagents	Volume (µL) per sample
Sample	Deepwell	Binding buffer PN	500
		Magnetic beads	20
Wash 1	Deepwell	Wash buffer PN 1	600
Wash 2	Deepwell	Wash buffer PN 1	600
Wash 3	Deepwell	Wash buffer PN 2	600
Wash 4	Deepwell	Molecular-grade water	600
Elution	Elution	Elution buffer	100

KingFisher program

Name plate	Steps within one plate
Tipholder	1. Pick-up plate (96 DW tip comb)
	2. Leave tip comb in plate 2 'Wash 1'
Plate 1 'Cell lysate'	1. Mix fast, 10 min
	2. Collect beads, 3 × 10 s
Plate 2 'Wash 1'	1. Release beads, bottom mix, 20 sec
	2. Mix fast, 10 min
	3. Collect beads, 3 × 10 s.

(continued)

[3]The speed recommended is as performed in the procedure developed by Naktuinbouw. It is recognized that some publications refer to a minimum speed of 5000–6000 × *g* (Gilbert *et al.*, 1995; Frei, 2011).

Name plate	Steps within one plate
Plate 3 'Wash 2'	1. Release beads, bottom mix, 20 sec 2. Mix fast, 10 min 3. Collect beads, 3 × 10 s
Plate 4 'Wash 3'	1. Release beads, bottom mix, 20 sec 2. Mix fast, 10 min 3. Collect beads, 3 × 10 s
Plate 5 'Wash 4'	1. Release beads, bottom mix, 20 sec 2. Mix fast, 10 min 3. Collect beads, 3 × 10 s
Plate 6 'Elution'	1. Release beads, bottom mix, 1 min 2. Preheat and mix fast at 65°C, 10 min 3. Collect beads, 5 × 10 s

2.2 TaqMan PCR

2.2.1 Multiplex PCR master mix

Triplex TaqMan qPCR mastermix

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	11.25	N.A.
PerfeCTa multiplex qPCR Tough Mix	5×	5.0	1×
Acit 1-F IS1002	20 µM	0.5	0.4 µM
Acit 1-R IS1002	20 µM	0.5	0.4 µM
Acit F Contig 22	20 µM	0.5	0.4 µM
Acit R2 Contig 22	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acit 1-probe IS1002 (FAM)	20 µM	0.25	0.2 µM
Acit probe Contig 22 (VIC)	20 µM	0.25	0.2 µM
Acat 1-probe (Texas Red)	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

2.2.2 Two duplex TaqMan PCR master mixes

Duplex TaqMan qPCR mix multiplex IS1002 and *A. cattleyae*

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.5	N.A.
5× PerfeCTa multiplex QPCR Tough Mix	5×	5.0	1×
Acit 1-F IS1002	20 µM	0.5	0.4 µM
Acit 1-R IS1002	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acit 1-probe IS1002 (FAM)	20 µM	0.25	0.2 µM
Acat 1-probe (Texas Red)	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

TaqMan qPCR mix multiplex Contig 22 and *A. cattleyae*

PCR mix multiplex qPCR	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	12.5	N.A.
5× PerfeCTa multiplex qPCR Tough Mix	5×	5.0	1×
Contig 22F	20 µM	0.5	0.4 µM
Contig 22R	20 µM	0.5	0.4 µM
Acat 2-F	20 µM	0.5	0.4 µM
Acat 2-R	20 µM	0.5	0.4 µM
Acat probe Contig 22 (VIC)	20 µM	0.25	0.2 µM
Acat 1-probe (Texas Red)	20 µM	0.25	0.2 µM
Subtotal		20.0	
DNA		5.0	
Total		25.0	

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

2.2.3 PCR conditions

Incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 48 s. The ramp speed should be 5°C s^{-1}.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected melon or watermelon seeds or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid

extraction and subsequent amplification of naturally infected melon or watermelon seeds or seeds spiked with *A. citrulli*. In addition to the external positive controls (PIC), internal positive controls (IPC) are used to monitor each individual sample separately. The seed extract should be spiked with another bacterium, *A. cattleyae*, to monitor isolation and amplification.

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of *A. citrulli*.

3.2 Interpretation of results

The Ct value given below is as established in Naktuinbouw. As a Ct cut-off value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

Verification of the controls

- The PIC, PAC and IPC amplification curves should be exponential.
- NIC and NAC should be negative.
- PIC and PAC should have a Ct value below 35 (see validation report).
- For each negative sample the *A. cattleyae* value (IPC) should be as expected.

When these conditions are met

- A sample will be considered positive if it produces at least an exponential amplification curve and a Ct value below 35.
- A sample will be considered negative if it produces no exponential amplification curve and a Ct value equal to or above 35 and the *A. cattleyae* value is as expected.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. The validation report can be found in the EPPO validation database.

4.1 Analytical sensitivity data

The detection limit of the test at 0.95 probability was found to be 9 cells mL^{-1} for primer set IS1002 and 46 cells mL^{-1} for primer set Contig 22.

4.2 Analytical specificity data

To determine specificity, 168 *A. citrulli* isolates were tested as well as 54 non-targets. All 168 *A. citrulli* isolates were detected by both primer sets. Primer set IS1002 showed some cross-reactivity with 14 non-targets, but Contig 22 only cross-reacted with one non-target isolate.

4.3 Data on repeatability

The repeatability was tested on eight samples and was found to be 100%.

4.4 Data on reproducibility

The reproducibility was tested on eight samples and was found to be 100%.

Appendix 7 – Sweat box grow-out test (Koenraadt *et al.*, 2005)

1. General information

1.1 The sweat box grow-out test (see Fig. 11) is performed to demonstrate the possible presence of *Acidovorax citrulli* on seeds of watermelon (*Citrullus lanatus*), melon (*Cucumis melo*) and cucumber (*Cucumis sativa*). Experience with other seeds is limited.

1.2 Treated seeds are sown in sealed sweat boxes (45 × 30 × 20 cm).

1.3 Seedlings are inspected visually for the presence of typical *A. citrulli* symptoms during the period from germination to seedling stage.

1.4 A PCR test is then performed to detect the presence of *A. citrulli* in the symptomatic seedlings.

1.5 Isolation should be also performed.

1.6 Finally, a bioassay is performed to verify whether test plants inoculated with extracts from seedlings which were PCR positive show symptoms, thus confirming the presence of *A. citrulli* in the sample.

1.7 The procedure described is as implemented by Naktuinbouw (NL) (Koenraadt *et al.*, 2005).

2. Preparation of samples

2.1 Determine the thousand seed weight of the seeds, and weigh 12× 833 seeds per sample (for the 10 000-seed test).

Fig 11 Illustration of the sweat box grow-out test.

2.2 Add (only when seeds are not yet treated) the equivalent of 6 g of thiram powder per 1000 g of seeds. This can be calculated as the weight of 833 seeds × 0.006 per subsample. Mix to distribute the fungicide evenly over the seeds.
2.3 Add 1 L of soil to ensures proper germination in each sweat box.
2.4 Distribute the seeds evenly over the sweat box.
2.5 Spread 2 L of vermiculite (medium coarseness) over the seeds.
2.6 Add 1000 mL of water per sweat box.
2.7 Close the sweat boxes well.
2.8 A positive control (PC) and a negative control (NC) should be included. For the PC take 827 healthy seeds per box. Treat the seeds with thiram, as described under 2.2. Add six artificially contaminated seeds at fixed places in the sweat box (four corners and the middle of long edges, Fig. 12). Also include a NC consisting of 833 healthy seeds.

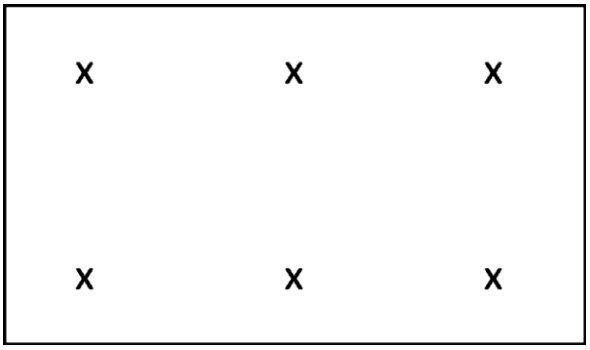


Fig. 12 Location of artificially contaminated seeds in a positive control.

2.9 Incubate the seeds for 14–21 days at 28°C and 16 h of light (24°C day per 28°C night).
2.10 5–7 days after sowing seeds for the seedling grow-out assay, sow seeds to produce seedlings that might eventually be used in the bioassay, if needed (see Appendix 8).

3. Examination of seedlings in sweat boxes

3.1 Examine the sweat boxes 14 days after emergence of the epicotyls, when cotyledons are spread. First check whether the PC seeds show clear symptoms without opening the sweat box.
3.2 If cotyledons are not spread, postpone the examination for a maximum of 7 days (until 21 days, often for triploid watermelon varieties).
3.3 Sweat boxes overgrown with fungi (>25%) and/or with poor germination (<75%) cannot be assessed.
3.4 Determine whether seedlings with water-soaked lesions or black/brown spots and/or edges on the cotyledons, sometimes causing collapse of the seedlings, are present (see Figs 13 and 14).
3.5 Sample the suspect seedlings. Pool a maximum of 5 cotyledons per sweat box in a bag and avoid sampling soil.
3.6 Check the NC for the absence of symptoms and lastly check the PC seed. Sample symptomatic material from PC seeds.
3.7 Confirmation of seedlings presenting suspicious symptoms should be performed as described in Appendix 8 and isolation should be attempted.

Fig 13 Symptoms on seedlings. Courtesy Geves (FR).

Fig 14 Symptoms on seedlings. Courtesy E. Stefani (IT).

Appendix 8 – Bioassay and pathogenicity test

Bioassay

A bioassay should be performed by inoculating plant extracts from symptomatic cotyledons in the sweat box grow-out test to test plants in order to isolate *Acidovorax citrulli*.

Test plants

Melon (cv. Charentais) and/or watermelon plants (cv. Crimson Sweet or Charleston Gray) should be used at the stage when the cotyledons are spread and the first true leaf is just visible, but no larger than 2 cm from base to tip.

For plant extracts obtained from watermelon seeds or plants, it is suggested to use watermelon plants.

For plant extracts obtained from melon seeds or plants it is suggested to use melon plants.

For other cases it is recommended to use both watermelon and melon plants.

Sample preparation

Use symptomatic cotyledons from the sweat box assay. Grind in 5 mL of sterile saline.

Inoculation

Inoculate the plants using a pipette with sterile filter tip with 10 µL of the extract. Insert the pipette tip vertically in the leaf axils between lobe leaves and poke a hole vertically in the leaf axils/stem and pipette. Depending on the hosts from which the isolates or plant extracts have been obtained (see above), inoculate, for each plant extract, 5 pots with two watermelon plants each or 5 pots with two melon plants each (or 5 pots with two watermelon plants each and 5 pots with two watermelon plants each).

Include one negative control (sterile saline buffer) and one positive control (a pure culture of a reference strain, preferably a Group I strain that is pathogenic to both melon and watermelon; see Walcott *et al.*, 2000) and the positive control seed from the sweat box test. For controls, 2 pots with two host plants each, are sufficient. Negative and positive controls should be separated by at least 0.5 m from plants inoculated with samples and negative controls should be positioned between them. Alternatively, negative and positive controls and suspected samples should be separated by a suitable separator for preventing infestation.

Incubation

Incubate inoculated plants at greenhouse temperatures of 29 ± 5°C and a relative humidity of 70–90% or in an incubator. Supplement light to 12 h per day if necessary. Pictures of symptoms are presented in Fig. 15.

Pathogenicity test

In critical cases a pathogenicity test should be performed to confirm the identity of putative *A. citrulli* isolates.

Test plants

Melon (cv. Charentais) and/or watermelon plants (cv. Crimson Sweet or Charleston Gray) should be used at the stage where the cotyledons are spread and the first true leaf is just visible, but no larger than 2 cm from base to tip.

For isolates obtained from watermelon seeds or plants, it is suggested to use watermelon plants.

For isolates obtained from melon seeds or plants it is suggested to use melon plants.

For other cases it is recommended to use both watermelon and melon plants.

Sample preparation

For putative *A. citrulli* isolates, prepare a suspension using a 48–72 h pure culture of the pathogen, at a concentration of approximately 10^6 cfu mL^{-1}.

Inoculation

Inoculate the plants using a pipette with sterile filter tip with 10 µL of the extract. Insert the pipette tip vertically in the leaf axils between lobe leaves and poke a hole vertically in the leaf axils/stem and pipette. Depending on the hosts of the isolates, for putative isolate/plant extract inoculate 5 pots with two watermelon plants each or 5 pots with two melon plants each (or 5 pots with two watermelon plants each and 5 pots with two watermelon plants each).

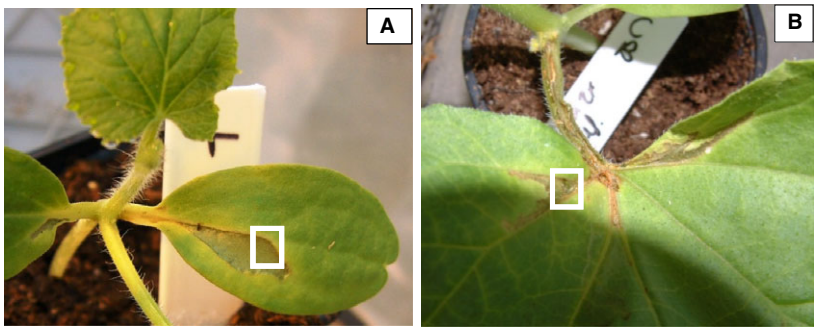


Fig. 15 *Acidovorax citrulli* colonizing cotyledon (A) and true leaf (B). The preferred tissue for sampling is indicated by a white square. Courtesy of Syngenta.

Include one negative control (sterile saline buffer) and one positive control (a pure culture of a reference strain, preferably a Group I strain pathogenic to both melon and watermelon; see Walcott *et al.*, 2000). For controls, 2 pots with two host plants each are sufficient. Negative and positive controls should be separated by at least 0.5 m from plants inoculated with samples and negative controls positioned between them. Alternatively, negative and positive controls and suspected samples should be separated by a suitable separator for preventing infestation.

Incubation

As for the bioassay.

Isolation of bacteria

Evaluate the inoculated plants and the controls after 5–6 days. Sample symptomatic tissue from both types of test plants if applicable. To obtain tissue in which *A. citrulli* is enriched compared with other bacteria select affected tissue distant from the point of inoculation (see Fig. 15). Transfer tissue to sample extraction bags. Proceed with isolation as indicated in Section 3.1.3.1 and identify suspect colonies as indicated in Section 4.