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EPPO STANDARD - DIAGNOSTICS

PM 7/120 (2) *Pseudomonas syringae* pv. *actinidiae*

Specific scope: This Standard describes a diagnostic protocol for *Pseudomonas syringae* pv. *actinidiae*. It should be used in conjunction with PM 7/76 *Use of EPPO Diagnostic Protocols*.[1]

Specific approval and amendment: Approved in 2014–09. Revised in 2021–06.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Pseudomonas syringae pv. *actinidiae* is the causal agent of the bacterial canker of *Actinidia* spp., the most damaging and severe disease of cultivated kiwifruits. *Actinidia* spp. are the only known cultivated host plant species of *P. syringae* pv. *actinidiae*. Wild reported hosts are *Alternanthera philoxeroides*, *Paulownia tomentosa* and *Setaria viridis* (EPPO, 2020). Since its first report in Japan in 1989 on *Actinidia* spp. (Serizawa et al., 1989; Takikawa et al., 1989) the disease has been reported in different countries where *Actinidia* is grown (EPPO, 2020), including major *Actinidia* producing countries such as Italy and New Zealand. *P. syringae* pv. *actinidiae* populations show genetic variability. Five different biovars, present in different areas of the world, are characterized by differences in virulence (Chapman et al., 2012; Vanneste et al., 2013; Sawada & Fujikawa, 2019). Biovar 1 groups the strains associated with the initial epidemics of bacterial canker observed in Japan (1984–1989) and Italy (1992). Biovar 2 has only been isolated in South Korea. Biovar 3 corresponds to a highly virulent population, responsible for severe outbreaks observed since 2008. Biovar 4, which originally included low virulence strains, was subsequently removed and a new pathovar of *P. syringae* pv. *actinidifoliorum* was established (Cunty et al., 2015). Strains differing from the four existing biovars were found in local areas of Japan and classified as biovars 5 (Fujikawa & Sawada, 2016) and 6 (Sawada et al., 2014).

This diagnostic protocol provides information on the diagnosis of *P. syringae* pv. *actinidiae* biovars 1, 2 and 3. When information is available on biovars 5 and 6 this is provided, but these are of limited distribution. The tests included allow a distinction of *P. syringae* pv. *actinidiae* from *P. syringae* pv. *actinidifoliorum*.

Flow diagrams describing the diagnostic procedure for *P. syringae* pv. *actinidiae* are presented in Figure 1a,b.

[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.

2 | IDENTITY

Name: *Pseudomonas syringae* pv. *actinidiae* (Takikawa et al., 1989).
Synonyms: None.
Taxonomic position: Proteobacteria, Gamma subdivision, Order Pseudomonadales, Family Pseudomonadaceae.
EPPO Code: PSDMAK.
Phytosanitary categorization: EPPO A2 list no. 370. EU emergency measures 2020/885 June 2020.

3 | DETECTION

P. syringae pv. *actinidiae* can be detected on both symptomatic and symptomless, latently infected, aerial plant parts. The pathogen may occasionally be detected in roots of symptomatic plants (Mazzaglia et al., 2010; Abelleira et al., 2014). The pathogen can be also detected on contaminated pollen, where it may survive epiphytically (Vanneste et al., 2011a; Stefani & Giovanardi, 2011; Gallelli et al., 2011b).

Commodities which may be infected by *P. syringae* pv. *actinidiae* are plants for planting (including plantlets and plant cuttings), bud chips and pollen.

Harvested fruits are very rarely infected, but this may happen when fruits are grown and harvested in heavily infected orchards (Gallelli et al., 2011b; Stefani & Giovanardi, 2011).

3.1 | Symptoms

Symptoms of the disease caused by *P. syringae* pv. *actinidiae* are easily observed on aerial parts, such as trunks, leaders, canes, leaves, flowers and fruits. Mazzaglia et al. (2010) reported that browning or darkening of the vascular tissues underlying the root cortex may occasionally be observed. On trunks, leaders and canes, cankers may be observed in autumn, late winter and throughout the growing season. Cankers are moist

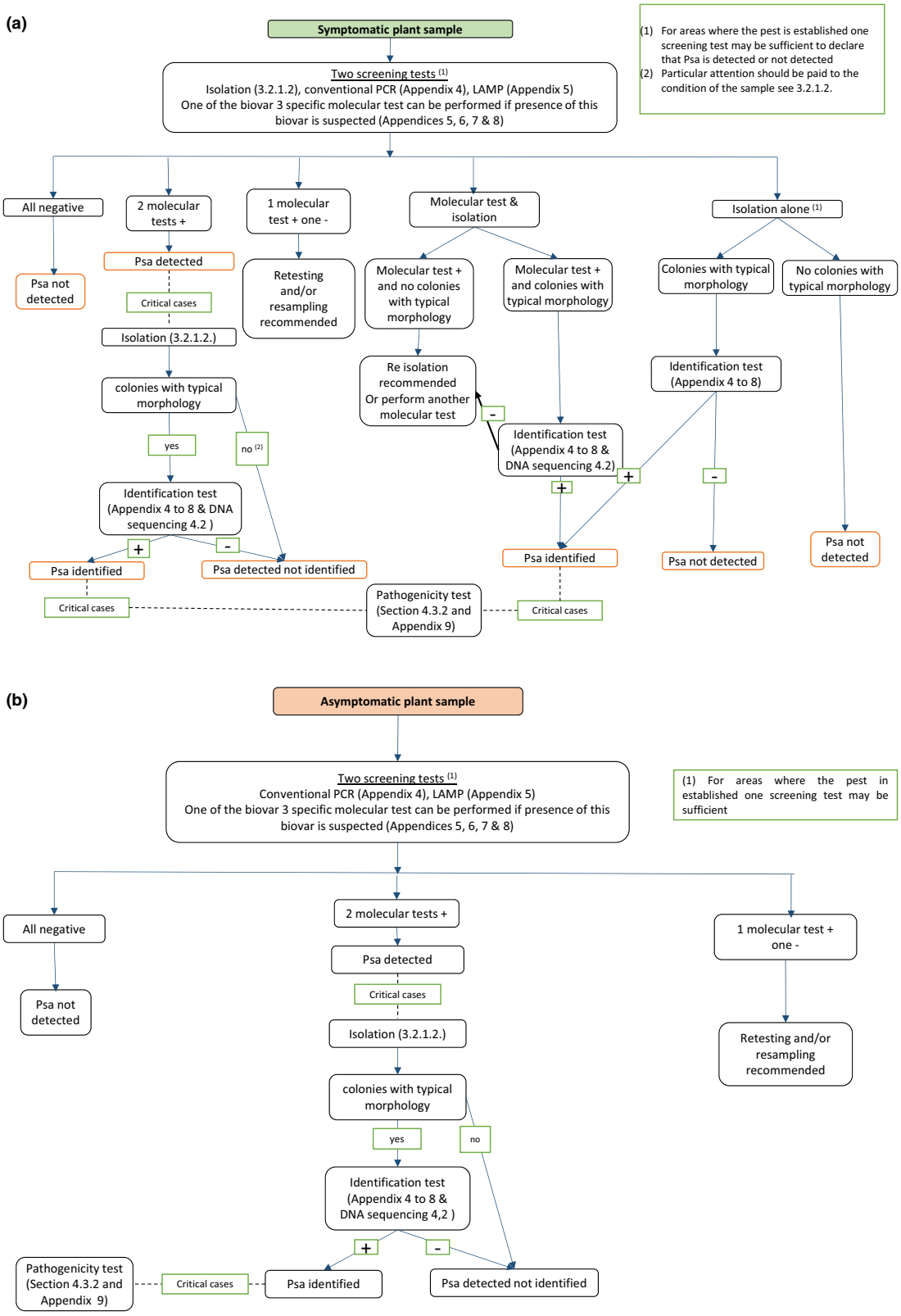

FIGURE 1 (a) Flow diagram for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* (Psa) in samples of symptomatic *Actinidia* spp. (b) Flow diagram for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* (Psa) in samples of asymptomatic *Actinidia* spp. [Colour figure can be viewed at wileyonlinelibrary.com]

in late winter and spring, and ooze bacterial exudates and plant sap. On trunks and leaders, the ooze turns from white to a reddish-brown colour. Infected canes and shoots may show wilting and blight of leaves. Lenticels on affected trunks, leaders and canes are usually larger and more elevated than in symptomless plants. On leaves, symptoms appear as small, angular water-soaked areas, which later become necrotic and turn dark brown; a chlorotic halo may sometimes be observed around the necrotic foliar spots. Similar symptoms might be caused on leaves by *P. syringae* pv. *syringae*, a common bacterial pathogen present in several areas worldwide on fruit crops, especially stone fruits and pome fruits. Another bacterium, *P. viridiflava*, has been reported to cause leaf necrotic spots, often coalescing in necrotic areas, but not showing chlorotic haloes. Affected buds and flowers may turn brown, become necrotic and then fall to the ground (Balestra et al., 2008). Similar browning and necrotising of buds and flowers may be caused by *P. syringae* pv. *syringae*. Affected fruitlets are misshapen, smaller in size than healthy fruits and may develop a necrotic apex; they usually fall during late spring or early summer or are manually detached and thrown away during pre-harvest selection. Fruits may collapse as a consequence of wilting of branches; affected fruits are not marketable. A description of symptoms according to the period of the year is available at https://upload.eppo.int/download/745oaa282c610.

3.2 | Screening tests

3.2.1 | Detection in symptomatic plants

Direct isolation (section 3.2.1.2) and molecular tests (section 3.2.1.3) can be used as screening tests for plant material showing symptoms. *P. syringae* pv. *actinidiae* is detected when positive results are obtained from two different tests. When the pest is established in an area, a single test may be performed to conclude if the pest is detected or not in the sample.

Two test performance studies showed that isolation has a lower analytical and diagnostic sensitivity than the molecular tests (Loreti et al., 2014, 2018).

3.2.1.1. Test sample requirements

Plant samples for the detection of *P. syringae* pv. *actinidiae* can include trunk and leader parts, canes and shoots, including those showing cankers or other lesions, leaves, buds, flowers or fruits with necrotic spots or necrotic lesions. Plant samples from the field or glasshouse should be processed as soon as possible after collection. 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 (as mentioned above) for up to 9 days to be processed for additional verification.

Extraction procedures for symptomatic material are presented in Appendix 1.

3.2.1.2. Isolation

Isolation of *P. syringae* pv. *actinidiae* from symptomatic samples is relatively easy because of the usually high number of culturable bacteria present. However, isolation may be difficult when symptoms are very advanced or when the environmental conditions after infection are not favourable for bacterial multiplication (e.g. sample collected in the middle of summer) or when a copper treatment has recently been performed in the orchard; in such cases it is preferable to use molecular tests.

In order to avoid the growth of too many saprophytes and contaminants, which may overgrow the agar plates within 36–48 h of plating, isolation should preferably be attempted on semi-selective medium, in particular modified nutrient sucrose agar (NSA) or modified King's B medium (KB) (Mohan & Schaad, 1987) in which NSA and KB are supplemented with boric acid and two antibiotics: cycloheximide and cephalexin. Media are described in Appendix 2. Colonies grow more slowly on media containing antibiotics, compared to the same media without them.

Isolation from plant extracts is done by dilution plating of 30–50 µL of the plant extract or solution prepared from bleeding cankers or bleeding sap obtained as indicated in Appendix 1 and its 10- and 100-fold dilutions onto agar plates (modified NSA and/or modified KB). Plates are then kept for 3–6 days at approximately 24°C in an incubator. As a control, 10–15 µL of a suspension of a *P. syringae* pv. *actinidiae* control strain (see reference material) at a concentration of approximately 10^4–10^5 cfu mL^{-1} should be plated onto the same medium. After 3 days, pale, pinhead colonies may appear on plates, which should be observed until the sixth day after plating. Suspect colonies may require a further purification step by re-streaking on modified NSA or modified KB before proceeding to further identification.

Colony morphology. On modified NSA colonies are smooth, elevated or convex, round and with entire margins, pearly whitish in colour (Figure 2a). Colonies are shiny when fresh, then becoming pale ivory whitish. *P. syringae* pv. *syringae* is difficult to distinguish just by visual observation of the agar plates, especially during the first days.

On modified King's B medium, colonies appear smooth, flat, with entire or slightly lobed margins, pearly whitish-yellowish in colour, 4–5 mm wide after 4–5 days, showing a tiny, white spot at the centre of the colony (Figure 2b).

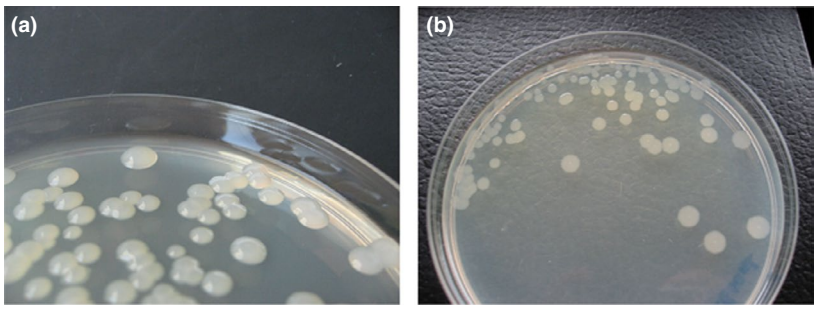

FIGURE 2 Morphology of *Pseudomonas syringae* pv. *actinidiae* colonies grown for 5 days on (a) NSA supplemented with antibiotics or (b) King's B medium supplemented with antibiotics. (Courtesy: E. Stefani, Universita di Modena e Reggio Emilia, IT)

3.2.1.3. Molecular tests

The molecular tests for screening recommended below have been selected based on test performance study results reported in Loreti et al. (2018) except for the LAMP Ruinelli et al. (2017). DNA extraction is described in Appendix 3.

Molecular tests together with the biovars detected by the tests are presented in Table 1.

Although performance characteristics are available for the PSA LAMP Ruinelli et al. (2017), unlike other tests mentioned in this section no interlaboratory comparison was performed. Based on in silico analysis this test can also detect biovars 5 and 6 (see Appendix 5). Although it was not evaluated during the TPS (Loreti et al., 2018) and is not widely used in the EPPO region, this test is included in the present Standard as (along with the test described above) it can detect a range of biovars.

- Tests detecting biovar 3

  When the presence of biovar 3 only is suspected the following tests can be performed.

  - Real-time PCR tests developed specifically for *P. syringae* pv. *actinidiae* biovar 3 (the most virulent biovar): Gallelli et al. (2014) described in Appendix 6 and Andersen et al. (2018) described in Appendix 7.
  - PSA3 LAMP Ruinelli et al. (2017) specific to biovar 3 described in Appendix 5.
  - Conventional simplex PCR Gallelli et al. (2014) described in Appendix 8.

The multiplex PCR (Balestra et al., 2013) and the nested PCR (Biondi et al., 2013) are not recommended as they gave several undetermined or false-positive results in an interlaboratory study (Loreti et al., 2018) when used as identification tests on colonies.

Conventional and real-time PCR kits have been developed by Qualiplante based on Gallelli et al. (2011a, 2014), respectively. A verification was performed by Qualiplante (data not published) and the performance characteristics of the kits are the same as the original publications (C. Chatillon, pers. comm., 2020).

3.2.1.4. Serological tests

A rapid serological test (immunochromatographic strips) based on the use of a polyclonal antiserum to the effector *Hopz5* was recently developed for *P. syringae* pv. *actinidiae* detection (Chen et al., 2018). However, no interlaboratory validation data is available and there is no experience in the region with this test.

Performance characteristics are reported in the publication.

Analytical sensitivity may reach 2.2×10^3 cfu mL^{-1}.

Analytical specificity.

Exclusivity 100% (based on 10 different bacterial species: *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *theae*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas koreensis*, *Bacillus subtilis*, *Bacillus megaterium*, *Ralstonia solanacearum*, *Erwinia rhapontici*, *Pseudomonas syringae* pv. *syringae*).

TABLE 1 Molecular tests and biovars detected

		Biovar 1	Biovar 2	Biovar 3
Conventional duplex PCR Gallelli et al. (2011a)	Appendix 4	X	X	X[a]
LAMP Ruinelli et al. (2017) PSA LAMP	Appendix 5	X	X	X[a]
LAMP Ruinelli et al. (2017) PSA3 LAMP	Appendix 5			X
Real-time PCR Gallelli et al. (2014)	Appendix 6			X
Real-time PCR Andersen et al. (2018)	Appendix 7			X
Conventional simplex PCR Gallelli et al. (2014)	Appendix 8			X

[a]Based on *in silico* analysis this test can also detect biovars 5 and 6.

Inclusivity 100% (evaluated on two *P. syringae* pv. *actinidiae* strains biovar 3).

Diagnostic sensitivity: 84%, 28 field samples gave a positive result out of 33 total samples tested (leaf and limb).

3.2.2 | Detection in symptomless plant material

Molecular tests (section 3.2.1.3) can be used as screening tests. *P. syringae* pv. *actinidiae* is detected when positive results are obtained from two different tests. When the pest is established in an area, a single test may be performed to conclude if the pest is detected or not in a sample.

3.2.2.1. Test sample requirements

Detection of *P. syringae* pv. *actinidiae* on plants without symptoms (latent infection) requires a composite sample. The use of a composite sample enhances detection of *P. syringae* pv. *actinidiae*. Dormant cuttings, budwood, shoots or twigs, plantlets and *in vitro* micro propagated plants (vitroplants) are susceptible to infection by *P. syringae* pv. *actinidiae*. Sampling in late winter has allowed detection of the pathogen in nurseries, but more information would be needed to establish the optimal period of sampling. Detection on fruit is erratic (Gallelli et al., 2011b; Stefani & Giovanardi, 2011). Pollen may carry *P. syringae* pv. *actinidiae* and disseminate it into kiwi orchards (Gallelli et al., 2011b; Stefani & Giovanardi, 2011; Vanneste et al., 2011). It has been demonstrated to spread the disease (Tontou et al., 2014) and can also be tested.

Detailed descriptions of different extraction procedures for different asymptomatic plant materials are given in Appendix 1.

Concentration step. A concentrated plant extract should be prepared as described in Appendix 1 to increase the concentration of viable *P. syringae* pv. *actinidiae* cells. The concentrated plant extract (usually 1.5–3 mL) should be divided into two parts: one is used to perform the detection tests and the second is stored for possible further confirmation analyses at –80°C by adding 15–25% of sterile glycerol.

3.2.2.2. Molecular tests

The molecular tests described in Section 3.2.1.3 can be performed on the concentrated plant extract. DNA extraction is described in Appendix 3.

4 | IDENTIFICATION

The identification of *P. syringae* pv. *actinidiae* should be performed on two to five colonies with typical morphology using at least one of the tests described below. In the case of critical samples two tests (4.1 and 4.2) should be performed based on different biological principles. At least five colonies with typical morphology should be tested before *P. syringae* pv. *actinidiae* is declared not identified.

4.1 | Molecular tests

The molecular tests presented in Table 1 together with the biovars detected can be used.

4.2 | DNA sequencing

Assignment of biovars 1, 2 and 3 of *P. syringae* pv. *actinidiae* is possible with a multilocus sequence analysis (MLSA) based on four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) (Cunty et al., 2015). The MLSA scheme based on seven genes (*acnB*, *cts*, *gapA*, *gyrB*, *pfk*, *pgi* and *rpoD*) (Sarkar & Guttman, 2004) can be used to discriminate all Psa biovars (Sawada et al., 2014).

4.3 | Other tests

4.3.1 | Biochemical and physiological tests

Arbutin and aesculin hydrolysis can be used to discriminate *P. syringae* pv. *syringae* (both positive) from *Pseudomonas syringae* pv. *actinidiae* (both negative) before performing molecular tests (Lelliot & Stead, 1987). Other biochemical and metabolic characteristics of *P. syringae* pv. *actinidiae* are described by Takikawa et al. (1989) (based on the American Society for Microbiology Committee on Bacteriological Technic, 1957).

4.3.2 | Pathogenicity tests

A pathogenicity test may be required for the completion of the diagnostic procedure, e.g. for critical cases, see PM 7/76 (EPPO, 2018), and may be conveniently carried out on *Actinidia chinensis* (cultivars Erica, Hort16A) or *Actinidia deliciosa* (cultivar Hayward or Soreli).

In vivo *tests*

Inoculation is done by spraying plantlets of an appropriate size. Information on pathogenicity tests is provided in Appendix 9.

In vitro *tests*

Two different *in vitro* procedures can be performed. Details of the procedures are reported in Appendix 9.

5 | REFERENCE MATERIAL

P. syringae pv. *actinidiae* (Takikawa et al., 1989).

Pathotype strain: NCPPB 3739 = ICMP 9617 = CFBP 4909 – Japanese isolate.

ISF 8.43 – Italian isolate, high virulent type.

Other strains which might be used as positive controls are CFBP 7285, CFBP 7286, CFBP 7287 and CRA-PAV 1530 (all Italian isolates).

The following collections can provide different *P. syringae* pv. *actinidiae* reference strains:

1. National Collection of Plant Pathogenic Bacteria (NCPPB), FERA, York, GB (https://www.fera.co.uk/ncppb)
2. International Center for Microbial Resources – French Collection for Plant-associated Bacteria (CIRM-CFBP), IRHS–INRAE Beaucouzé (FR) (https://www6.inrae.fr/cirm/CFBP-Bacteries-associees-aux-Plantes).

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

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

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

E. Stefani, Universita di Modena e Reggio Emilia (IT), e-mail: emilio.stefani@unimore.it.

S. Loreti, Research Centre for Plant Protection and Certification (CREA-DC), via C.G. Bertero 22, 00156 Roma (IT), e-mail: stefania.loreti@crea.gov.it.

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A. Cunty, ANSES, Laboratoire pour la Santé des Végétaux, 7 rue Jean Dixméras, 44049 Angers Cedex (FR), e-mail: francoise.poliakoff@anses.fr.

9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Standard, or any of the tests included, or if you can provide additional validation data for tests included in this standard that you wish to share, please contact diagnostics@eppo.int.

10 | STANDARD REVISION

An annual review process is in place to identify the need for revision of Diagnostic Standards. Standards 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 E. Stefani (Department of Life Sciences, University of Modena & Reggio Emilia, Reggio Emilia, IT) and further reviewed by S. Loreti (CRA, IT). The present revision was prepared by S. Loreti (CREA-DC), J. Costa (IPN, PT), A. Cunty (ANSES, FR), E. Stefani (University of Modena & Reggio Emilia, IT) and J.F. Pothier (ZHAW, CH), and was reviewed by the Panel on Diagnostics in Bacteriology.

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APPENDIX 1 −EXTRACTION PROCEDURES FOR DIFFERENT PLANT MATERIALS

(see Appendix 2 for buffers)

1. Extraction from symptomatic plant material

1.1 Symptomatic material

- Bleeding cankers: A sample may be easily taken by collecting a few ooze drops with a cotton swab. The cotton swab is dipped into a tube containing 5 mL of sterile saline solution or sterile water and the tube is shaken for a few seconds. This suspension can be used for direct isolation on agar medium (see 3.2.1.2). Old exudates (generally rusty red to brown) are not suitable for an effective *P. syringae* pv. *actinidiae* isolation.
- Lesions (margins of a canker, flowers, leaves, buds or fruits): Three to five small pieces 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. Alternatively, fragments of infected tissues can be ground in a sterile mortar containing 2–5 mL of sterile saline solution (or sterile water) and allowed to soak for 2–3 min. From leaves, large, necrotic areas are generally not suitable for pathogen isolation:

preference should be given to small spots with a water-soaked halo. These small leaf pieces should be excised with a sterile scalpel and then either comminuted in a mortar or shredded with a few drops of sterile saline solution (or sterile water) or put into a tube containing 1–2 mL of sterile saline solution (or sterile water) and allowed to soak for 5–10 min.

1.2 Bleeding sap

Bleeding sap (particularly useful for early detection at the end of winter/early spring): About 10 mL of bleeding sap is collected from a single plant by removing the distal 2–3 cm segment of a branch, previously sterilized with ethanol, and by collecting the sap from the open wound in a sterile 15 mL tube. Samples should be maintained in a cold container till centrifugation at 10 000*g* for 20 min to recover a pellet of bacterial cells, which is resuspended in 1 mL of sterile distilled water.

2. Extraction from asymptomatic plants

2.1 For dormant cuttings, budwood, shoots or twigs different procedures are described hereafter

2.1.1 Dormant cuttings

Different sampling procedures are in place in countries where the pest occurs. The procedure presented here is the one adopted in Italy. One hundred bud chips are collected from 20 dormant cuttings of 30 cm in length and crushed for 2 min. Crushing can be done with a stomacher or a hammer. Fifteen millilitres of phosphate buffered saline solution (PBS) are added and the plant material is crushed again for an additional 1 min. Again, 15 mL of PBS is added followed by a last crushing step for an additional 1 min (three crushing steps for a total of 4 min). The fluid is then filtered through a sterile gauze and centrifuged at 10 000 g for 20 min at approximately 4°C. The supernatant is discarded and the pellet resuspended in 1–2 mL of sterile PBS.

2.1.2 Nursery plants and plants growing in the field

The laboratory sample consists of 30 shoots/twigs of 10 cm in length randomly selected; disinfection of cutting tools (i.e. using quaternary ammonium salts or 70% ethanol) should be carried out between plants. If more than one cultivar is available each should be tested separately. Leaves should be removed, and each shoot/twig cut into small pieces (total 100 pieces of about 2–3 cm). These should be shaken in 300 mL of PBS-Tween in Erlenmeyer flasks on a rotary shaker at 125 rpm for 1.5 h at room temperature. The washing fluid should then be filtered through a sterile gauze and centrifuged at 10 000 g for 20 min at approximately 4°C. The resulting pellet should then be suspended in 1–2 mL of PBS.

Dormant cuttings can also be processed in the same way.

Samples of bleeding sap may also be collected (see 1.2 above).

2.2 *In vitro* plants

A laboratory sample consists of three jars (15–25 plantlets each jar). Ten plantlets should be randomly chosen from three jars to make the test sample, then the basal part of the chosen plantlets should be cut away and the upper part finely chopped and put into an Erlenmeyer flask with an appropriate volume of PBS-Tween to cover the plant tissue. Flasks should then be put on a rotary shaker (125 rpm) for 60 min at room temperature. The fluid should then be filtered through a sterile gauze and centrifuged as described in Section 2.1. The supernatant should be discarded and the pellet resuspended in 1–2 mL of sterile PBS.

2.3 Plantlets

The laboratory sample is composed of 100 plantlets out of a lot of up to 10 000. Plantlets should be cut at the base, thoroughly washed under tap water, rinsed with sterile deionized water and blotted dry. For each plantlet a segment of 1 cm is cut from the lower part of the stem and the test sample consists of the resulting 100 segments. Segments are roughly crushed in a sealed bag after adding 5–10 mL of sterile saline solution. After crushing, an additional 20 mL of sterile saline solution should be added and the sample left to soak for 5–10 min. The plant extract should then be filtered through a sterile gauze and then centrifuged at 10 000 g for 20 min at approximately 4°C. After centrifugation, the supernatant is discharged and the pellet resuspended in 1–2 mL of sterile saline solution.

2.4 Pollen

From pollen lots up to 500 g the laboratory sample should be approximately 1.5 g. The 1.5 g sample should be put into a 50 mL vial and 20 mL of sterile saline solution added. The vial should be shaken at 120 rpm for 60 min and allowed to settle for 20 min or centrifuged at 180 g for 5 min. The supernatant should then be gently filtered into a clean vial through a sterile gauze and centrifuged at 10 000 g for 20 min at approximately 4°C. After centrifugation the supernatant is discharged and the pellet resuspended in 1–2 mL of sterile saline solution.

APPENDIX 2 – BUFFERS AND MEDIA

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

1. Buffers

Sterile saline solution 0.90 w/v of NaCl. Dissolve 9 g of NaCl in 1 L of deionized water. Sterilize by autoclaving.

10 mM phosphate buffered saline (PBS) solution

NaCl	8 g
KCl	0.2 g
$Na_2HPO_4 \cdot 12\ H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 and autoclave. If Tween is to be added to PBS (0.1%) then add 1 mL of sterile Tween 20 to 1 L of PBS and shake the buffer on a rotary shaker for 1 h at room temperature.

2. Media

Nutrient sucrose agar (NSA) (Crosse, 1959)

Nutrient broth	8 g
Sucrose	50 g
Bacteriological agar	18 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 and autoclave. If antibiotics are to be added then prepare NSA in 900 mL of distilled water, autoclave, cool to about 50°C and then add 100 mL of a sterile 1.5% boric acid aqueous solution, 8 mL of a 25 mg mL^{-1} solution of cycloheximide in 70% ethanol and 8 mL of a 10 mg mL^{-1} aqueous solution of cephalexin.

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

King's medium B (KB) (King et al., *1954), modified according to Mohan and Schaad (1987).*

Proteose peptone N. 3	20 g
Glycerol	10 mL
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Bacteriological agar	15 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 and autoclave.

If antibiotics are to be added then prepare KB in 900 mL of distilled water, autoclave, cool at about 50°C and then add 100 mL of a sterile 1.5% boric acid aqueous solution, 8 mL of a 25 mg mL^{-1} solution of cycloheximide in 70% ethanol and 8 mL of a 10 mg mL^{-1} aqueous solution of cephalexin.

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

Nutrient glucose agar (NGA) (Schaad & Forsters, 1985), modified

Nutrient agar	28 g
Glucose	2.5 g
Distilled water	To make up to 1 L

Adjust pH to 7.2 and autoclave.

APPENDIX 3 – DNA EXTRACTION

Plant extracts

Extraction of DNA from plant extracts and from ooze drops or bleeding sap for molecular analyses can be achieved using standard commercial kits.

Several commercial kits are widely used and validated in several European laboratories to process samples from different plant species such as DNeasy Plant Mini Kit-based extraction (Qiagen), Macherey-Nagel NucleoSpin Plant II, etc. Manufacturer's instructions should be followed.

Single colonies

For identification purposes of putative *P. syringae* pv. *actinidiae*, a single colony of a fresh pure culture is suspended in 1 mL of PCR grade water or 0.9 mL of PCR grade water, supplemented with 0.1 mL of a 0.5 M NaOH solution. The suspension is then boiled for 4 min at approx. 95°C and immediately chilled on ice for 10 min.

Alternatively, DNA extraction kits can be used.

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

APPENDIX 4 – CONVENTIONAL DUPLEX PCR (GALLELLI ET AL., 2011A)

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

1. General information

1.1 This test distinguishes *P. syringae* pv. *actinidiae* from other genetically related *P. syringae* pathovars (Gallelli et al., 2011a). The test detects biovars 1, 2 and 3. An *in silico* analysis showed that the primers KN-F/R have a 100% homology with a deposited homologous sequence of biovar 5, whereas the avrD1 target (primers AvrDdpxF/R) has a 99% homology shared between biovar 1, 2, 3 and 6.

1.2 The target sequences for primer pair KN-F/R were obtained by RAPD and RFLP analyses of *P. syringae* pv. *actinidiae* genome, and for the primer pair AvrDdpxF/R from the sequence of the *avrD1* gene.

1.3 Oligonucleotides

Primer	Sequence	Amplicon size
KN-F	5′-CACGATACATGGGC TTATGC-3′	492 bp
KN-R	5′-CTTTTCATCCACA CACTCCG-3′	
AvrDdpx-F	5′-TTTCGGTGGTAAC GTTGGCA-3′	230 bp
AvrDdpx-R	5′-TTCCGCTAGGTGAA AAATGGG-3′	

1.4 Enzyme

The test performance study was performed with polymerases Platinum Taq (Invitrogen) and Immolase DNA Polymerase (Bioline).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: plant, ooze drops or bleeding sap extracts, or pure culture suspension

2.1.2 See Appendix 3 for DNA extraction procedures from plant, ooze drops or bleeding sap and culture suspension

2.2 Polymerase chain reaction (PCR)

For identification purposes, template DNA is 2 µL of boiled bacterial suspension (at about 10^8 cfu mL^{-1}). For plant, ooze drops or bleeding sap extracts, obtained as explained above, template is 5 µL of DNA extract and a 10-fold dilution.

2.2.1 Master mix prepared according Gallelli et al. (2011a).

The DNA polymerase should always be a 'hot start' polymerase.

2.2.2 PCR cycling conditions: 95°C for 3 min, 30 cycles of 94°C for 30 s, 63°C for 45 s, 72°C for 50 s and a final step of 72°C for 5 min. PCR amplification is illustrated in Figure 3.

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 *Actinidia* plant matrix or if not available clean extraction buffer.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	To make up to 50 µL	NA
PCR buffer	10×	5.0	1×
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	10 mM	1.0	0.2 mM
Primer KN-F	25 µM	1.0	0.5 µM
Primer KN-R	25 µM	1.0	0.5 µM
Primer AvrDdpx-F	25 µM	0.8	0.4 µM
Primer AvrDdpx-R	25 µM	0.8	0.4 µM
Platinum Taq DNA polymerase	5 U μL^{-1}	0.5	0.05 U μL^{-1}
DNA (bacterial suspensions)		5	15–25 ng μL^{-1}
Or DNA (plant, ooze drops or bleeding sap extract)		2	20 ng μL^{-1}
Total		50.0	

Abbreviation: NA, not applicable.

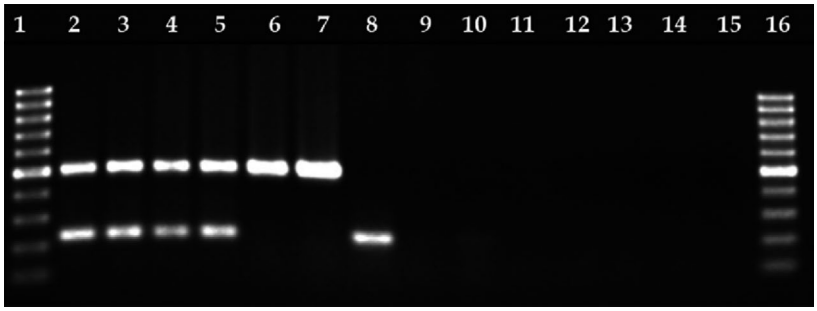

FIGURE 3 Gel electrophoresis analysis of duplex-PCR products from bacterial suspension (10^8 cfu mL^{-1}). Lanes 2–5, *P. syringae* pv. *actinidiae* ISF Act.1, ISPaVe 019, ISPaVe 020, NCPPB 3740; lanes 6–7, *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *theae* CFBP 4097; lane 8, *Pseudomonas avellanae* NCPPB 3872; lane 9–14, *P. syringae* pv. *phaseolicola* 1448A, *P. syringae* pv. *glycinea* ISPaVe 1155, *P. syringae* pv. *syringae* OMP-BO 4250,1, *P. syringae* pv. *papulans* NCPPB 2848, *P. viridiflava* OMP-BO 4254A,1, *P. syringae* pv. *syringae* OMP-BO3909B,1; lane 15, water control. Lanes 1 and 16: M, molecular markers (Gene RulerTM 100 bp DNA ladder, Fermentas). From Gallelli et al. (2011a)

- 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 an *Actinidia* plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC) two bands of 492 and 230 bp are visualized and if relevant two bands of 492 and 230 bp are visualized for the IC.

When these conditions are met:

- A test will be considered positive if two bands of 492 and 230 bp are visualized.
- A test will be considered negative if no band or a band of a different size, or only one band of the two expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data are provided by Loreti et al. (2014, 2018). Data from Loreti et al. (2014) were produced in a TPS performed with seven Italian laboratories. The matrices tested were leaves, pollen and canker tissue from *Actinidia chinensis* and the test used plant extracts and pure cultures.

Data from Loreti et al. (2018) were produced by a TPS performed within the PSA-DID project (ERA-NET 266505 EUPHRESCO II). The matrices tested were woody tissue from *Actinidia deliciosa* cv. Hayward and the tests were assessed on plant extracts and pure cultures. The TPS involved 12 laboratories (nine European, two from New Zealand and one from Turkey). The sample extracts consisted of woody samples spiked (or not) with suspensions of the bacterial strain Psa ISF 8.43 (biovar 3) containing 10^7 cfu mL^{-1} (two replicates), 10^5 cfu mL^{-1}, 10^4 cfu mL^{-1}, 10^3 cfu mL^{-1} (three replicates) and 0 cfu mL^{-1} (two replicates).

TABLE 2 The proportion of the number of samples testing positive for *P. syringae* pv. *actinidiae* with the total number of each strain tested (seven laboratories participating in the test performance study)

Psa CRA-FRU 8.53	6/7
Psa CFBP 7287	6/7
Psa CRA-PAV 1583	7/7
Psa NCPPB 3740	7/7
P. viridiflava OMP-BO 4254A,1	0/7
P. s. pv. *syringae* OMP-BO 3909B,1	0/7
P. s. pv. *tomato* NCPPB 2563	0/7
P. s. pv. *theae* CFBP 4097	0/7
P. avellanae NCPPB 3872	0/7
CRA-PAV 1686	0/7
CRA-PAV 1687	0/7
CRA-PAV 1688	0/7
CRA-PAV 1689	0/7

4.1 Analytical sensitivity data

- After Gallelli et al. (2011a): 2 × 10 cfu per PCR reaction.

4.2 Analytical sensitivity data

- From Loreti et al. (2014): 10^3 cfu mL^{-1} for pollen extracts.
- From Loreti et al. (2018): 10^4 cfu mL^{-1} for wood extracts.

4.3 Analytical specificity data

- From Loreti et al. (2014): see Table 2.
- From Loreti et al. (2018)
  Inclusivity: 100% (tested on 30 *P. syringae* pv. *actinidiae* strains).
  Exclusivity: 95% (tested on 20 bacterial strains of different Pseudomonads and of *P. syringae* strains associated with kiwifruit matrices). A cross-reaction was observed with *Pseudomonas avellanae*, a pest of *Corylus avellana*.
- From Gallelli et al. (2011a)
  Inclusivity: 100% (tested on 14 *P. syringae* pv. *actinidiae* strains).
  Exclusivity: 100% (tested on 37 bacterial strains of different bacterial species).

4.4 Data on repeatability

- From Loreti et al. (2018): 93%

4.5 Data on reproducibility

- From Loreti et al. (2014)

Duplex PCR from plant extracts (pollen and leaf): 98%
Duplex PCR from bacterial cultures: 95.5%

- From Loreti et al. (2018)

Duplex PCR from wood extracts: 86%

4.6 Data on diagnostic sensitivity: 90.9%

4.7 Data on diagnostic specificity: 95.5%

4.8 Data on accuracy: 93.2%

APPENDIX 5 –LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) (RUINELLI ET AL., 2017)

The test below is described as it was carried out to generate the validation data from Ruinelli et al. *(2017) provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.*

1. General information

1.1 Two LAMP tests have been developed for the detection and identification of *P. syringae* pv. *actinidiae* biovars 1, 2 and 3 (PSA LAMP) and for the specific discrimination of *P. syringae* pv. *actinidiae* biovar 3 (PSA3 LAMP), respectively. Based on *in silico* analysis the PSA LAMP test would detect biovars 5 and 6 as the target is 100% identical in the two biovars genomes (MAFF 212056, MAFF 212061, MAFF 212134 and MAFF 212141)

1.2 The tests were developed by Ruinelli et al. (2017).

1.3 The target sequences are coding DNA sequence (CDS) for hypothetical proteins.

1.4 Primers used for the LAMP tests are shown in Table 3.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: leaves, lenticel, buds and 1-year-old twigs, colonies

2.1.1.1 Plant material

Two different procedures have been performed in Ruinelli et al. (2017):

(i) Plant materials collected from kiwifruit plants are placed into nutrient broth in agitation overnight at 220 rpm at 24°C. From this suspension, 1 mL is transferred to a 1.5 mL Eppendorf tube and centrifuged at 20 000 *g* for 5 min. The pellet is resuspended in 200 µL of 50 mM NaOH. Tubes are kept in a water bath at 95°C for 15 min and centrifuged at 20 000 *g* for 5 min. The supernatant is used to perform the LAMP tests.

(ii) DNA is extracted from 1 cm^2 of leaf tissue using the ZR Fungal/Bacterial DNA MiniPrep (Zymo-Research) according to the manufacturer's instructions.

2.1.1.2 Colonies see Appendix 3.

2.2 LAMP

For identification purposes use 1.25 µL of template DNA from boiled bacterial suspension (at about 10^8 cfu mL^{-1}). For plant extracts, obtained as explained above, 1.25 µL of DNA extract and a 10-fold dilution should be used as a template.

2.2.1 Master mix: Isothermal MMX (Optigene Ltd) was used in the development of this test (see Ruinelli et al., 2017).

TABLE 3 LAMP primers for the detection of all *P. syringae* pv. *actinidiae* biovars (PSA LAMP) and the discrimination of *P. syringae* pv. *actinidiae* biovar 3 (PSA3 LAMP)

Target	Primer	Sequence (5′–3′)
PSA LAMP	F3	GGCTCTCCTAGCAAGCATAC
	B3	TGAGAAGGGACGCAACCA
	FIP	CGGATTCGCAACGCTCCAAGATCTG CTGAGCACGTTGGTC
	BIP	ACTCTTCCGCAACGAGTTTGGGTCC CCACATGGAGTTGTCT
	loopF	TGCCGATCGAGTATCCATTTCCT
	loopR	AGCCTTTTCCGAACGGTCT
PSA3 LAMP	F3	GCTATGGAATCCATTGCGGT
	B3	CGCATCTGCTGGATCATCC
	FIP	ATCCCTTGCCCAGCACGAACATGAG GTCGAGGTGTCTGA
	BIP	ATCCACAGTGGGTACACGGACGGGG GCACCTTCTTTCTTGG
	loopF	GGCCTTTTCAATGCGGTCAATATCC
	loopR	GGTGTCAATCTGGTGGTGCAT

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	Adjust the quantity to make up to 12.5 µL	NA
Isothermal MMX (Optigene Ltd)		7.5	1×
Outers primers (F3-B3)	100 µM	0.02	0.16 µM
Inner primers (FIP-BIP)	100 µM	0.2	1.6 µM
Loop primers (loopF-loopR)	100 µM	0.1	0.8 µM
DNA obtained from bacterial suspensions		1.25	
Or DNA obtained from plant extract		1.25	
Total		12.5	

Abbreviation: NA, not applicable.

2.2.2 PCR cycling conditions: 65°C for 30 min on a Light Cycler 480 Real-Time PCR system (Roche), on GenieII (OptiGene Ltd) or on Illumina Eco Real-Time PCR system (Illumina Inc.); melting curve analysis during cooling from 92°C to 82°C with a temperature decrease of 0.05°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 *Actinidia* plant matrix or, if not available, sterile 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 an *Actinidia* plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons nor fluorescence.
- PIC and PAC (and if relevant IC) should produce an exponential curve.

When these conditions are met

- A test will be considered positive if it produces an exponential curve and revealed a specific peak with a melting temperature of 90.4°C (±0.1) and 88.8°C (±0.1), respectively, for PSA LAMP and PSA3 LAMP.
- A test will be considered negative if it does not produce an amplification curve or if it produces no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data are provided in Ruinelli et al. (2017).

4.1 Analytical sensitivity

Tested on kiwifruit leaves spiked with serial dilutions of *P. syringae* pv. *actinidiae* strain CFBP 7 287 using boiled cells ranging from 10^8 to 10^1 cfu mL^{-1}, 125 cfu per reaction (about 10^4 cfu mL^{-1}).

4.2 Analytical specificity

PSA LAMP

Inclusivity: 100% (tested on 31 *P. syringae* pv. *actinidiae* strains).

Exclusivity: 100% (tested on 56 strains of several *P. syringae* pathovars and on *P. syringae* pv. *actinidifoliorum*).

PSA3 LAMP

Inclusivity: 100% (tested on 20 *P. syringae* pv. *actinidiae* biovar 3 strains).

Exclusivity: 100% (tested on 67 strains of several *P. syringae* pathovars, on *P. syringae* pv. *actinidifoliorum* and on *P. syringae* pv. *actinidiae* biovars 1 and 2).

APPENDIX 6 – REAL-TIME PCR (GALLELLI ET AL., 2014)

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

1. General information

1.1 This real-time PCR is suitable for the detection and identification of *P. syringae* pv. *actinidiae* biovar 3.

1.2 The test was developed by Gallelli et al. (2014).

1.3 The target sequence is a region of the *hrpW* gene.

1.4 Oligonucleotides

Primer name	Sequence	Amplicon size
P3F	5′-GGTTTCGGACACCGCA GGTTCTACCGAG-3′	147 bp
P5R1	5′-CTTCCTGATCCCCGTT ACCCATCGAC-3′	

1.5 The test performance study was performed with various real-time PCR systems.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: plants, ooze drops, bleeding sap or pure culture suspension.

2.1.2 See Appendix 3 for DNA extraction procedures from plant, ooze and culture suspension.

2.2 Real-time polymerase chain reaction (real time PCR)

For identification purposes, template DNA is 2 µL of boiled bacterial suspension (at about 10^8 cfu mL^{-1}). For plant ooze drops or bleeding sap extracts, obtained as explained above, template is 2 µL of DNA extract and a 10-fold dilution.

2.2.1 Master mix: the SsoFast EvaGreen supermix (Bio-Rad) was used in the development of this test (see Gallelli et al., 2014).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	NA	Adjust the quantity to 20 µL depending on DNA concentration	NA
SsoFast EvaGreen supermixes (Bio-Rad)	10×	5.0	2.5×
Primer P3F	25 µM	0.4	0.5 µM
Primer P5R1	25 µM	0.4	0.5 µM
DNA bacterial suspension or plant, ooze drops or bleeding sap		2	20 ng
Total		20.00	

Abbreviation: NA, not applicable.

2.2.2 PCR cycling conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, 72°C for 20 s and a final step of 72°C for 5 min. This programme was optimised for use with a CFX96 real-time PCR (Bio-Rad) thermal cycler. A melting curve analysis is generated during a dissociation run from 55°C to 95°C with a temperature increase of 0.5°C per 0.05 s.

Samples (boiled suspension and boiled plant extract) can be stored at approximately –20°C for several months.

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 *Actinidia* plant matrix or, if not available, sterile 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 an *Actinidia* plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

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

When these conditions are met

- A test will be considered positive if it produces an exponential curve and reveals a specific peak with a melting temperature of 87°C.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data are provided by Gallelli et al. (2014) and by Loreti et al. (2018).

Data from Gallelli et al. (2014) were produced during the development of this test.

Data from Loreti et al. (2018) were produced by a TPS performed within the PSA-DID project (ERA-NET 266505 EUPHRESCO II). The matrices tested was woody tissue from *Actinidia deliciosa* cv. Hayward and the test was assessed on plant extracts and pure cultures. The TPS involved nine European laboratories, two from New Zealand and one from Turkey for a total of 12 laboratories. The sample extract consisted of woody samples spiked (or not) with suspensions of the bacterial strain Psa ISF 8.43 (biovar 3) containing 10^7 cfu mL^{-1} (two replicates), 10^5 cfu mL^{-1}, 10^4 cfu mL^{-1}, 10^3 cfu mL^{-1} (three replicates) and 0 cfu mL^{-1} (two replicates).

4.1 Analytical sensitivity data (from Loreti et al., 2018)

10^3 cfu mL^{-1}

4.2 Analytical specificity data

- From Gallelli et al. (2014)
  Inclusivity: 100% (tested on 40 *P. syringae* pv. *actinidiae* strains).
  Exclusivity: 100% (tested on 43 strains of different bacterial species).
- From Loreti et al. (2018)
  Inclusivity: 100% (tested on seven *P. syringae* pv. *actinidiae* strains).

Exclusivity: 100% (tested on five bacterial strains of different *Pseudomonas* species and of *P. syringae* strains associated with kiwifruit matrices).

4.3 Data on repeatability (from Loreti et al., 2018): 94%

4.4 Data on reproducibility (from Loreti et al., 2018): 91%

4.5 Data on diagnostic sensitivity (from Loreti et al., 2018): 96.4%

4.6 Data on diagnostic specificity (from Loreti et al., 2018): 90%

4.7 Data on accuracy (from Loreti et al., 2018): 93.2%

APPENDIX 7 −REAL-TIME PCR (ANDERSEN ET AL., 2018)

The test below is described as it was carried out to generate the validation data from Andersen et al. *(2018) provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.*

1. General information

1.1 This real-time PCR is suitable for the detection and identification of *P. syringae* pv. *actinidiae* biovar 3. The two target regions permit the specific detection of biovar 3 and enable it to be distinguished from other *P. syringae* pv. *actinidiae* biovars and from *P. syringae* pv. *actinidifoliorum*.

1.2 The test was developed by Andersen et al. (2018).

1.3 The target sequences corresponded to the *hopA1* and *hrpZ5* genes.

1.4 Oligonucleotides.

Target gene	Primer name	Sequence	Amplicon size
hopA1	Psa_A1F2	5′-GCCTCGATGTCGGCGC-3′	132 bp
	Psa_A1 R1	5′-ATTCGATAGAAGAACTTC TTTGCGTTT-3′	
hrpZ5	Psa_Z5 F2	5′-ACAACTTCAGGCTACAAT ACTTACGC-3′	102 bp
	Psa_Z5 R2	5′-CTCAGGATGCGTTTCGG TTAC-3′	

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: plants and pure culture suspension.

2.1.2 DNA extraction procedures from plant and culture suspension was performed by Andersen et al. (2018):

(i) DNA was extracted by either DNeasy Blood and Tissue kit (Qiagen) or Pure gene Yeast/Bacteria kit B: protocol for Gram-negative bacteria (Qiagen), according to the manufacturer's instructions, from a pure culture of *P. syringae* pv. *actinidiae* grown overnight at 28°C.

(ii) Sections of symptomatic leaf tissue (0.1 ± 0.02 g) and cane tissue (0.05 ± 0.005 g) are excised and transferred into sterile 2 mL screw-top tubes. Samples were lysed by mechanical disruption in the presence of 1 mL cetyltrimethylammoniumbromide (CTAB) buffer (3% (w/v) CTAB, 100 mM Tris-HCl, 50 mM EDTA, 1.4 M NaCl and 2% (w/v) PVP-40) and DNA extracted using the InviMag Plant DNA mini kit (Stratec Molecular) and MagMAX Express 96 Instrument (Applied Biosystems) according to the manufacturer's instructions.

2.2 Real time polymerase chain reaction (real time PCR)

2.2.1 Master mix.

The SsoFast EvaGreen supermixes (Bio-Rad) and the LightCycler 480 SYBR Green I Master (Roche Diagnostics) were used in the development of this test (see Andersen et al., 2018).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	Adjust to 10 µL* depending on DNA input	NA
SsoFast EvaGreen supermixes (Bio-Rad) or LightCycler 480 SYBR Green I Master (Roche Diagnostics)	10×	5.0	0.5×
Primer F (Psa_A1F2 or Psa_Z5 F2)	25 µM	0.2	0.5 µM
Primer R (Psa_A1 R1 or Psa_Z5 R2)	25 µM	0.2	0.5 µM
DNA obtained from plant extract or DNA obtained from bacterial suspensions		1–2.5 (depending on the DNA concentration)	
Total		10.00	

Abbreviation: NA, not applicable.

This programme was developed in a LightCycler 480 II (Roche Diagnostic) or an Eco Real-Time PCR system (Illumina) thermal cycler, following two different PCR conditions.

2.3 PCR cycling conditions

LightCycler 480 II: 95°C for 10 min, 45 cycles of 95°C for 5 s, 65°C for 7 s, 72°C for 7 s.

Eco Real-Time PCR system: 95°C for 3 min, 40 cycles of 95°C for 5 s, 65°C for 7 s and 72°C for 10 s.

Melting curve spectra for melting curve analysis (MCA) were determined by heating to 95°C for 15 s, cooling to 55°C for 15 s and then heating to 95°C at the machine programmed ramp rate of 0.25°C s^{-1}, holding at that temperature for 15 s.

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 *Actinidia* plant matrix or, if not available, sterile 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 an *Actinidia* plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

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

When these conditions are met

- A test will be considered positive if it produces an exponential curve and revealed a specific peak with melting temperatures of, respectively, 85°C (primers combination Psa_A1 F2/R1, targeting on *hopA1* gene) and 80.6°C (primers combination Psa_Z5 F2/R2, targeting on *hrpZ5* gene).
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data are provided in Andersen et al. (2018).

4.1 Analytical specificity data

Inclusivity: 100% (tested on 13 *P. syringae* pv. *actinidiae* strains).

Exclusivity: 100% (tested on 15 strains of *P. syringae* pv. *actinidiae* biovars 2 and 5 and on *P. syringae* pv. *actinidifoliorum*).

APPENDIX 8 –SIMPLEX PCR GALLELLI ET AL. (2014)

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

1. General information

1.1 This PCR is suitable for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* biovar 3.

1.2 The test was developed by Gallelli et al. (2014).

1.3 The target sequence is a region of *hrpW* gene.

1.4 Oligonucleotides.

Primer name	Sequence	Amplicon size
Forward primer (P0F)	5′-CTGCAACAGGCGACG GCGAGGC-3′	243 bp
Reverse primer (P6R)	5′-CATAGGCTTCTGGTTTTCTTC CTGATCC-3′	

1.5 PCR: The test performance study was performed with various PCR systems.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Matrices: plants, ooze drops, bleeding sap extract or pure culture suspension.

2.1.2 See Appendix 3 for DNA extraction procedures from plant, ooze drops, bleeding sap and culture suspension.

2.2 Polymerase chain reaction (PCR)

For identification purposes, template DNA is 2 µL of boiled bacterial suspension (at about 10^8 cfu mL^{-1}). For plant extracts, obtained as explained above, template is 5 µL of DNA extract and a 10-fold dilution.

2.2.1 Master mix: the DNA polymerase should always be a ‘hot start’ polymerase.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	Adjust the quantity to make up to 50 µL depending on DNA concentration	NA
Taq DNA polymerase buffer (Bioline)	10×	5.0	1×
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	10 mM	1.0	0.2 mM
Primer P0F	25 µM	0.4	0.2 µM
Primer P6R	25 µM	0.4	0.2 µM
Immolase Taq DNA polymerase (Bioline)[a]	2.5 U μL^{-1}	0.5	0.025 U μL^{-1}
gDNA obtained from bacterial suspension or boiled bacterial suspension		– 2	20 ng
Or DNA obtained from plant extract		5	
Total		50.00	

Abbreviation: NA, not applicable.[a] As an alternative, Platinum Taq DNA Polymerase (Invitrogen) was used in the PCR reaction in the development of this assay (see Gallelli et al., 2014).

2.2.2 PCR cycling conditions: 95°C for 2 min, 30 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 30 s and a final step of 72°C for 5 min.

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 *Actinidia* plant matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or an *Actinidia* plant matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC): a band of 243 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 243 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data are provided by Gallelli et al. (2014) and by Loreti et al. (2018). Data from Gallelli et al. (2014) are produced during the developing of this method.

Data from Loreti et al. (2018) were produced by a TPS performed within the PSA-DID project (ERA-NET 266505 EUPHRESCO II). The matrices tested were woody tissue from *Actinidia deliciosa* cv. Hayward and the test was assessed on plant extracts and pure cultures. The TPS involved nine European laboratories, two from New Zealand and one from Turkey for a total of 12 laboratories. The samples extract consisted of woody samples spiked (or not) with suspensions of the bacterial strain Psa ISF 8.43 (biovar 3) containing 10^7 cfu mL^{-1} (two replicates), 10^5 cfu mL^{-1}, 10^4 cfu mL^{-1}, 10^3 cfu mL^{-1} (three replicates) and 0 cfu mL^{-1} (two replicates).

4.1 Analytical sensitivity data (from Loreti et al., 2018): 10^4 cfu mL^{-1}.

4.2 Analytical specificity data.

- From Gallelli et al. (2014)
  Inclusivity: 100% (tested on 40 *P. syringae* pv. *actinidiae* strains).
  Exclusivity: 100% (tested on 43 strains of different bacterial species).
- From Loreti et al. (2018)
  Inclusivity: 100% (tested on 18 *P. syringae* pv. *actinidiae* strains).
  Exclusivity: 78% (tested on 18 bacterial strains of different Pseudomonas species and of *P. syringae* strains associated with kiwifruit matrices). Cross-reaction

was noted with *P. syringae* pv. *theae* (strain NCPPB 2598), *P. avellanae* (strain NCPPB 3487), *P. syringae* pv. *actinidifoliorum* (strain CFBP 7951), *P. syringae* (strain LSV 43.31).

4.3 Data on repeatability (from Loreti et al., 2018): 94%
4.4 Data on reproducibility (from Loreti et al., 2018): 85%
4.5 Data on diagnostic sensitivity: 88.9%
4.6 Data on diagnostic specificity: 94.4%
4.7 Data on accuracy: 91.7%

APPENDIX 9 –PATHOGENICITY TEST

A pathogenicity test to verify Koch's postulates and confirm the pathogenicity of the isolates can be performed as an identification test. This should be (especially) applied in critical cases (see PM 7/76).

In vivo test

For each isolate to be tested, a minimum of three plantlets of *Actinidia deliciosa* cv. Hayward or *A. chinensis* cultivars such as cv. Hort16A and Soreli should be inoculated. The plantlets should be approximately 15–20 cm tall and kept in single pots. At least three plantlets should be used as a positive control (inoculated with a known *P. syringae* pv. *actinidiae* strain, i.e. ISF 8.43 or CFBP 7285) and three other plantlets should be used as a negative control (inoculated with sterile, PBS pH7.2). Inoculated plantlets should be kept in a climatic chamber or in a conditioned greenhouse at approximately 80% RH and 24 ± 3°C and a photoperiod of 14 h at 14 000 lux. Inoculated plantlets may start to show typical symptoms 6–10 days after inoculation and should be observed for symptom development for up to 3 weeks after inoculation.

Inoculation procedure

Suspensions of the putative *P. syringae* pv. *actinidiae* isolates are used to prepare a suspension in sterile PBS at a concentration of approximately 10^8 cfu mL^{-1}. Each bacterial suspension is then sprayed onto the abaxial sides of the leaves of plantlets, making sure to carefully wet all the leaves. After spraying, a transparent polythene bag is put on each single plant to allow maximum humidity around the plantlets, thus helping bacterial penetration into leaves. The polythene bag is kept on the plantlets overnight.

Symptoms

Small chlorotic spots appear on leaves 6–10 days after bacterial inoculation. During the following days the spots enlarge and necrotic angular lesions appear at the centre, eventually surrounded by a chlorotic halo. After 12–15 days, the chlorotic-necrotic lesions observed on leaves coalesce into large necrotising areas. Symptom severity is usually higher on *A. chinensis* than on *A. deliciosa*. The plantlets should be observed for symptoms (including blight) for a period of up to 3 weeks. The virulence of *P. syringae* pv. *actinidiae* strains tested for their identification may vary and therefore disease severity observed on test plants may vary accordingly.

Re-isolation and identification should be done from symptomatic plants as soon as symptoms appear. Despite differences in its virulence and symptoms caused in test plants, re-isolation of the pathogen will confirm its identity.

In vitro tests

Two different procedures are reported for leaf-disc *in vitro* assay.

- Procedure of Prencipe et al. (2018):

Leaf discs (2 cm diameter) prepared from 14-day-old leaves of *A. chinensis* (1-year-old plant) are placed in 10 mL of sterile water in not sealed three-sector Petri dishes. The leaf discs are inoculated with bacterial suspension (10^8 cfu mL^{-1}) at three equidistant points (three 30 µL drops per leaf disc). Control leaves are prepared similarly with sterile deionized water. Three replicates for each strain should be assessed (nine leaf discs per strain). The first symptoms, necrotic spots, are observed approximately 6 days after inoculation. Controls should not show symptoms.

- Procedure of Brunetti et al. (2020):

Fully expanded leaves are collected from nursery-grown equal-in-age-trees, taking care to collect homogeneous

FIGURE 4 Symptom appearance and progression on leaf discs inoculated with *Pseudomonas syringae* pv. *syringae* (from Brunetti et al., 2020)

leaves (e.g. degree of tenderness) from the fifth to eighth nodes of actively growing twigs. Immediately after leaf detachment, discs of about 2 cm diameter are punched out from the leaf blade, randomized and inoculated by immersing in 10^8 cfu $mL^{-1}$of bacterial suspension containing 0.01% (v/v) silwet L-77 (adjuvant), for 1 h at 23°C under gentle agitation, with the abaxial face uppermost. Leaf discs are deposed in 90 mm diameter Petri plates containing 25 mL of water-agar (12 g L^{-1} of agar). Five discs per plate, abaxial surface uppermost, are incubated at 15°C in a growth chamber (CFT 1200, Piardi, ITA) with a photoperiod of 14–15 h/day and 9–10 h/night. Each strain is assessed on 10 discs, distributed in two Petri dishes. Symptoms of black necrotic lesions will appear nearly simultaneously within 48 h. Symptoms develop from 5 to 8 days after inoculation. Over time necrotic lesions progressively increase in size and number until coalescence (Figure 4). Within 14–20 days, spreading necrosis affects the entire leaf disc surface. Control leaf discs should not show symptoms or senescence.

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CORRIGENDUM

Corrigendum - PM 7/120 (2) *Pseudomonas syringae* pv. *actinidiae*

It has been brought to our attention that in the EPPO Standard PM 7/120 (2) *Pseudomonas syringae* pv. *actinidiae* (EPPO, 2021) there is an error in the master mix description for the real time PCR (Gallelli et al., 2014) described in Appendix 6. The correct values for the SsoFast EvaGreen supermix (Bio-Rad), and adjusted volume for molecular grade water, are given in bold below.

APPENDIX 6

Point 2.2.1 master mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	NA	**7.2**	NA
SsoFast EvaGreen supermix (Bio-Rad)	**2×**	**10.0**	**1×**
Primer P3F	25 µM	0.4	0.5 µM
Primer P5R1	25 µM	0.4	0.5 µM
DNA bacterial suspension or plant, ooze drops or bleeding sap		2.0	20 ng
Total		20.0	

The EPPO Secretariat apologizes for any inconvenience and thanks the expert that brought this to our attention.

REFERENCE

EPPO (2021) PM 7/120 (2) *Pseudomonas syringae* pv. *actinidiae*, *EPPO Bulletin*, 51, 549–567.