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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/120 (1)

Diagnostics
Diagnostic

PM 7/120 (1) *Pseudomonas syringae* pv. *actinidiae*

Specific scope

This Standard describes a diagnostic protocol for *Pseudomonas syringae* pv. *actinidiae*.[1]

Specific approval and amendment

Approved in 2014–09.

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 host plant species of *P. syringae* pv. *actinidiae*. The disease was first reported in Japan in 1989 on *Actinidia* sp. (Serizawa *et al.*, 1989; Takikawa *et al.*, 1989). The disease was later reported in Korea (Koh *et al.*, 1994) and China (Li *et al.*, 2004). In the EPPO region the disease appeared for the first time in 1992 on *A. deliciosa* in Central Italy (Scortichini, 1994). From 2008 to date, severe epidemics of the bacterial canker were described in Central Italy, first on *A. chinensis* and later on *A. deliciosa* (Balestra *et al.*, 2008b; Ferrante & Scortichini, 2009). The disease and its causal agent were then detected in France (Vanneste *et al.*, 2011b), Germany (EPPO, 2013), Portugal (Balestra *et al.*, 2010), Spain (Abelleira Argibay *et al.*, 2011; Balestra *et al.*, 2011), Switzerland (EPPO, 2011a) and Turkey (Başta& Karakaya, 2012). Outside the EPPO region the disease was reported in New Zealand (Biosecurity New Zealand, 2010), Chile (EPPO, 2011c; SAG, 2011), Australia (EPPO, 2011b) and Korea (Koh *et al.*, 2012). *P. syringae* pv. *actinidiae* populations show genetic variability and strains may be differentiated by the presence of genes coding for phaseolotoxin, coronatine and effector proteins (Ferrante & Scortichini, 2010; Mazzaglia *et al.*, 2010) and by the DNA sequences of several genes (Vanneste *et al.*, 2010; Gallelli *et al.*, 2011a). The recent severe outbreaks of the disease in the EPPO region and in New Zealand have been related to the local presence of more aggressive strains (Balestra *et al.*, 2009; Ferrante & Scortichini, 2010; Vanneste *et al.*, 2010; Vanneste *et al.*, 2011). Four different biovars have also been previously described, characterised by different virulence (Chapman *et al.*, 2012; Vanneste *et al.*, 2013). Among them, biovar 4 was represented by low virulence strains originally isolated in New Zealand and Australia. An accurate redefinition of the global population of *P. syringae* pv. *actinidiae* made by Ferrante & Scortichini (2014) was able to confirm that biovar 4 does not belong to the pathovar *actinidiae*. This was further confirmed by Cunty *et al.* with the strains of biovar 4 isolated in France, therefore, it has been proposed to rename them as *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty *et al.*, in press). Therefore, only three genetically distinct populations pathogenic to *Actinidia* spp., are currently described. Based on the analysis of sequence data of strains from China, Japan, Korea, Italy and Portugal, Mazzaglia *et al.* (2012), suggest a possible Chinese origin of the European and New Zealand outbreaks. Indeed, analyses of draft genomes from three strains of different origin revealed features involved in adaptation and virulence (Marcelletti *et al.*, 2011). More recently, it has been shown that *P. syringae* pv. *actinidiae* from recent outbreaks belong to different clones originating in China (Butler *et al.*, 2013).

A flow diagram describing the diagnostic procedure for *Pseudomonas syringae* pv. *actinidiae* is presented in Fig. 1.

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.

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

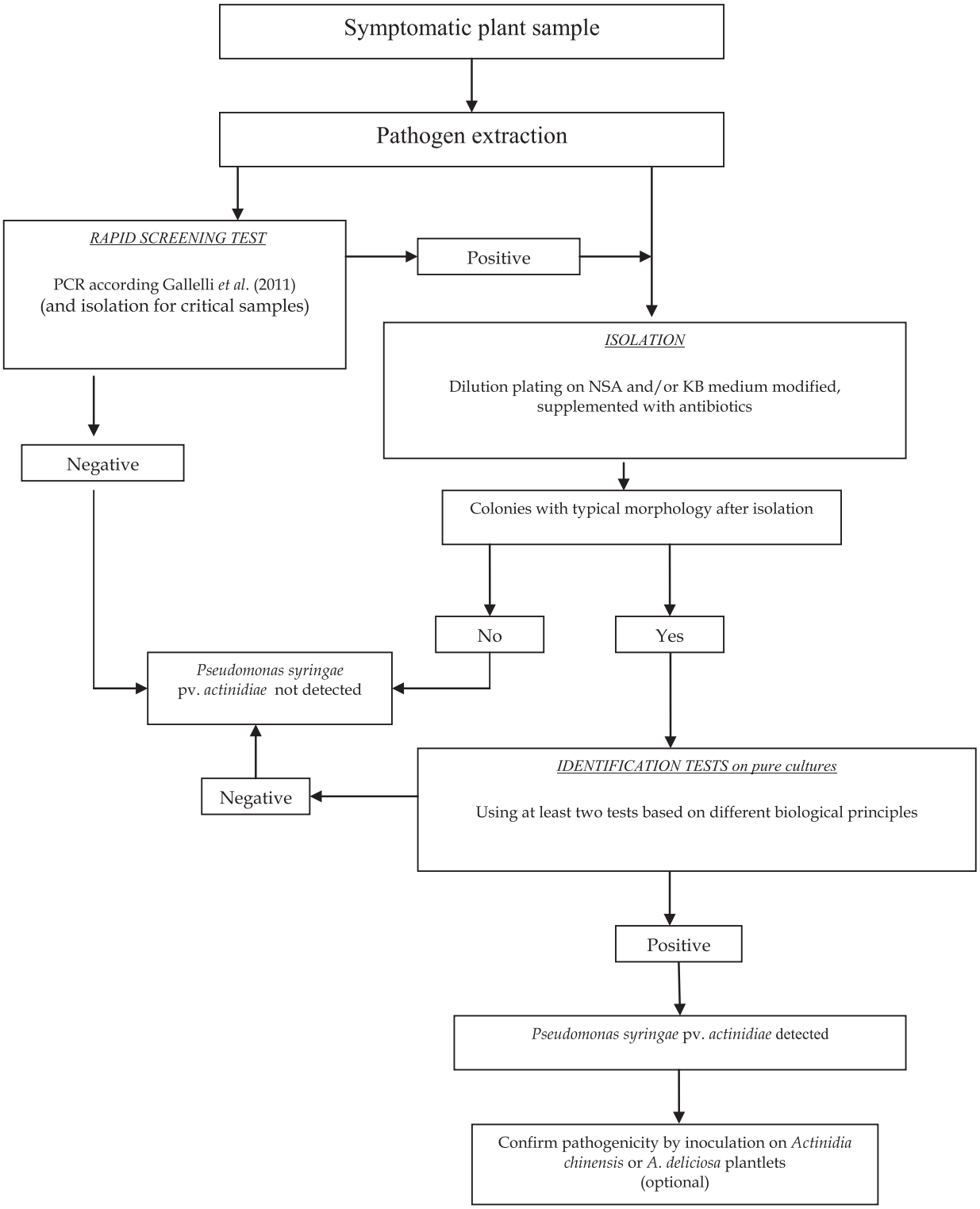

Fig. 1a (a) Flow diagram for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* in samples of symptomatic *Actinidia* spp. *Warning*: in some EPPO countries not well defined atypical strains have been observed. When symptoms are present on *Actinidiae* plants, isolation should be performed in case of negative rapid screening test. http://www.euphresco.net/.

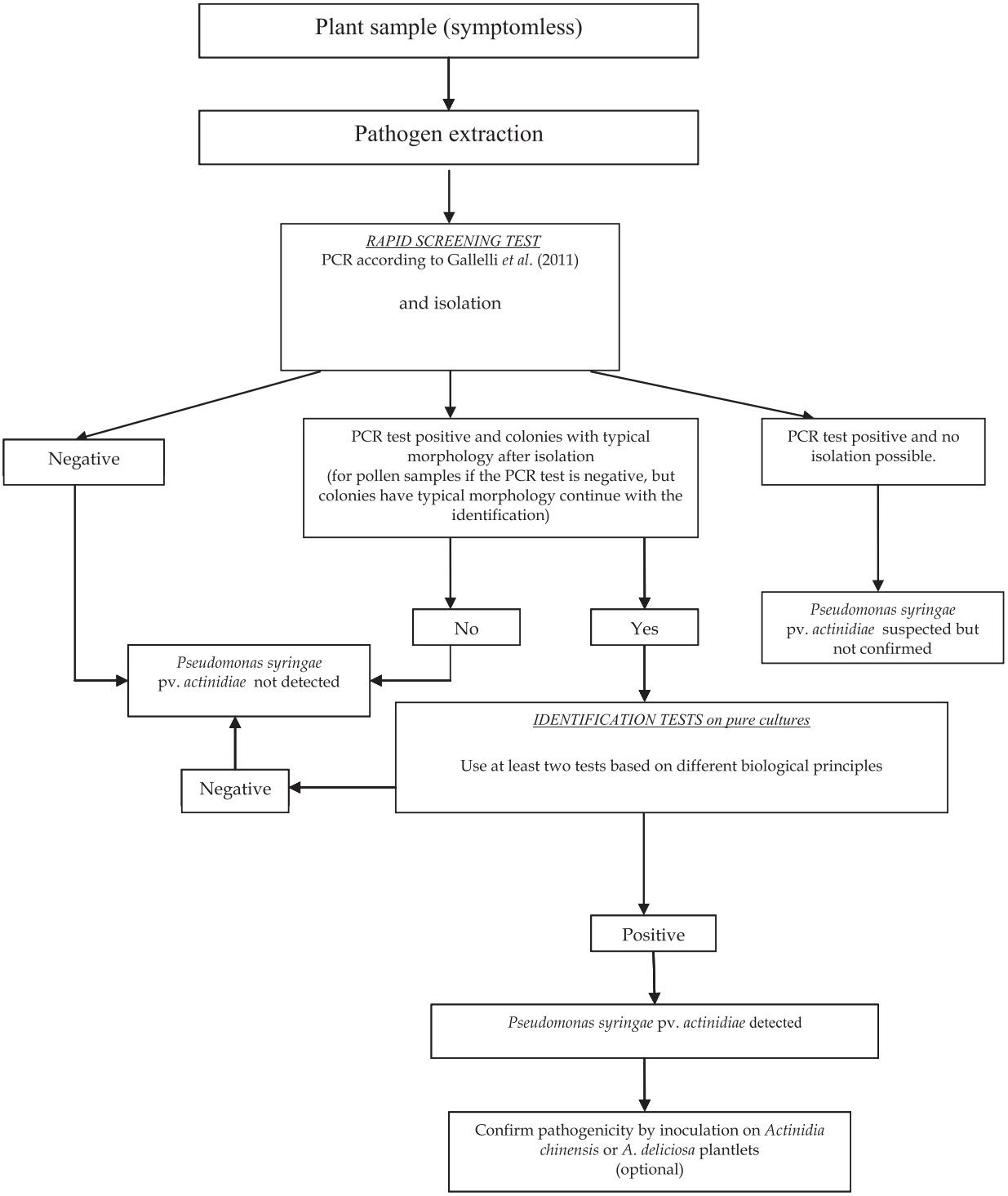

Fig. 1b (b) Flow diagram for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* in samples of symptomless *Actinidia* spp.

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.*, in press). 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 infested, however this may happen when fruits are grown and harvested in heavily infected orchards (Stefani & Giovanardi, 2011; Gallelli *et al.*, 2011b).

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 in late winter and spring and ooze bacterial exudates and plant sap. On trunks and leaders the ooze turns from dark 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 *Pseudomonas syringae* pv. *syringae*, a common bacterial pathogen present in several areas worldwide on fruit crops, especially stone fruits and pome fruits. Another bacterium, *Pseudomonas 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.*, 2008a). 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; wilted fruits are not marketable. A description of symptoms according to the period of the year is available at http://www.eppo.int/MEETINGS/2011_meetings/11-17237_psa_symptoms.pdf.

Screening tests

Direct isolation, and/or PCR according to Gallelli *et al.* (2011a) can be used as screening tests for plant material showing symptoms. For critical symptomatic samples and for all asymptomatic samples, both PCR and isolation should be performed. The PCR tests are described in Appendix 2. No specific serological test is available for *P. syringae* pv. *actinidiae*. Commercial PCR kits, based on the protocol developed by Rees-George *et al.* (2010), are currently available and may be used to screen symptomatic plant material, although cross reactions with other pseudomonads have been noted. The kits use either a) an end-point PCR or b) a real-time PCR, using the SYBR® green chemistry. More information is available from Qualiplante.

Detection in symptomatic plants

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 isolation of *P. syringae* pv. *actinidiae* from symptomatic samples is relatively easy because the number of culturable bacteria in them is usually high. A semi-selective medium is suggested in order to avoid the growth of too many saprophytes and contaminants, which may overgrow the agar plates within 36–48 h of plating. The remaining plant samples can be cold-stored (as mentioned above) for up to 9 days to be processed for additional verification.

Isolation of *P. syringae* pv. *actinidiae* should preferably be attempted on modified Nutrient Sucrose Agar (NSA) or modified King's B medium (KB) (Mohan & Schaad, 1987): NSA and KB are modified by adding boric acid and the two antibiotics: cycloheximide and cephalexin (Appendix 1). The use of media supplemented with antibiotics is strongly recommended for *P. syringae* pv. *actinidiae* isolation from symptomless organs such as pollen, cuttings, buds or when symptoms become old, to avoid the growth of contaminants that can limit the capacity to recover *P. syringae* pv. *actinidiae* by isolation. Colonies grow slower on media containing antibiotics, compared to the same media without them.

From symptomatic material a few fragments may be used for detection and/or isolation of the pathogen:

- From bleeding cankers a sample may be taken simply by collecting a few ooze drops with a cotton swab, dipping the swab in a tube containing 5 mL of sterile saline solution or sterile water and shaking for a few seconds and using this suspension for direct isolation on agar medium (NSA or KB). Old exudates (generally rusty red to brown) are not suitable for an effective *P. syringae* pv. *actinidiae* isolation.
- From the lesion (margins of a canker, flowers, leaves, buds or fruits), 3–5 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 sterile mortars containing 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 used for direct isolation. From leaves, large, necrotizing 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 cut out 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.

Isolation

Isolation from plant extracts is done by dilution plating of 30–50 µL of the plant extract or plant washing solution obtained as above 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, a suspension of a *P. syringae* pv. *actinidiae* control strain (see reference material) at a concentration of approx. 10^4–10^5 cfu mL^{-1} is plated onto the same medium. After 3 days, pale and pinhead colonies may appear on plates, which should be observed until the 6th day after plating. Suspect colonies may require a further purification step by re-streaking on NSA or KB before proceeding to further identification.

Detection in symptomless plant material

Detection and identification 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*. Plant material, which may be contaminated or have a latent infection of *P. syringae* pv. *actinidiae* are: dormant cuttings, budwood, shoots or twigs, plantlets and *in vitro* micropropagated plants (vitroplants). Sampling in nurseries in late winter has allowed detection of the pathogen in nurseries, however more information would be needed to establish the optimal period of sampling. Detection on fruit is erratic (Stefani & Giovanardi, 2011; Gallelli *et al.*, 2011b). Pollen may carry *P. syringae* pv. *actinidiae* and disseminate it into kiwi orchards (Vanneste *et al.*, 2011; Stefani & Giovanardi, 2011; Gallelli *et al.*, 2011b) and has recently been demonstrated to spread the disease (Tontou *et al.*, 2014).

Concentration step

From the laboratory sample a test sample is prepared and used as described below to increase the concentration of viable *P. syringae* pv. *actinidiae* cells possibly present. A concentrated plant extract is prepared as described below. It is recommended that the concentrated plant extract (usually 1.5–3 mL) is divided into two parts: one is used to perform the different isolation or other detection tests envisaged in this diagnostic protocol and the second one is to have 15–25% of sterile glycerol added and to be kept at −80°C for possible further confirmation analyses.

The text below provides a detailed description of different detection procedures for different asymptomatic plant material. Appendix 1 contains a description of buffers.

- For dormant cuttings, budwood, shoots or twigs different procedures are described hereafter.
  - i) 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 twenty dormant cuttings of 30 cm in length and crushed for 2 min. Crushing can be done with a stomacher or a hammer. Fifteen mL 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 are 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 discharged and the pellet is resuspended in 1–2 mL of sterile PBS.
  - ii) Nursery plants and plants growing in the field:
    The laboratory sample consists of 30 shoots/twigs of 10 cm in length randomly selected; disinfection (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 PBS.
    Dormant cuttings can also be processed in the same way.
- 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 ii). The supernatant should be discharged and the pellet resuspended in 1–2 mL of sterile PBS.
- Plantlets:
  the laboratory sample is composed of one hundred 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 deionised 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.

- 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, alternatively, 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.

Isolation and colony purification should be carried out starting from the concentrated plant extract, as described above for symptomatic plant material.

Identification

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

Warning: in some EPPO countries not well defined atypical strains have been observed. When symptoms are present on *Actinidiae* plants, isolation should be performed in the case of negative rapid screening test.

Colony morphology

On modified NSA colonies are smooth, elevated or convex, round and with entire margin, pearly whitish in colour (Fig. 2). Colonies are shiny when fresh, then becoming pale ivory whitish. *P. syringae* pv. *syringae* is quite common in *Actinidia* spp. orchards and it 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 (Fig. 3). Unlike *P. syringae* pv. *syringae*, *P. syringae* pv. *actinidiae* colonies are usually not fluorescent on King's B medium although Everett *et al.* (2011) reported the possibility that some isolates might produce fluorescence.

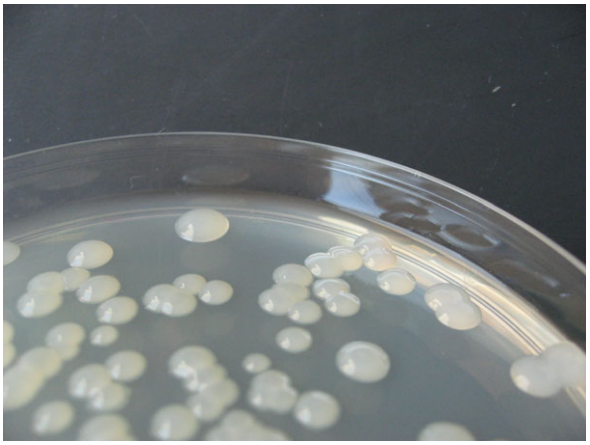

Fig. 2 Morphology of *Pseudomonas syringae* pv. *actinidiae* colonies grown for 5 days on NSA medium, supplemented with antibiotics (*Source*: E. Stefani, Dept. of Life Sciences, Reggio Emilia, Italy).

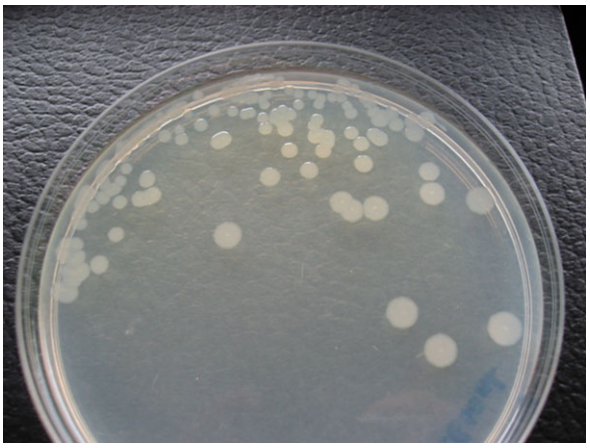

Fig. 3 Morphology of *Pseudomonas syringae* pv. *actinidiae* colonies grown for 5 days on King's medium B, supplemented with antibiotics. (*Source*: E. Stefani, Dept. of Life Sciences, Reggio Emilia, Italy).

Hypersensitive Reaction (HR)

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

Biochemical and physiological tests

P. syringae pv. *actinidiae* strains are aerobic rods, motile by a polar tuft of flagella (Scortichini *et al.*, 2012). Biochemical and physiological characteristics to be tested for *P. syringae* pv. *actinidiae* strains are given in Table 1. Metabolic tests to distinguish *P. syringae* pv. *actinidiae* from *P. syringae* pv. *syringae* are shown in Table 2.

Table 1 Biochemical and metabolic characteristics of *Pseudomonas syringae* pv. *actinidiae* according to Takikawa *et al.* (1989) (based on: American Society for Microbiology. Committee on bacteriological technic, 1957)

Tests	Results
Gram's reaction	−
Cytochrome C oxidase reaction	−
Fluorescent pigment on King's medium B	−
Gelatin liquefaction	−
Levan	+
Oxidation/fermentation test (Hugh & Leifson, 1953)	Oxydative
Hydrolysis of:	
Aesculine	−/+−
Arbutin (facultative)	−
Casein (facultative)	+
Urease	−
Utilisation of:	
D-xylose	−
L-Arabinose	+
Inositol	+
Erythritol	−
DL-Lactate	−
n-Caprate	−
L-Hystidine	−
L-Serine	+
L-Arginine	+
Trigonelline	−
L-Leucine	+
L-Tyrosine	+

Table 2 Differences between *Pseudomonas syringae* pv. *actinidiae* and *Pseudomonas syringae* pv. *syringae* concerning a set of biochemical and metabolic tests

Tests	Results for *P. syringae* pv. *actinidiae*	Results for *P. syringae* pv. *syringae*
Hydrolysis of:		
Arbutin	−	+
Aesculin	−	+
Gelatin	−	+
Urease production	−	+
Acid production from:		
Xylose	−	+
Erythritol	−	+
DL-Lactate	−	+
n-Caprate	−	+
L-Histidine	−	+
Trigonellin	−	+
L-Tyrosin	+	−
Fluorescence production on KB medium	−	+

Biochemical identification can be performed using the Biolog system.

The performance of the LOPAT tests, as described by Lelliott *et al.* (1966) are sufficient for a first characterisation of the isolates, but insufficient to discriminate *Pseudomonas syringae* pv. *actinidiae* from *P. syringae* pv. *syringae*. If *P. syringae* pv. *actinidiae* putative colonies are transferred onto King's B media, they are not able to produce any fluorescent pigment, as *P. syringae* pv. *syringae* does (Scortichini *et al.*, 2002): this test, although not predictive on the identity of *P. syringae* pv. *actinidiae*, can discriminate *Pseudomonas syringae* pv. *actinidiae* from *P. syringae* pv. *syringae*. Colonies resembling *P. syringae* pv. *actinidiae* are purified on King's medium B, NSA or NGA and identified through different tests, such as: hypersensitivity reaction (HR) on tobacco, which is positive in most cases, and a number of biochemical tests aimed to discriminate *P. syringae* pv. *actinidiae* from *P. syringae* pv. *syringae* (Crosse & Garrett, 1963; Schaad *et al.*, 2001).

The identity of putative *P. syringae* pv. *actinidiae* colonies should be checked with simplex or duplex PCR, as described below. Colony identity can be further confirmed by repetitive-PCR fingerprinting, using the BOX, primers (Ferrante & Scortichini, 2009, 2010).

Molecular tests

PCR

A suspension containing approximately 10^8 cfu mL^{-1} in molecular grade sterile water is prepared from a 24 h growing culture on NSA or KB. Appropriate PCR procedures are applied to amplify specific amplicons of *P. syringae* pv. *actinidiae,* as described in Appendix 2 (Rees-George *et al.*, 2010; Gallelli *et al.*, 2011a).

DNA fingerprinting methods

Repetitive PCR (rep-PCR) has been shown to be highly reliable for strain identification of pseudomonads, xanthomonads and other bacteria at the species and infraspecies level (Louws *et al.*, 1994; Rademaker & DeBruijn, 1997) by means of amplification of interspersed repetitive DNA sequences present in bacterial genomes. One or more primers (REP, BOX, ERIC) might be used during this genotyping technique (Ferrante & Scortichini, 2010; Mazzaglia *et al.*, 2011). Guidance on how to perform rep-PCR tests for the identification of bacterial isolates is given in PM 7/100 *Rep-PCR tests for identification of bacteria*. An illustration of an agarose gel obtained after BOX PCR with several strains is provided in Appendix 3.

DNA sequencing

Comparisons of commercially sequenced PCR products amplified from selected housekeeping genes allow identification of *P. syringae* pv. *actinidiae* from other members of the *P. syringae* complex. For example, all isolates of *P. syringae* pv. *actinidiae* tested to date were clonally related, *i.e.* with 100% sequence similarity of partial *rpoD* genes using the method of Parkinson *et al.* (2011). *P. syringae* pv. *actinidiae* clusters in a distinct phylogroup to *P. syringae* pv. *syringae* and the two pathogens can be clearly distinguished using this method with a minimum

rpoD sequence similarity less than or equal to 95% between their respective phylogroups. The species type and pathotype strain for *Pseudomonas syringae* pv. *syringae* is NCPPB 281 (=CFBP 1392, LMG 1247) and the pathotype strain for *Pseudomonas syringae* pv. *actinidiae* is NCPPB 3739.

Pathogenicity tests

A pathogenicity test may be required for the completion of the diagnostic procedure (i.e. in the case of first findings, or in doubtful cases) and may be conveniently carried out on *Actinidia chinensis*. Inoculation is done by spraying plantlets of an appropriate size. Information on pathogenicity tests is provided in Appendix 4.

Reference material

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

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

ISF ACT1 – Italian isolate, high virulent type.

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

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

(1) National Collection of Plant Pathogenic Bacteria (NCPPB), FERA, York, GB (http://www.nppb.com);
(2) Collection Française de Bactéries associées aux Plantes (CIRM-CFBP), INRA Station Phytobactériologie, Angers, France (http://www-intranet.angers.inra.fr/cfbp/catalogue).

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

Reporting and documentation

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

Performance criteria

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

Further information

Further information on this organism can be obtained from: E. Stefani, Dept. of Life Sciences, via Amendola 2, Pad. Besta, 42122 Reggio Emilia (IT) E-mail: emilio.stefani @unimore.it

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Feedback on this Diagnostic Protocol

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

Protocol revision

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

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

Acknowledgements

This protocol was originally drafted by: E Stefani, (Department of Life Sciences, University of Modena & Reggio Emilia, via Amendola 2, 42122 Reggio Emilia, IT) and further reviewed by S Loreti (CRA, IT).

References

Abelleira Argibay AA, López MM, Peñalver Navarro J, Casal OA, Mansilla Vázquez JP, Montoussé AP *et al.* (2011) First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Spain. *Plant Disease* **95**(12), 1593 [doi: 10.1094/PDIS-06-11-0537].

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Appendix 1 – Buffers and media

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

1. Buffers

Physiological solution (saline): 0.90 w/v of NaCl. Dissolve 9 g of NaCl in 1 L of deionised water. Sterilise 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	1 L

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

5X TBE buffer:

Tris 54 g, 20 mL of a 0.5 M Na_2-EDTA solution at pH 8.0, 27.5 g boric acid, distilled water to 1 L. This buffer does not need autoclaving.

2. Media

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 and autoclaving is needed.

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 solution of cycloheximide in 70% ethanol and 8 mL of a 10 mg mL^{-1} aqueous solution of cephalexin.

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

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

Proteose peptone N. 3	20.0 g
Glycerol	10.0 mL
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Bacteriological agar	15.0 g
Distilled water	1.0 L

The pH is adjusted to 7.2 and autoclaving is needed.

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 one month in the dark and refrigerated.

Nutrient Glucose Agar (NGA) (Schaad & Forsters, 1985), modified

Nutrient agar	28.0 g
Glucose	2.5 g
Distilled water	1.0 L

The pH is adjusted to 7.2 and sterilization is done by autoclaving.

Appendix 2 – Polymerase chain reaction (PCR)

Overview

PCR may be used in two different steps along the detection procedure for *P. syringae* pv. *actinidiae*: the first step may be a presumptive diagnosis by PCR using the plant extract (or final concentrate) and its dilutions as a template. A second step may be the identification of a pure culture, after its isolation and purification on agar media to confirm the identity of the isolate as *P. syringae* pv. *actinidiae*. A two-step PCR-based method can yield reliable results. A preliminary screening of the pure culture may be done by applying the PCR technique described by Gallelli *et al.* (2011a). Another, earlier PCR test was described by Rees-George *et al.* (2010): this test used in several laboratories proved to be less specific than the one of Gallelli *et al.*, (2010) Validation data are available for both procedures. Sometimes non-specific bands may appear if a recombinant Taq polymerase, other than the Taq 'hot start', is used, and amplification from *Pseudomonas syringae* pv. *theae* can be obtained by Rees-George *et al.* (2010) PCR test. The main difference between the two proposed PCR tests resides in the fact that the first (Rees-George *et al.*, 2010) is a simplex-PCR, i.e. using one primer pair, whereas the latter (Gallelli *et al.*, 2011a) is a duplex-PCR, i.e. using two primer pairs targeting different chromosomal regions. The advantages of a duplex-PCR versus a simplex-PCR are that it has a higher specificity. The use of a 'hot start' Taq polymerase is highly recommended because it allows an increase in the sensitivity and specificity of the reaction. This is particularly important for duplex-PCR because it decreases the nonspecific coupling of primers and the formation of dimers.

A) PCR – according to Rees-George *et al.* (2010) (Simplex PCR)

1. General information

1.1. Rees-George *et al.* (2010) published two sets of PCR primers, PsaF1/R2 and PsaF3/R4. In the original paper a number of actinidia saprophytes gave a non-specific amplicon of 175 bp with primers PsaF3/R4 (Stefani & Loreti, unpublished), therefore simplex PCR should be performed with the primer pair PsaF1/R2. It should be noted that some *Pseudomonas syringae* pvs (i.e. pv. *theae* and pv. *tomato,* not present on *Actinidiae* plants) reacted positively during the test performance study to this PCR protocol (Rees-George *et al.*, 2010; Loreti *et al.*, submitted).

1.2 The test can be applied to the plant extract (or final concentrate) and its dilutions or to a pure culture, after isolation and purification on agar media.

1.3. The target sequences are located in 16S-23S rDNA internal transcribed spacer (ITS) regions.

1.4. Oligonucleotides:

PsaF1 (5′ – TTTTGCTTTGCACACCCGATTTT – 3′)

PsaR2 (5′ – CACGCACCCTTCAATCAGGATG – 3′)

1.5. The amplicon size in base pairs from PsaF1/R2 primers: 280 bp.

1.6. Enzyme: The test performance study was performed with various polymerases - Platinum® *Taq* polymerase (Invitrogen) and GoTaq® Hot Start DNA Polymerase (Promega), following manufacturers' instructions.

2. Methods

2.1 Nucleic Acid Extraction and Purification: 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.

Detection of *P. syringae* pv. *actinidiae* in the plant extracts obtained from plant samples and in 10-, and 100-fold dilutions of these extracts: The DNeasy Plant Mini Kit (Qiagen) can be used for extraction before amplification.[1]

2.2 Polymerase chain reaction – PCR.

The reaction is prepared for a total volume of 25 µL in each reaction tube. For identification purposes, template DNA is 0.5–1 µL of boiled bacterial suspension. For plant extracts, obtained as explained above, template DNA is 2–5 µL of the final extract and its dilutions. The master mix is prepared as explained in Table 3. The DNA polymerase should always be a 'hot start' polymerase; the master mix can be conveniently prepared also using the GoTaq® Hot Start Polymerase (Promega).

2.2.1. 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.

2.3 Samples (boiled suspension and boiled plant extract) can be conveniently stored at approx. −18°C for several months.

[1]Other DNA extraction procedures are being tested as part of a EUPHRESCO project.

Table 3 Master mix prepared according to Rees-George *et al.* (2010)

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
PCR grade water	N.A.	Adjust the quantity to make up to 25 μL depending on DNA input (see below)	N.A.
Taq DNA polymerase buffer (Invitrogen)	10 x	2.5	1x
$MgCl_2$	50 mM	0.75	1.5 mM
dNTPs	10 mM	0.5	0.2 mM
Primer PsaF1	20 μM	0.625	0.5 μM
Primer PsaR2	20 μM	0.625	0.5 μM
Platinum Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.062	0.0125 U μL^{-1}
DNA/cDNA obtained from bacterial suspensions		0.5–1	1–10 ng μL^{-1}
Or DNA obtained from plant extract		2–5	
Total		25.00	

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 should produce no amplicons.
- PIC, PAC (and if relevant IC) should produce amplicons of the expected size.

When these conditions are met:

- A test will be considered positive if amplicons of 280 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.

Table 4 The proportion of the number of samples testing positive for *P. syringae* pv. *actinidiae* with the total number of each strain tested (7 laboratories participating in the test performance study) is reported

Psa CRA-FRU 8.53	7/7
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	3/7
P. s. pv. *theae* CFBP 4097	7/7
P. avellanae NCPPB 3872	6/7
CRA-PAV 1686	0/7
CRA-PAV 1687	0/7
CRA-PAV 1688	0/7
CRA-PAV 1689	0/7

4. Performance criteria available

Results presented below are from CRA-PAV (Research Centre on Plant Pathology, Rome, Italy). The matrices tested were leaves, pollen and canker tissue from *Actinidia chinensis* and the test used plant extracts and pure cultures. The analytical specificity data are from a test performance study, including 7 Italian laboratories.

4.1 Analytical sensitivity data (Gallelli *et al.*, 2011a)
10^3 cfu/mL for pollen

4.2 Analytical specificity data (see Table 4)

4.3 Data on Repeatability

Not available
4.4 Data on Reproducibility
Simplex PCR from plant extract: 98%
Simplex PCR from bacterial cultures: 94%

B) PCR – according to Gallelli *et al.* (2011a) (Duplex PCR)

1. General information

1.1 This method distinguishes *P. syringae* pv. *actinidiae* from other genetically related *P. syringae* pathovars (Gallelli *et al.*, 2011a).
1.2 The test can be applied to the plant extract (or final concentrate) and its dilutions or to a pure culture, after isolation and purification on agar media.
1.3 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.
1.4 Oligonucleotides:
KN-F (5′ – CACGATACATGGGCTTATGC – 3′)
KN-R (5′ – CTTTTCATCCACACACTCCG – 3′)
AvrDdpx-F (5′ – TTTCGGTGGTAACGTTGGCA – 3′)
AvrDdpx-R (5′ – TTCCGCTAGGTGAAAAATGGG – 3′)
1.5 The amplicon size in base pairs for KN-F/R primers: 492 bp; for AvrDdpxF/R primers: 230 bp.
1.6 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: 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.

Detection of *P. syringae* pv. *actinidiae* in the plant extracts obtained from plant samples and its 10-, and 100-fold dilutions are treated in the same way as above indicated in A, to allow the DNA extraction of the target bacteria.

2.2 Polymerase chain reaction – PCR
Table 5 describes the Master mix.
2.2.1 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 Fig. 4.
2.3 Samples (boiled suspension and boiled plant extract) can be conveniently stored at approximately −18°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 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 con-

Table 5 Master mix prepared according to Gallelli *et al.* (2011a).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR grade water	N.A.	Adjust the quantity to make up to 50 µL depending on DNA input (See below)	N.A.
PCR buffer	10 x	5.0	1x
$MgCl_2$	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/cDNA obtained from bacterial suspensions		5	15–25 ng μL^{-1}
DNA obtained from plant extract		2	20 ng μL^{-1}
Total		50.00	

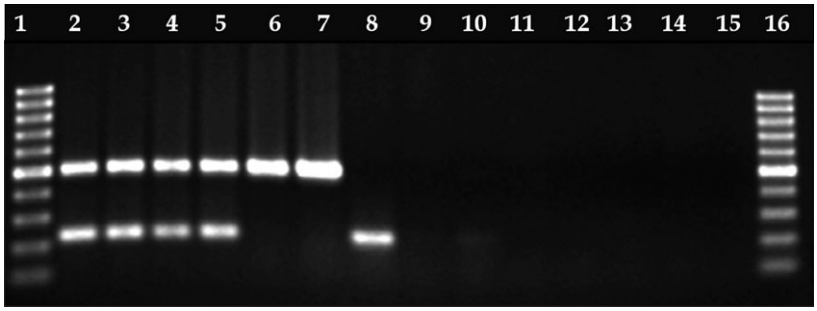

Fig. 4 Gel electrophoresis analysis of polymerase chain reaction products amplified by duplex-PCR from bacterial suspension (10^8 CFU mL^{-1}). Lanes 2–5: *Pseudomonas syringae* pv. *actinidiae* ISF Act.1, ISPaVe 019, ISPaVe 020, NCPPB 3740; lanes 6–7: *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *theae* CFBP 4097; lane 8: *Pseudomonas avellanae* NCPPB 3872; lane 9–14: *P. syringae* pv. p*haseolicola* 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).

tains 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 should produce no amplicons.
- PIC and PAC should produce two amplicons of 492 and 230 bp and if relevant IC should produce two amplicons of 492 and 230 bp.

When these conditions are met:

- A test will be considered positive if two amplicons of 492 and 230 bp are produced.
- A test will be considered negative, if it produces no band, or a band of a different size, or only one band, as in case of detection of the formerly known biovar 4 described by Vanneste *et al.* (2013) now proposed as *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty *et al.*, in press).
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data after Gallelli *et al.* (2011a): 2×10 cfu per PCR reaction

Results presented below are from CRA-PAV (Research Centre on Plant Pathology, Rome, Italy). The matrices tested were leaves, pollen and canker tissue from *Actinidia chinensis* and the test used plant extracts and pure cultures. The analytical specificity data is from a test performance study with seven Italian laboratories. Analytical sensitivity data:

10^3 cfu mL^{-1} for pollen.

4.2 Analytical specificity data

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

The proportion of the number of samples testing positive for *P. syringae* pv. *actinidiae* with the total number of each strain tested (7 laboratories participating in the test performance study) is reported.

4.3 Data on Repeatability

Not available

4.4 Data on Reproducibility

Duplex PCR from plant extracts: 98%

Duplex PCR from bacterial cultures: 95.5%

Appendix 3 – BOX Polymerase Chain Reaction

Figure 5 provides an illustration of an agarose gel after BOX PCR with several strains of *Pseudomonas*. The strains of *Pseudomonas* used in this study are listed in Table 6.

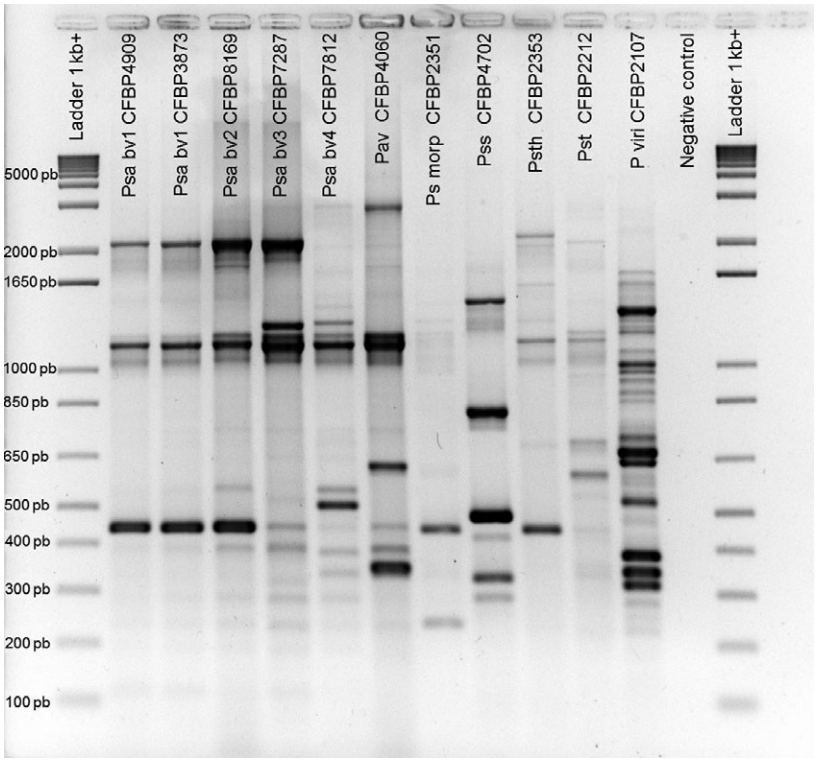

Fig. 5 Agarose gel electrophoresis of strains of *Pseudomonas* after BOX polymerase chain reaction (Courtesy ANSES, LSV, FR). Please, note that Psa bv4, which shows a very distinct BOX fingerprinting profile, does not belong to the pv. *actinidiae* any more (Ferrante & Scortichini, 2014).

Table 6 Strains collection

CFBP reference strain	Classification	*Psa* biovar	Host	Country of isolation	Year of isolation
4909	*Pseudomonas syringae* pv. *actinidiae*	1	*Actinidia deliciosa*	Japan	1984
3873	*Pseudomonas syringae* pv. *actinidiae*	1	*Actinidia deliciosa* « Hayward »	Italy	1994
8169	*Pseudomonas syringae* pv. *actinidiae*	2	*Actinidia sp.*	Korea	1994
7287	*Pseudomonas syringae* pv. *actinidiae*	3	*Actinidia deliciosa* « Hayward »	Italy	2008
7812	*Pseudomonas syringae* pv. *actinidiae*	4*	*Actinidia deliciosa* «Hayward»	New-Zealand	2010
4060	*Pseudomonas avellanae*	.	*Corylus avellana*	Greece	1976
2351	*Pseudomonas syringae* pv. *morsprunorum*	.	*Prunus domestica*	USA	NA
4702	*Pseudomonas syringae* pv. *syringae*	.	*Syringa vulgaris*	United-Kingdom	1950
2353	*Pseudomonas syringae* pv. *theae*	.	*Thea sinensis*	Japan	1970
2212	*Pseudomonas syringae* pv. *tomato*		*Lycopersicon esculentum*	United-Kingdom	1960
2107	*Pseudomonas viridiflava*	.	*Phaseolus* sp.	Switzerland	1927

NA: Not Available. * Now proposed to be renamed as *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty, in press).

Appendix 4 – Pathogenicity test

A pathogenicity test is done to verify Koch's postulates and confirm identity of isolates and their pathogenicity. For each isolate to be tested, 10 plantlets of *Actinidia deliciosa*, cv. Hayward or *A. chinensis* cultivars such as cv. Hort16A, Soreli or G3 should be inoculated. The plantlets should be approx. 15–20 cm tall and kept in single pots. At least five plantlets should be used as a positive control (inoculated with a known *P. syringae* pv. *actinidiae* strain, i.e. ISF ACT1 or CFBP 7285) and five other plantlets are used as a negative control (inoculated with sterile, physiological solution). Inoculated plantlets should be kept in a climatic chamber or in a conditioned greenhouse at approx. 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 to be used in the pathogenicity test are used to prepare a suspension in sterile, physiological solution at a concentration of approximately 10^8 cfu mL^{-1}. Each bacterial suspension is then sprayed onto the abaxial sides of the leaves of the 10 plantlets, paying attention 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 may appear on leaves 6–10 days after bacterial inoculation. During the following days the spots may enlarge and necroticangular lesions may appear at the centre, eventually surrounded by a chlorotic halo. After 12–15 days, the chlorotic-necrotic lesions observed on leaves may coalesce into large necrotising areas. Symptom severity is usually higher on *A. chinensis* than on *A. deliciosa*. The plants should be observed for symptoms (including blight) for a period of up to 3 weeks. 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.