Phytopathol. Mediterr. (2011) 50, 462–472

Detection of *Pseudomonas syringae* pv. *actinidiae*, causal agent of bacterial canker of kiwifruit, from symptomless fruits and twigs, and from pollen

Angela GALLELLI, Silvia TALOCCI, Alessia L'AURORA and Stefania LORETI

C.R.A. - Centro di Ricerca per la Patologia Vegetale, Via C.G. Bertero, 22, 00156 Roma, Italy

Summary. *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, was monitored in symptomless fruits, twigs and pollen of the host using bacterial isolation and DNA-extraction followed by two PCR-assays (direct-PCRs). A procedure for Psa detection from symptomless twigs was established. Out of 16 symptomless twigs samples, Psa was detected in 12 samples by isolation and 13 samples by direct-PCR. Thirteen pollen samples were treated using two different procedures; Psa was detected in eight samples by isolation and ten samples by direct-PCR. By washing 108 samples of fruits, Psa was detected by isolation in only two samples, collected from severely affected orchards. However, one of these samples contained wilted fruits, whereas for the other, only one colony was isolate. From 60 bulk-samples of fruits, endophytic Psa was detected in six samples by isolation and ten samples by direct-PCRs. A Psa-positive bulk-sample of fruits was analyzed separately as individual fruits: there was a faint signal in five or seven fruits out of 50 depending on the PCR assay used. Isolation was negative for these samples. Presence of the pathogen on bulk-fruit samples could be due to low amounts of inoculum distributed over many fruits: as a consequence, there is a negligible risk of introducing the pathogen into countries free of bacterial canker by symptomless fruits. This integrated approach (isolation plus PCR) is proposed as a tool for the analysis of symptomless kiwifruit material for the presence of Psa.

Key words: *Actinidia deliciosa*, *Actinidia chinensis*, duplex-PCR, symptomatic kiwifruit, pollen, fruit.

Introduction

Kiwifruit (Actinidia deliciosa Liang and Ferguson) is one of the main species of fresh fruit produced in Italy, covering a total area of 24,000 ha (Source: ISTAT 2010 http://www3.istat.it/dati/catalogo/20111216_00/PDF/cap13.pdf). Cultivation of kiwifruit is mainly located in Latium, Piedmont, Emilia Romagna, Veneto, Calabria and Campania regions of Italy. Traditionally *A. deliciosa* has been the species cultivated, but in recent years there has been an increase in golden or yellow kiwifruit (*A. chinensis* Planchon).

Over the last 4 years severe outbreaks of bacterial canker, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), have been observed on *Actinidia chinensis* 'Hort 16A' and 'Jin Tao' in the province of Latina (central Italy) (Balestra *et al.,* 2008; Ferrante and Scortichini, 2009) and also during the winter of 2009–2010 on *A. deliciosa* cultivars (Balestra *et al.,* 2009). Currently, the disease is present in Latium, Piedmont, Friuli Venezia Giulia, Emilia Romagna, Veneto, Campania and Calabria regions of Italy. The pathogen has previously caused significant economic losses in Japan (Serizawa *et al.,* 1989), where it was first described (in 1984), in Korea (Koh *et al.,* 1994) and in China (CABI, 2008). In 2010 it was reported in Portugal (Balestra *et al.,* 2010), France (Vanneste *et al.,* 2011a), New Zealand (Everett *et al.,* 2011), and Chile (htpp://www.promedmail.org); in 2011 it was reported in Spain (Argibay *et al.,* 2011; Balestra *et al.,* 2011), Australia and Switzerland (EPPO, 2011a, b) and Turkey (Bastas and Karakaya, 2011). Bacterial

Corresponding author: S. Loreti
Fax +39 06 82070370
E-mail: stefania.loreti@entecra.it

canker is characterized by an oozing of whitish or reddish exudates on the trunks and branches of affected vines, leaf spots (possibly surrounded by chlorotic halos), browning of buds and flowers, and the wilting of branches, twigs and of entire plants. Affected fruits are misshapen, smaller than healthy fruits, and may collapse as a consequence of the wilting of the branches. Wilted fruits are not marketable. As Psa is currently appearing in the Mediterranean region, the EPPO Secretariat decided to include it in the Pest Alert. In Italy, a ministerial (MIPAAF) decree (D.M. 7.2.2011) was issued defining the emergency measures for the prevention, control and eradication of the disease. However, the absence of a national certification scheme for kiwifruit has complicated the effective control of bacterial canker.

The disease may also have socio-economic repercussions, because its presence in a kiwifruit-producing area influences the trade of materials, such as fruit, pollen, cuttings or plantlets that are exported to other countries.

A PCR-based approach has previously been applied to detect this bacterium both in bacterial cultures and in experimentally contaminated fruit and pollen samples (Gallelli *et al.,* 2011). In addition, the presence of Psa in association with pollen, symptomatic leaves and canes, and symptomless leaves or flowers has recently been reported (Loreti *et al.,* 2011; Vanneste *et al.,* 2011b; Vanneste *et al.,* 2011c).

In the present study an integrated approach based on bacterial isolation and DNA extraction followed by two conventional PCR assays (direct-PCRs) (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011) was used to detect Psa in symptomless fruit, twigs and pollen, and to estimate the usefulness of this approach for the analysis of these kiwifruit matrices.

Materials and methods

Sample preparation

Symptomless fruits were processed either immediately after harvest or after storage at 4°C, or in controlled atmospheric conditions, from June 2009 to October 2011. They were sourced from the province of Latina (Latium, Italy), directly from producers, from local markets, and from three affected orchards (Table 1). Harvested fruits were evaluated for the epiphytic presence of Psa on the fruit surfaces and as endophytes in the *columellae* tissues. Isolation from the fruit surfaces was performed on a total of 108 samples (Table 2), each consisting of five-15 symptomless kiwifruits; the only exceptions were three samples that also contained wilting fruits collected from a severely affected area of cultivation (orchard B). Samples (five-15 fruits) were washed in 0.2 to 1 L of sterile phosphate buffer (PBS) with shaking for 90 min. Washing solution from each sample (200 mL) was filtered through sterile filter paper and then centrifuged for 20 min at 10,000 *g*. The pellet obtained was suspended in 5 mL of sterile PBS (final concentrate) and divided into two halves: sterile glycerol was added to one of these, to a final concentration of 20–30%, and this was kept at -80°C for further confirmation analyses. Aliquots (50 µL) from the second half, and of two ten-fold dilutions were plated on nutrient sucrose agar (NSA) and incubated at 25–-27°C for 3 days. Only the samples collected in October 2010 and 2011 were assessed by isolation on NSA supplemented with 0.2 mg mL^{-1} cycloheximide (Sigma Aldrich, Steimheim, Germany), 0.08 mg mL^{-1} cephalexin (Sigma Aldrich) and boric acid (1.5 mg mL^{-1}) (NSA-AB) and on modified King's medium B (KBC; Mohan and Schaad, 1987).

To check for endophytic Psa, 60 samples, each consisting of 50 fruits, were assessed. From each individual fruit, a cone of *columellae* tissue was cut from the fruit apex, and the resulting 50 cones represented the test sample, which was treated as described by Gallelli *et al.* (2011). One of these samples, collected in October 2010, was treated as follows: each cone cut from a single fruit was divided into two halves, one was used to constitute the bulk-sample, the other was macerated in 1 mL of a sterile physiological saline solution (SPS) (0.85% NaCl in distilled water). Aliquots (50 µL) of these suspensions and of two ten-fold dilutions were plated on an NSA and incubated at 25–27°C for 3 days; the remaining 900 µL were used for DNA extraction (DNeasy Plant Mini Kit; Qiagen, Dorking, UK).

Asymptomatic twigs were collected in April 2011 from two different orchards (W and P). Orchard W showed severe symptoms of the disease, while orchard P did not show any evident symptoms of bacterial canker. In orchard W, thirteen samples

Table 1. Lists of fruit samples: their sampling date, origin, total number of tested samples, storage conditions and *Actinidia* species.

Sampling date	Origin [a]	*Actinidia* sp.	Samples (Total No.) [b]		Storage [c]
			EP	EN	
June 2009	Producers (LT)	*A. chinensis*	6	-	6–7 months
October 2009	Producers (LT)	*A. chinensis*	40	8	NP
		A. deliciosa	20	4	
February 2010	Producers (LT)	*A. chinensis*	5	5	3–4 months
		A. deliciosa	8	8	
June 2010	(1) Affected orchard*-B (LT)	*A. chinensis*	3	3	NP
	(2) Local markets (RM)	*A. chinensis*	-	2	-
		A. deliciosa		2	
October 2010	Affected orchard*-M (LT)	*A. chinensis*	-	14	NP
		A. deliciosa	6	8	
June 2011	Local markets (RM)	*A. chinensis*	-	1	-
		A. deliciosa		5	
October 2011	Affected orchard* -W (LT)	*A. deliciosa*	20	-	NP
Total			108	60	

[a] LT, province of Latina (Latium region); RM, province of Rome. * Affected orchard: B and W, orchards located in LT with severe disease symptoms on leaves and branches (positive in diagnostic analysis), only fruits of orchard B showed wilting symptoms; M, orchard located in LT with old symptoms on branches, fruits were symptomless.
[b] EP, number of fruit samples processed for epiphytic Psa; EN, number of fruit samples processed for endophytic Psa.
[c] NP, storage not performed, fruits were processed immediately after harvest.

Table 2. *Pseudomonas syringae* pv. *actinidiae* (Psa) detection in symptomless fruits from 2009 to 2011 as epiphytes from washing (A) and as endophytes from cones of *columellae* cuts from the apices of fruit (B). The number of positive samples divided by the total number of tested samples is reported for each sampling. For endophytic Psa detection, the results of comparisons between isolation and direct-PCR are also reported. RG-PCR refers to Rees-George *et al*. (2010), d-PCR to Gallelli *et al*. (2011).

Sampling date[a]	(A) Epiphytes	(B) Endophytes		
	Isolation	Isolation	Direct-PCRs	
			d-PCR	RG-PCR
June 2009	0/6	---	---	---
October 2009	0/60	3/12	3/12	3/12
February 2010	0/13	0/13	0/13	0/13
June 2010 (1)	1/3	1/3	1/3	1/3
June 2010 (2)	---	0/4	1/4	1/4
October 2010	0/6	2/22	5[b]/22	6[c]/22
June 2011	---	0/6	0/6	0/6
October 2011	1[d]/20	---	---	---
Total	2/108	6/60	10/60	11/60

[a] See Table 1 for the origin of the samples.
[b] One faint signal by d-PCR.
[c] Two faint signals by RG-PCR.
[d] One colony of Psa was recovered (see the results).

were collected randomly both from symptomless (eight) and from symptomatic plants (five), and each sample was processed separately. In orchard P, samples were randomly collected from several plants distributed over the entire orchard. Each sample consisted of 100 twigs of approx. 10 cm length. For each sample, 30 twigs were randomly selected, leaves were removed after collection and each twig was cut into four pieces (total 120 stem pieces). These were then washed in 300 mL of sterile PBS buffer supplemented with 0.1% Tween 20, for 1.5 h in a rotary shaker at room temperature. The washing solution was filtered through a sterile gauze and the filtrate was centrifuged for 20 min at 10,000 *g*. The pellet was suspended in 5 mL of sterile PBS (final concentrate) and treated with the same procedure as fruits for isolation (as medium NSA-AB was used only), and DNA extraction. In parallel, 0.9 mL of final concentrate were enriched in liquid NSA-AB for 72 h at 25–27°C with gentle shaking and used for DNA extraction and PCR amplification following the above described procedures. Leaves (150–300 g) collected from six out of 12 samples were washed (0.5–1 L) as previously described for twigs, and 250 mL of washing solution were treated with the previously described procedure for isolation, enrichment and DNA extraction.

Thirteen pollen samples were analyzed, which came from six kiwifruit producers located in the province of Latina, and three from New Zealand. Two different procedures were applied for DNA extraction of the pollen samples. The first (procedure (i) was as described by Gallelli *et al.* (2011): pollen (1.5 g) was washed by gentle shaking (120 rpm) in SPS (10–20 mL) (0.85% NaCl), for 1 h at 4°C. After a spin (5 min at 180 rpm), the supernatant was divided into two sub-samples of approximately 5 mL, and both were centrifuged at 10,000 rpm for 10 min at 4°C. The pellets obtained were each suspended in 1 mL of sterile distilled water. One subsample was conserved at -80°C in 20–30% sterile glycerol for subsequent checking. Aliquots (50 µL) from the second sub-sample and of two ten-fold dilutions were plated on NSA and NSA-AB and incubated at 25–27°C for 3 days. The remaining 900 µL were centrifuged at 10,000 rpm for 10 min and the pellet was used for DNA extraction (DNeasy Plant Mini Kit; Qiagen, Dorking, UK). The second procedure (procedure (ii)) was as follows: 0.2–0.5 g of pollen were macerated in liquid nitrogen and treated with DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

To ascertain whether Psa contaminated the surface or resided inside the pollen grains, one Psa-positive pollen sample was washed repeatedly. The following procedure was used: 1.5 g of pollen grains were vortexed for 1 min in 10 mL of SPS solution with or without 0.1% Tween 20. They were then washed for 30 min at 4°C by gentle shaking and centrifuged at 10,000 g for 10 min (4°C). This step was repeated - in two different experiments - 3 and 8 times, and the pellet (washed pollen) was saved. With each step the supernatant (washing solution) was also recovered and collected as a single sample, and centrifuged at 10,000 rpm for 10 min (4°C). The two pellets obtained, corresponding to washed pollen and washing solution, were treated as described above for procedure (i).

Detection and identification procedures

Detection of Psa was performed both from axenic Psa-like colonies or by direct-PCR; direct-PCR used DNA extracted from vegetal tissues (fruit, pollen, twigs) followed by the two PCR methods of Rees-George *et al.* (2010) and of Gallelli *et al.* (2011). Aliquots (10 µL) of heat denatured bacterial suspension (corresponding to about 5×10^7 CFU mL^{-1}) prepared in sterile double distilled water or 10–20 µL of DNA were used in PCR assays (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011). The strain CRA-PAV 1530 was isolated in 2009 from *A. chinensis* plants showing symptoms of bacterial canker, and was identified as *P. syringae* pv. *actinidiae* by PCR (Rees-George *et al*., 2010; Gallelli *et al*., 2011) and by rep-PCR (primer ERIC) (Louws *et al*., 1994) using the strain CRA-FRU 8.43 (provided by M. Scortichini) as positive control. Both the strains CRA-PAV 1530 and CRA-FRU 8.43 were used as reference strains for this work. All Psa isolates recovered from fruits were compared with reference strains by means of repetitive sequence PCR (rep-PCR) (Louws *et al.,* 1994) using a primer BOX and a bacterial suspension prepared as described above. Levan-positive, fluorescence-positive colonies frequently recovered in isolation by washing the fruits, which yielded negative Psa-specific PCR assays (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011), were characterized by applying the following tests according to Lelliot and Stead

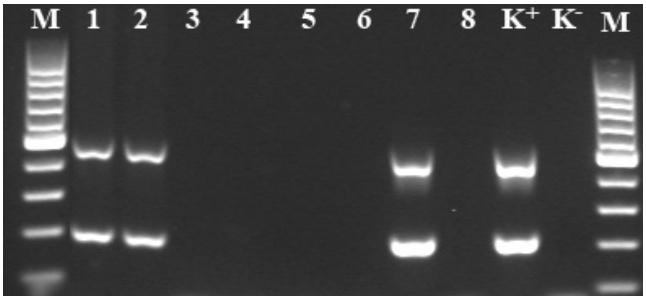

Figure 1. Detection of *Pseudomonas syringae* pv. *actinidiae* in bulk fruit samples by duplex-PCR. K^-: negative control (water); lanes 1 to 8: bulk-fruit samples; K^+: bacterial suspension (5×10^7 cfu mL^{-1}) of *Pseudomonas syringae* pv. *actinidiae* CRA-PAV 1530; M: molecular marker 100 bp DNA Ladder Plus (Fermentas, Lithuania).

(1987): the presence of oxidase, soft rot in potato slices, the presence of arginine dihydrolase, hypersensitivity reactions in tobacco leaves (LOPAT tests), aesculin and arbutin hydrolysis. The presence of the *syrB* gene, which is required for the synthesis of syringomycin, was tested according to Sorensen *et al.,* 1998. *Pseudomonas syringae* pv. *syringae* NCPPB 3869 was used as a reference strain.

Pathogenicity test

A pathogenicity test on *A. deliciosa* cv. Hayward plantlets (3–4 leaves stage) was performed as described by Minardi *et al.* (2011). Bacterial suspensions (10^8 cfu mL^{-1}) of three isolates recovered from symptomless fruits (CRA-PAV 1527, 1528 and 1583) were sprayed with a hand-held sprayer onto adaxial and abaxial leaf surfaces of ten plants; four control plants were inoculated with sterile distilled water (negative control) or with the reference Psa strains CRA-FRU 8.43 and CRA-PAV1530 (positive controls). Re-isolations were performed after symptoms of chlorotic spots on leaves appeared, and Psa-like colonies were identified as previously described.

Results

Detection of Psa in fruits, twigs and pollen

Psa was isolated from fruit washings only in two samples harvested from two severely affected bacterial canker orchards (B and W, Table 2). Moreover, some fruits of the bulk-sample collected in orchard B were not symptomless but showed typical wilting symptoms. In the positive sample collected in October 2011 in orchard W the isolation on NSA-AB and KBC media allowed selection of 40 Psa-like colonies, only one of which was identified as Psa (Table 2A). The pathogen was not isolated on NSA or on NSA-AB or KBC on any of the other samples tested (Table 2A).

Conversely, *P. syringae*-like colonies were detected in about 22% of the tested samples. These isolates were fluorescent on the King's B medium (King *et al.*, 1954), and degraded aesculin and arbutin, but gave negative results from duplex-PCR (Gallelli *et al*., 2011) and RG-PCR (Rees-George *et al*., 2010). LOPAT tests gave the following results: isolates produced levan and were oxidase-negative, were potato soft rot-negative, arginine dihydrolase-negative and tobacco hypersensitivity-positive. Approximately 40% of the *P. syringae*-like isolates gave the *syrB* amplicon by PCR amplification (data not presented). The analysis to check the endophytic presence of Psa in fruits showed that the pathogen was detected in six out of 60 samples tested by isolation (followed by bacterial identification of Psa-like colonies) (Table 2B; Figure 1). Using direct-PCR assays (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011) with DNA extracts from fruit samples, ten samples out of 60 were positive by duplex-PCR, and 11 were positive by RG-PCR. The individual fruit analysis of one PCR-positive bulk showed a faint signal in several fruits: seven out of 50 were Psa-positive by duplex-PCR and five out of 50 were Psa-positive by d-PCR (Figure 2). The isolation procedure gave negative results.The described procedure detected Psa in asymptomatic twigs (Figure 3, Table 3). In orchard W, which showed disease symptoms, Psa was isolated from 12 out of 13 samples tested, whereas all 13 samples were positive in direct PCR assays. All samples

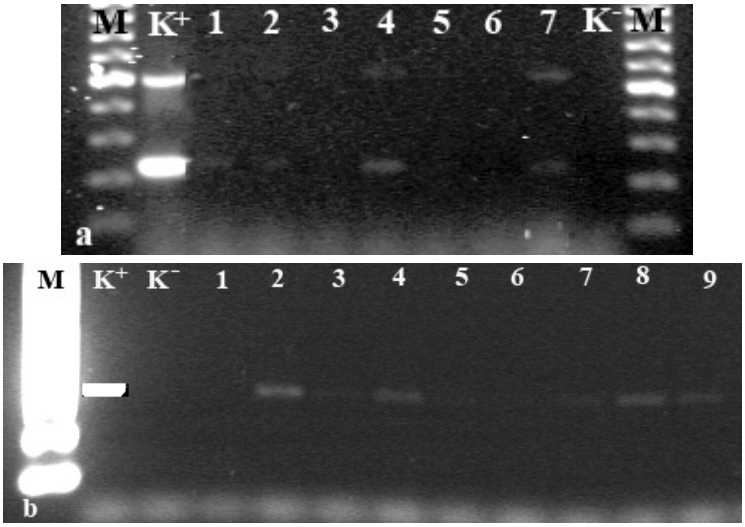

Figure 2. Gel electrophoresis analysis of polymerase chain reaction products amplified by duplex-PCR (a) and RG-PCR (b) performed on individual cones of the *columellae* of fruits. K^+, bacterial suspension (10^8 cfu mL^{-1}) of *Pseudomonas syringae* pv. *actinidiae* CRA-PAV 1530; M, molecular marker (100 pb DNA Ladder Plus; Fermentas, Lithuania); K^-, negative control (water).

collected in orchard P were negative for Psa, both by isolation and by PCR. For these latter samples, the enrichment followed by direct-PCR also gave negative results. The pathogen was detected in washings of symptomless leaves. Three samples collected from orchard W were all positive both by isolation and direct-PCR assays, whereas all the three samples from orchard P were negative with both procedures (Table 3).

The results of the Psa detection in pollen are shown in Table 4. Using procedure (i) by isolation in NSA medium, five out of 13 samples were positive. However, DNA extraction performed according to procedures (i) and (ii), followed by PCR assays (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011), detected ten Psa-positive out of the 13 te-

Table 3. *Pseudomonas syringae* pv. *actinidiae* (Psa) detection by isolation and direct-PCR in symptomless twigs collected in 2011 from orchard W and orchard P. The number of positive samples divided by the total number of tested samples is reported for isolation and direct-PCR.

Plant tissue	Origin[a]	No. samples		Isolation	Direct-PCRs
Twigs	Orchard W	Symptomatic plants	5	5/13	5/13
		Symptomless plants	8	7/13	8/13
	Orchard P		3	0/3	0/3
	Total No.		16	12/16	13/16
Leaves	Orchard W		3	3/3	3/3
	Orchard P		3	0/3	0/3
	Total No.		6	3/6	3/6

[a] W, orchards located in Latina province which showed severe disease symptoms on leaves and branches (positive in diagnostic analysis); P, orchard located in Latina province that did not show any symptoms of bacterial canker

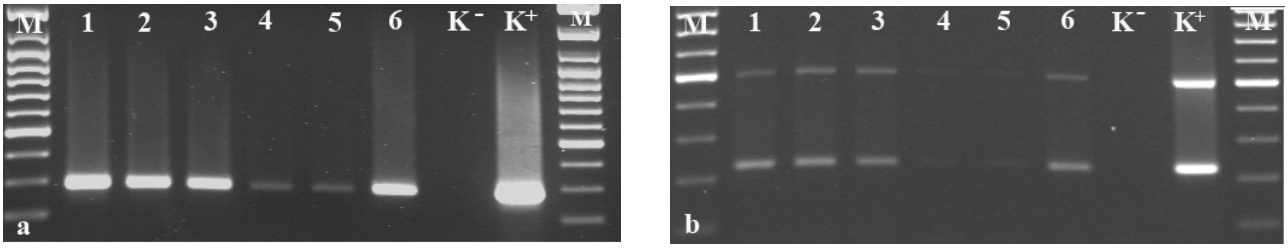

Figure 3. Gel electrophoresis analysis of polymerase chain reaction products amplified by RG-PCR (a) and by duplex-PCR (b) obtained from several twig samples treated as described in the text and collected in orchard W. K^-: negative control (water); lanes 1 to 6: twig samples; K^-: negative control (water); K^+: bacterial suspension (10^8 cfu mL^{-1}) of *Pseudomonas syringae* pv. *actinidiae* CRA-PAV 1530; M: molecular marker (100 bp DNA Ladder Plus; Fermentas, Lithuania).

sted samples. Three samples were negative with all the procedures (samples 5, 12, 13). Of the negative samples with NSA isolation, but positive with PCR-based procedures, three (samples 8, 9, 10) were again assessed for isolation on NSA supplemented with antibiotics (NSA-AB), thus permitting the isolation of Psa (producing eight positive samples out of 13 tested by isolation).

The procedure performed to test whether Psa was either within or on pollen grains enabled us to detect the bacterium both in washed pollen and in the washing solution (Figure 4).

Identification and pathogenicity test

All isolates recovered from fruits, pollen and twigs that were positive with duplex-PCR and RG-PCR showed the same rep-PCR fingerprinting profile as the reference strains used for comparison. Three isolates recovered from symptomless fruit (CRA-PAV 1527, 1528 and 1583), tested for pathogenicity on *Actinidia* plantlets, developed leaf spot symptoms within 10-15 days. Re-isolates were identified as Psa by the two PCR methods (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011).

Discussion

Pseudomonas syringae pv. *actinidiae* causes severe epidemics in kiwifruit crops in central Italy. Consequently, a detection system for this pathogen needs to be optimized for the analysis of kiwifruit materials. A strategy comprising isolation and DNA extraction followed by two PCR assays (Rees-George *et al.,* 2010; Gallelli *et al.,* 2011), based on primer sets from different genes, was recently proposed by Gallelli *et al.* (2011).

Our study has assessed the practical applicability of this integrated approach for the detection of Psa in symptomless samples of fruit, twigs and pollen collected from areas affected by bacterial canker, with fruits of different origin. This study

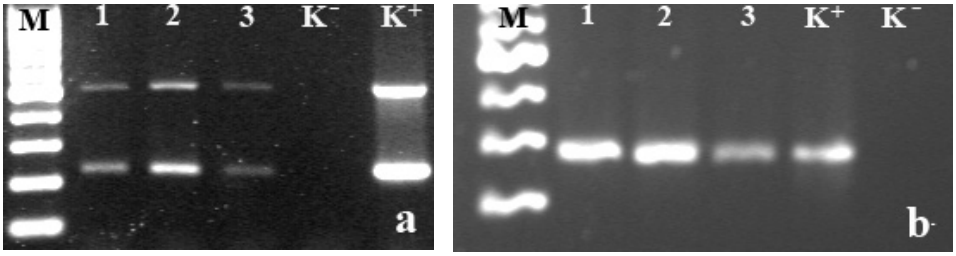

Figure 4. Gel electrophoresis analysis of polymerase chain reaction products amplified by duplex-PCR (a) and by RG-PCR (b) of pollen samples washed eight times and water used for washing without Tween 20; lane 1, washed pollen; lane 2, water collected after the first four washings (washing water); lane 3, water collected from the fifth to the eighth washing (washing water). K^-, negative control (water); K^+, bacterial suspension (10^8 cfu mL^{-1}) of *Pseudomonas syringae* pv. *actinidiae* CRAPAV-1530; M, molecular marker (Gene Ruler ™ 100 pb DNA ladder, Fermentas, Lithuania).

Table 4. Comparison of the results obtained from the two procedures (procedures (i) and (ii); refer text) used for the extraction and detection of *Pseudomonas syringae* pv. *actinidiae* from pollen of *Actinidia chinensis*. RG-PCR refers to the method of Rees-George *et al.* (2010), d-PCR refers to the duplex-PCR method of Gallelli *et al.* (2011).

Sample [a]	Procedure (i)				Procedure (ii) [b]	
	Isolation NSA	Isolation NSA-AB	RG-PCR	d-PCR	RG-PCR	d-PCR
1 (IT)	+	-	+	+	+ [f]	+ [f]
2(IT)	0	-	+ [f]	+ [f]	+ [f]	0
3 (IT)	+	-	+	+	+	+
4 (IT)	+	-	+	+	+	+
5 (NZ)	0	-	0	0	0	0
6 (IT)	0	-	+ [f]	+ [f]	+	+
7 (IT)	+	-	+	+	+	+
8 (IT)	0	+	+	+	+	+ [f]
9 (IT)	0	+	+	+	+ [f]	+ [f]
10 (IT)	0	+	+	+	+	+
11(IT)	+	-	+	+	+	+
12 (NZ)	0	0	0	0	0	0
13 (NZ)	0	0	0	0	0	0
Total	5/13	8/13	10/13	10/13	10/13	9/13

[a] IT, Italian origin; NZ, New Zealand origin.
[b] + [f], faint positive signal by PCR; –, missing data; 0, negative result.

has confirmed the reliability of the protocol of Gallelli *et al.* (2011) for Psa detection in symptomless fruit and pollen, and proposes a protocol for the extraction and detection of Psa from symptomless twigs. A comparison of two procedures (procedures (i) and (ii) above) for pollen analysis showed that both were equally efficient for detecting the presence of the bacterium in pollen. Procedure (i) is more time-consuming, but also includes isolation procedures, which are advisable in order to determine if the bacterium is viable; procedure (ii) should be used for the preliminary screening of pollen samples.

Other authors (Vanneste *et al.,* 2011b) have also recently found pollen samples contaminated with Psa, and the risk of disseminating the pathogen via pollen, was recently confirmed (Stefani *et al*., 2011).

Our attempts to establish if some external or internal contamination occurred, suggest that Psa was located both externally and internally. This agrees with the findings of other authors for detecting other pathogens (Aparicio *et al*., 1999; Barba *et al*., 2007). It is also possible that cells of Psa remain adhered to the pollen grains, despite intensive washing. However, transmissibility of the bacterium by pollen has yet to be established. For example, *Tobacco mosaic virus* can be detected in pollen, but is not pollen-transmitted (Brunt *et al.,* 1996).

Our analyses on fruit samples revealed that Psa was an epiphyte only in two samples (of 108 tested), which were collected from orchards where severe disease symptoms occurred on leaves and branches. However, it is important to emphasize that among the fruit samples tested for the presence of Psa on the fruit surfaces, the bacterium was isolated in one of the three samples containing non-marketable wilted fruits (June 2010), and in one sample collected in the severely affected orchard W (October 2011). This orchard was not subjected to treatments to prevent bacterial canker, and contained a high pathogen inoculum load. Stefani *et al.* (2011) recently reported the epiphytic contamination of Psa on fruit collected

in a untreated orchard with low disease incidence in the Emilia Romagna region of Italy, indicating that Psa was no more detectable until early August. It is also possible that Psa cells associated with symptomless fruit are numerically below the sensitivity threshold of the detection technique, in particular isolation. This was also suggested by Minardi *et al*. (2011), who did not isolate Psa, either endophytically or epiphytically, from a bulk of 15 fruit harvested from affected orchards in the Emilia Romagna region. Our ability to recover Psa, as an endophyte in the *columellae* tissues of fruit may also have depended on the use of a bulk of 50 fruits, instead of 15, permitting the reaching of the the sensitivity thresholds of the detection methods. This evidence is also supported by the analysis of individual fruits of the Psa-positive bulk sample. The faint amplicons obtained with just over one-tenth of the individual fruit constituting the bulk, suggests that the specific amplicon observed with the complete bulk-sample could be due to the summation of small bacterial inoculum from several fruit, rather than the detection of a large bacterial charge from a single fruit.

As a consequence, in our opinion, there is negligible risk of introducing Psa into countries free of bacterial canker by means of symptomless kiwifruit. These observations must be taken into consideration in order to avoid unnecessary alarm, which would have negative socio-economic impacts. As a consequence, as reported also by Stefani *et al*. (2011), kiwifruit probably does not represent a significant pathway for Psa dissemination.

Another interesting aspect was the inability to recover Psa from the 13 samples stored in a controlled atmosphere or in a cold chamber at 4°C. For a variety of Gram-negative bacteria, it is known that stress conditions, including incubation at low temperatures, can induce the VNBC state (Oliver, 1993; Ghezzi and Steck 1999). This has been evaluated for *Xanthomonas axonopodis* pv. *citri* from fruit lesions for which it has also been speculated that the difficulties in the growth of bacterial cells could be due to treating the fruit (washing, disinfection, chemical treatments, transport, and storage at low temperatures for variable periods of time) (Golmohammadi *et al.,* 2007). During cold storage the population of *Erwinia amylovora* also progressively loses its ability to be cultured (Taylor *et al*., 2003; Temple *et al*., 2007).

The decline of a bacterial population on plant material due to effects of low temperature on the survival and growth, has also been reported by other authors (Van Vuurde and de Vries, 1994; Feil and Purcel, 2001). The opposite conclusion was reached by Ordax *et al*. (2009) who detected viable *E. amylovora* cells at low levels in symptomless apples, and showed an increase in the survival of the pathogen in the cold conditions used for the long distance transport of fruits. In any case, it should be emphasized that our stored fruit samples were negative for Psa both in isolation, and direct-PCR assays. However, further analysis on stored fruit samples should be carried out in order to draw firm conclusions. Interestingly, studies that have evaluated the phytosanitary risk associated with the movement of export-quality fruit to countries free from fire-blight have highlighted that the risks of importing the disease or establishing new outbreaks is so small as to be insignificant (Roberts and Sawyer, 2008).

Our study has detected Psa from asymptomatic twigs and from washing of symptomless leaves from samples collected from a bacterial canker-affected kiwifruit orchard. This result is not surprising as the bacterium has been assumed to survive as an epiphyte on asymptomatic leaves (Stefani *et al*., 2011; Vanneste *et al.,* 2011c).

In general, of the all tested samples that we collected from the different plant organs, several samples were only positive by direct-PCR, although the PCR signal was sometimes faint. A faint PCR signal also corresponded with the recovery of only one to two Psa colonies by isolation. Some samples showed a clear positive PCR signal, but Psa was not isolated. In our opinion, these results were not due to a lack of specificity of the PCR-based methods, as both of the PCR assays always gave the same results. Instead, this may have been due to the presence of the target bacteria at the detection level limit in these symptomless samples, and to the competiveness with contaminants in isolation. The sensitivity of these protocols, which had been tested previously by experimentally calibrated inoculations with bacterial suspensions, showed that the isolation and PCR had the same detection levels (Gallelli *et al.,* 2011). However it is possible that naturally infected samples may have infestation levels that are close to the limit of detection, not included in the serial dilutions of

the bacterial suspension used in experimentally calibrated inoculations. The evidence obtained in our work has confirmed that several techniques need to be used in conjunction when diagnosing asymptomatic plant material. This has also been suggested by Gallelli *et al.* (2011), and has been concluded for other pathosystems (Loreti *et al.,* 2002; Loreti *et al.,* 2009).

Although isolation of Psa should always be carried out, we believe that the use of both PCR methods on DNA extracts obtained from plant materials (fruit, pollen, twigs) is advisable as a preliminary screening, and can also be applied for Psa detection in latent infections. Also our results from processing of individual fruit which belonged to a bulk positive sample, confirm the usefulness of direct-PCRs. This procedure enabled the detection of Psa in individual fruits (although the signal resulted was very faint).

Successful Psa detection in symptomless twigs, collected from an infected orchard, suggests the possibility of using the procedure here described for the detection of latent infections. This is particularly important for healthy nursery plant production and for the inspection of kiwifruit material traded between countries. These findings are also likely to be useful in the development of national certification schemes to safeguard the health of the kiwifruit plant material.

Acknowledgements

This research was supported by the Regione Lazio, Project "Cancro batterico dell'actinidia (*Pseudomonas syringae* pv. *actinidiae)*: messa a punto di strategie di difesa". We thank all the kiwifruit producers who supported us in collecting fruit, in particular Apofruit.

Literature cited

Argibay A.A., M.M. López, J. Peñalver Navarro, O.A. Casal, J.P. Mansilla Vázquez, A.P. Montoussé and M.J. Garcia Fernandez, 2011. First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Spain. *Plant Disease* 95, 1583.Aparicio F., M.A. Sanchez-Pina, J.A. Sanchez-Navarro and V. Pallas, 1999. Location of Prunus necrotic ringspot ilarvirus within pollen grains of infected nectarine trees: evidence from RT-PCR, dot-blot and in situ hybridisation. *European Journal of Plant Pathology* 105, 623–627.

Balestra G.M., A. Mazzaglia, R. Spinelli, S. Graziani, A. Quattrucci and A. Rossetti, 2008. Cancro batterico su *Actinidia chinensis*. *L'Informatore Agrario* 38**,** 7576.

Balestra G.M., A. Mazzaglia, A. Quattrucci, M. Renzi, L. Ricci and A. Rossetti, 2009. Cresce la diffusione in Italia del cancro batterico dell'actinidia. *L'Informatore Agrario* 24**,** 58–62.

Balestra G.M., M. Renzi and A. Mazzaglia, 2010. First report of bacterial canker of *Actinidia deliciosa* caused by *Pseudomonas syringae* pv. *actinidiae* in Portugal. *New Disease Report* 22, 10.

Balestra G.M., M. Renzi and A. Mazzaglia, 2011. First report of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit plants in Spain. *New Disease Report* 24, 10. (http://dx.doi.org/10.5197/j.2044-0588.2011.024.010).

Barba M., E. Ragozzino and F. Faggioli, 2007. Pollen transmission of Peach latent mosaic viroid. *Journal of Plant Pathology* 89, 287–289.

Bastas K.K. and A. Karakaya, 2012. First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Turkey. *Plant Disease* (doi: 10.1094/PDIS-08-11-0675).

Brunt A., K. Crabtree, M. Dallwitz, A. Gibbs and L. Watson (ed.), 1996. *Viruses of Plants: Descriptions and Lists from the VIDE Database*. CABI Publishing, CAB International, Wallingford, UK.

CABI, 2008. *Pseudomonas syringae* pv. *actinidiae*. [Distribution map]. Distribution maps of plant diseases, October (edition 1) map 1043. CABI, Wallingford, UK.

EPPO, 2011. First record of *Pseudomonas syringae* pv. *actinidiae* in Australia. *EPPO Reporting Service* 6, 130.

Everett K.R., R.K. Taylor, M.K. Romberg, J. Rees-George, R.A. Fullerton, J.L. Vanneste and M.A. Manning, 2011. First report of *Pseudomonas syringae* pv. *actinidiae* causing kiwifruit bacterial canker in New Zealand. *Australasian Plant Disease Notes* 6, 67–71.

Feil H. and A.H. Purcel, 2001. Temperature-dependent growth and survival of *Xylella fastidiosa* in vitro and in potted grapevines. *Plant Disease* 85, 1230–1234.

Ferrante P. and M. Scortichini, 2009. Identification of *Pseudomonas syringae* pv. *actinidiae* as causal agent of bacterial canker of yellow kiwifruit (*Actinidia chinensis* Planchon) in central Italy. *Journal of Phytopathology* 157, 768–770.

Gallelli A., A. L'Aurora and S. Loreti, 2011. Gene sequence analysis for the molecular detection of *Pseudomonas syringae* pv. *actinidiae*: developing diagnostic protocols. *Journal of Plant Pathology* 93, 425–435.

Ghezzi J.I. and T.R. Steck, 1999. Induction of the viable but non-culturable condition in *Xanthomonas campestris* pv. *campestris* in liquid microcosms and sterile soil. *FEMS Microbiology Ecology* 30, 203–208.

Golmohammadi M., J. Cubero, J. Penalver, J.M. Quesada, M.M. Lopez and P. Llop, 2007. Diagnosis of *Xanthomonas axonopodis* pv. *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods. *Journal of Applied Microbiology* 103, 2309–2315.

King E.O., M.K. Raney and D.E. Ward, 1954. Two simple media for the demonstration of pyocianin and fluore-

scin. *Journal of Laboratory and Clinical Medicine,* 44, 301–307.

Koh J.K., B.J. Cha, H.J. Chung. and D.H. Lee, 1994. Outbreak and spread of bacterial canker in kiwifruit. *Korean Journal of Plant Pathology* 10, 68–72.

Lelliot R.A. and D.E. Stead (ed.), 1987. Methods for the diagnosis of bacterial diseases of plants. In: *Methods in Plant Pathology*, Vol. 2. Blackwell Scientific Publications for British Society for Plant Pathology, Oxford, UK.

Loreti S. and A. Gallelli, 2002. Rapid and specific detection of virulent *Pseudomonas avellanae* strains by PCR amplification. *European Journal of Plant Pathology* 108, 237–244.

Loreti S., A. Gallelli, D. De Simone and A. Bosco, 2009. Detection of *Pseudomonas avellanae* and occurrence of the bacteria microflora of hazelnut affected by 'moria' in central Italy. *Journal of Plant Pathology* 91, 365–374.

Loreti S., A. Gallelli, A. L'Aurora and S. Talocci, 2011. Bacterial canker of kiwifruit by *Pseudomonas syringae* pv. *actinidiae*: detection of the bacterium from fruits and pollen. *Proceedings XVII Convegno Annuale della Società Italiana Patologia Vegetale (SIPaV),* 12–15 Settembre, 2011, Bologna, Italia (abstract).

Louws F.J., D.W. Fullbright, C.T. Stephens and F.J. De Bruijn, 1994. Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomona*s pathovars and strains generated with repetitive sequence and PCR. *Applied and Environmental Microbiology* 60, 2286–2295.

Minardi P., C. Lucchese, S. Ardizzi and U. Mazzucchi, 2011. Evidence against the presence of *Pseudomonas syringae* pv. *actinidiae* in fruits of actinidia orchards affected by bacterial canker. *Proceedings XVII Convegno Annuale della Società Italiana Patologia Vegetale (SIPaV).* 12–15 Settembre, 2011, Bologna Italia (abstract).

Mohan S.K. and N.W. Schaad, 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* 77, 1390–1395.

Ordax M., E.G. Biosca, S.C. Wimalajeewa, M.M. Lopez and E. Marco-Noales 2009. Survival of *Erwinia amylovora* in mature apple fruit calyces through the viable but nonculturable (VNBC) state. *Journal of Applied Microbiology* 107, 106–116.

Oliver J.D., 1993. Formation of viable but nonculturable cells. In: *Starvation in Bacteria* (Kjelleberg S., ed.), Plenum, New York, NY, USA, 239–272.

Rees-George J., J.L. Vanneste, D.A. Cornish, I.P.S. Pushparajah, J. Yu, M.D. Templeton and K.R. Everett 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S-23S rDNA intertrascribed spacer region and comparison with PCR primers based on other gene regions. *Plant Pathology* 59**,** 453–464.

Roberts R.G. and A.J. Sawyer, 2008. An updated pest risk assessment for spread of *Erwinia amylovora* and fire blight via commercial apple fruit. *Crop Protection* 27, 362–368.

Serizawa S., T. Ichikawa, Y. Takikawa, S. Tsuyumu and M. Goto, 1989. Occurrence of bacterial canker of kiwifruit in Japan: description of symptoms, isolation of the pathogen and screening of bactericides. *Annals of the Phytopathological Society of Japan* 55, 427–436.

Sorensen K.N., K.H. Kim and J.Y. Takemoto, 1998. PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Applied and Environmental Microbiology* 64, 226–230.

Stefani E., D. Giovanardi and F. Ferrari, 2011. Dissemination of *Pseudomonas syringae* pv. *actinidiae* through pollen and its epiphytic life on leaves and fruits. *Phytopathologia Mediterranea*. 489–496.

Taylor R.K., C.N. Hale, F.A. Gunson and J.W. Marshall, 2003. Survival of the fire blight pathogen, *Erwinia amylovora*, in calyxes of apple fruit discarded in an orchard. *Crop Protection* 22, 603–608.

Temple T.N., V.O. Stockwell, P. Lawrence Pusey and K.B. Johnson, 2007. Evaluation of likelihood of co-occurrence of *Erwinia amylovora* with mature fruit of winter pear. *Phytopathology* 97, 1263–1273.

Van Vuurde J.W.L. and P.H.M. de Vries, 1994. Population dynamics of *Erwinia carotovora* subsp. *atroseptica* on the surface of intact and wounded seed potatoes during storage. *Journal of Applied Bacteriology* 76, 568–575.

Vanneste J.L., F. Poliakoff, C. Audusseau, D.A. Cornish, S. Paillard, C. Rivoal and J. Yu., 2011a. First Report of *Pseudomonas syringae* pv. *actinidiae* the causal agent of bacterial canker of kiwifruit in France. *Plant Disease* 95, 1311.

Vanneste J.L., D. Giovanardi, J. Yu, D.A. Cornish, C. Kay, F. Spinelli and E. Stefani, 2011b. Detection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen samples. *New Zealand Plant Protection* 64, 246–251.

Vanneste J.L., J. Yu, D.A. Cornish, S. Max and G. Clark, 2011c. Presence of *Pseudomonas syringae* pv. *actinidiae* the causal agent of bacterial canker of kiwifruit, on symptomatic and asymptomatic tissues of kiwifruit. *New Zealand Plant Protection* 64, 241–245.

Accepted for publication: November 11, 2011