GENE SEQUENCE ANALYSIS FOR THE MOLECULAR DETECTION OF *PSEUDOMONAS SYRINGAE* pv. *ACTINIDIAE*: DEVELOPING DIAGNOSTIC PROTOCOLS

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SUMMARY

Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*) is seriously damaging *Actinidia deliciosa* and *A. chinensis* in central Italy. Since this severe outbreak of the disease may reflect on the trade of kiwifruit pollen and fruits, standardized protocols are needed for the extraction of the bacterium from different matrices and for its detection and identification. Current PCR detection of *Psa* is aspecific, as the amplified product has the same size as that of *P. syringae* pv. *theae.* To improve the specificity of molecular detection of *Psa*, a gene-sequence analysis was done to identify new specific DNA markers. This enabled us to develop a duplex-PCR that distinguishes *Psa* from *P. syringae* pv. *theae* and from other genetically related *P. syringae* pathovars. This method was also successfully applied to detect *Psa* directly in infected kiwifruit matrices such as leaves, wood, flowers and in experimentally contaminated pollen and fruits. We propose two protocols for *Psa* extraction and detection from pollen and fruits. These protocols can be used for epidemiological studies, to establish whether symptomless fruits or pollen can harbour *Psa,* and can help diagnostic laboratories in the analysis of these type of materials.

Key words: *Actinidia deliciosa*, *A. chinensis*, duplex-PCR, pollen, fruits, diagnosis, disease outbreak.

INTRODUCTION

Over the last three years, severe outbreaks of bacterial canker induced by *P. syringae* pv. *actinidiae* (*Psa*) have been observed in the province of Latina (central Italy), first on *Actinidia chinensis* cvs Hort16A and Jin Tao, then on *A. deliciosa* (Balestra *et al.*, 2008, 2009a; Ferrante and Scortichini, 2009). *Psa* has caused serious damage in Japan (Serizawa *et al.*, 1989), where it was first described (in 1984), and in Korea (Koh *et al.*, 1994). In 1992, it was detected on *A. deliciosa* in the province of Latina (Scortichini, 1994) where, until 2008, damages were minor.

The main symptoms of the disease are: oozing of reddish exudates from the trunk and branches, spots surrounded by yellow halos on the leaves, twig dieback, leaf and plant wilting. Because *Psa* is currently emerging in the Mediterranean area, EPPO has decided to add it to its alert list. The importance of the disease also has a socioeconomic impact, because its presence in a producing area may hinder fruit export.

Psa detection has mainly been based on traditional techniques (isolation and purification), followed by identification of pure cultures by rep-PCR (Ferrante and Scortichini, 2009) or sequencing of 16S rDNA (Balestra *et al.*, 2009b). Molecular identification by PCR has been reported by Koh and Nou (2002) (KN-PCR) and by Rees-George *et al.* (2010) (RG-PCR). However, both these methods are aspecific, as they yield an amplicon of the same size as that of *P. syringae* pv. *theae* (Rees-George *et al.*, 2010). Moreover, these methods have been tested on experimentally contaminated extracts from buds (Rees-George *et al.*, 2010), but not on naturally infected kiwifruit plants.

To improve the specificity of *Psa* molecular detection, a gene-sequence analysis was performed to identify new specific DNA markers capable of identifying this bacterium but not other *P. syringae* pathovars or related pseudomonads. It is well known that *Psa* is genetically related to *P. syringae* pv. *theae* (Sawada *et al.*, 1999) and *P. avellanae* (Scortichini *et al.*, 2002; Manceau and Brin, 2003). These species belong to genomospecies 8 *sensu* Gardan *et al.* (1999) and *P. syringae* pv. *tomato* (belonging to genomospecies 3) is most closely related to *Psa*, *P. syringae* pv. *theae* and *P. avellanae* (Gardan *et al.*, 1999; Scortichini *et al.*, 2002; Sarkar and Guttman, 2004). Recent studies based on rpoD phylogeny grouped these Pseudomonas in phylogroup 1 (Parkinson *et al.*, 2011). Taking the strong relationship among these pseudomonads into consideration, we focused on the following genes of representative strains of *Psa*, *P. syringae* pv. *theae, P. syringae* pv. *tomato* and *P. avellanae: avrD1*, *hrpW*, *hrpL, hopAB1* were investigated along with the housekeeping *rpoD* gene and the 492 bp sequence of

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the Koh and Nou (2002) (KN-) amplicon. Thus, we developed a duplex-PCR based on the amplification of two bands, in the same PCR reaction, for the specific detection of *Psa.*

The aim of this study was to apply this specific molecular method for the rapid identification of suspected *Psa* colonies and for the specific, sensitive, and rapid detection of the bacterium in infected kiwifruit tissues, fruits and pollen.

MATERIALS AND METHODS

Gene amplification and sequencing. The amplification of homologues of *hrpW*, *hrpL, hopAB1, rpoD* and of the 492 bp KN-amplicon of Koh and Nou (2002) was done by PCR using the primers reported in Table 1. Primers for *avrD1* were provided by Robert Jackson (University of Reading, UK) and are available upon request. The PCR reaction was carried out in a final volume of 50 µl containing 1X PCR buffer (MBI Fermentas, Lithuania), 0.05 U/µl *Pfu* DNA polymerase (MBI Fermentas, Lithuania), 0.2 mM dNTPs, 0.04 ng/µl BSA, 0.1 µM each primer, 25 ng genomic DNA extracted from 1.5 ml bacterial cultures by a Puregene DNA isolation kit (Gentra System-Flowgen, UK). The thermal profile consisted of an initial hot-start step 70°C for 3 min and an initial denaturation step 94°C for 3 min, followed by 30 cycles at 94°C (1 min), annealing (1 min) at the appropriate temperature for each gene (*hrpW,* 56°C; *hrpL,* 60°C; *hopAB1,* 50°C; *avrD1,* 58°C; *rpoD*, 57°C; KN-amplicon, 57°C) and an extension at 72°C (from 1 min to 2 min, depending on gene length). Nucleotide sequences were deposited in GenBank, as reported below.

Sequence analysis. Multiple alignment of nucleotide sequences was performed using a Clustal W algorithm analysis (Higgins and Sharp, 1988). Phylogenetic and molecular analyses were conducted using MEGA version 4.0 (Tamura *et al.*, 2007; Kumar *et al.*, 2008) and the neighbor joining (NJ) clustering algorithm. Bootstrap analyses were performed to estimate the significance level of the NJ tree internal branches (Hedges, 1992).

Development of duplex-PCR assay. The duplex-PCR assays were carried out in a 50 µl reaction mixture containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM each KN-F and KN-R primers (Koh and Nou, 2002), 0.4 µM each primer AvrDdpx-F and -R primers (reported in Table 2), 0.05 U/µl Platinum *Taq* DNA Polymerase (Invitrogen, Life Technologies, UK), 1X PCR buffer (Invitrogen, Life Technologies, UK). As an alternative to the hot-start Platinum *Taq* DNA Polymerase, Biotaq™ DNA Polymerases (Bioline, UK) was used in the PCR reaction. Bacterial genomic DNA (20 ng), or an aliquot (5 µl) of the bacterial suspension at a concentration of 2×10^7 CFU ml^{-1} were used as template. AvrDdpx primers were designed by comparing by Clustal W (Higgins and Sharp, 1988) the *avrD1* nucleotide sequences of *Psa* ISF Act.1 (accession No. FR734159), *Psa* ISF 8.57 (FR734161), *Psa* ISPaVe 019 (FR734160) and *P. syringae* pv. *tomato* NCPPB 2563 (AFR734162), obtained in this study, with the following known NCBI sequences: *Psa* NCPPB 3740 (AM410556.1), *P. avellanae* NCPPB 3872 (AM410894), *P. syringae* pv. *tomato* PT1 (ZP03400273), *P. syringae* pv. *coryli* ISPaVe 598 (AM410554.1) and DPP51 (AM410553.1). AvrDdpx primers were used in the same PCR reaction with KN-PCR primers of Koh and Nou (2002). All primers were synthesized by Invitrogen (Life Technologies, UK). The PCR thermal profile consisted of an initial denaturation step (95°C for 3 min), followed by 30 cycles at 94°C for 30 sec, 63°C for 45 sec, 72°C for 50 sec and a final elongation step of 5 min at 72°C. All amplification products (15-20 µl) were analyzed on 1% (w/v) agarose gel cast and run in TAE buffer (0.04 M Tris, 0.001 M EDTA and 0.02 M acetic acid) stained with ethidium bromide and photographed under UV light.

The specificity of duplex-PCR was assessed by testing bacterial suspensions (approximately $5x10^7$ CFU ml^{-1}) of all strains in Table 2. The sensitivity threshold of duplex-PCR was determined from bacterial cells and genomic DNA. Cell suspensions of two *Psa* strains (ISF Act.1 and NCPPB 3740) were serially ten-fold diluted from a 2×10^4 CFU/PCR reaction (corresponding to 10^8 CFU ml^{-1}) to 10^{-5}. For genomic DNA, ten-fold dilutions in the range of 50 ng to 50 fg were used for PCR. Bacterial genomic DNA was extracted from 1.5 ml broth cultures of *Psa* ISF Act.1 and NCPPB 3740 using the Puregene DNA isolation kit (Gentra System-Flowgen, UK). DNA quantity was estimated by comparing known standards in ethidium bromide-stained 1% agarose gel.

Sample preparation from symptomatic kiwifruit. Leaves, petioles, sepals, trunk or branches were sampled during 2010 from diseased *A. deliciosa* cv. Hayward, and *A. chinensis* cvs Hort 16A and Soreli. Symptomatic samples were collected from adult trees from April to June depending on the developmental stage of the plants. Small portions of tissue (3-5 mm) were aseptically removed around necrotic areas and crushed in 1-2 ml of sterile 0.85% NaCl solution in distilled water (SPS). Aliquots (50-100 µl) of this suspension and of two ten-fold dilutions (10^{-1} and 10^{-2}) were plated on nutrient agar with 5% sucrose (NSA) medium and incubated at 25-27°C for 3 days. The residual suspension was filtered and centrifuged for 10 min at 10,000 rpm to concentrate bacterial cells. The pellet was treated for DNA extraction with a DNeasy Plant Mini Kit (Qiagen, Italy) in accordance with the manufacturer's instructions.

Table 1. PCR primers used in this study.

Primer target	Primer name	Primer sequence	Reference
hopAB1	AvrPtoB-LF	5'-GGAGAGGATCAGCATATG-3'	This study
	AvrPtoB-LR	5'-TCAGGGGACTATTCTAAAAG-3'	
hrpL	L1	5'-ACCTGGTTGTGTGGCATTGC-3'	Cournoyer *et al.*, 1996
	L2	5'-CCGTGAGCGGACGGTGCC-3'	
rpoD	PsrpoD FNP1	5'-TGAAGGCGARATCGAAATCGCCAA-3'	Parkinson *et al.*, 2011
	PsrpoDnprpcr1	5'-YGCMGWCAGCTTYTGCTGGCA-3'	
hrpW	WthFor	5' -AAGCGGCAAGAGTCCTCAAC -3'	
	WthRev	5'- GATGCCTGGGTTTTATCGTAG -3'	This study
	WthFint	5'- ACAGCCTGATAGCCAGGCTC -3'	
	Wthfint	5'- GCGTGCACGTTGTCAATGGT -3'	
KN-PCR amplicon	KN-F	5'-CACGATACATGGGCTTATGC-3'	Koh and Nou, 2002
	KN-R	5'-CTTTTCATCCACACACTCCG-3'	
	KN-F/ R and		Koh and Nou, 2002
KN-amplicon and *avrD1*	AvrDdpx-F	5'-TTTCGGTGGTAACGTTGGCA-3'	
	AvrDdpx-R	5'-TTCCGCTAGGTGAAAAATGGG-3'	This study

Sample preparation from pollen and fruit. *Pollen.* For bacterial isolation and DNA extraction, 1.5 g of pollen were washed by gentle shaking in SPS (10 ml) for 1 h at 4°C (washing step). After a spin (5 min at 180 rpm), the supernatant was divided into two sub-samples of approximately 5 ml, each of which was centrifuged at 10,000 rpm for 10 min at 4°C (concentration step). The pellets were each suspended in 1 ml of sterile distilled water (final-suspension step). One sub-sample was conserved at -80°C in 30% sterile glycerol for subsequent check. Aliquots (50-100 µl) of the other sub-sample and of two ten-fold dilutions were plated on NSA supplemented with 0.2 mg ml^{-1} cycloheximide (Sigma, USA) and 0.08 mg ml^{-1} cephalexin (Sigma, USA), and incubated at 25-27°C for 3 days. The remaining 850 µl were centrifuged at 10,000 rpm for 10 min and the pellet was used for DNA extraction (DNeasy Plant Mini Kit, Qiagen, Italy). The detection level was determined with calibrated dilutions of *Psa* cell suspensions mixed with pollen samples. Contaminated samples were prepared by adding 1 ml of bacterial suspension to the washing step of the procedure. A ten-fold dilution suspension was inoculated to obtain a theoretical initial bacterial concentration between 10^5 and 10 CFU ml^{-1}. A pollen extract without inoculum served as negative control. All samples were processed as described above for bacterial isolation and DNA extraction.

Fruits. The protocol for *Psa* extraction and detection in symptomless fruits consisted in processing a bulk-sample of 50 fruits as follows. After washing, a small portion (a cone) of the columella tissue without peel was aseptically removed from the petiolar end of each fruit. Each sample (made up of 50 cones) was macerated in 30 ml of PBS in a Stomacher bag and left to settle for 15 min on ice (soaking step). The supernatant was filtered and centrifuged at 10,000 rpm for 10 min (concentration step), and the pellet was suspended in 1 ml PBS. Aliquots (50-100 µl) of this suspension and of two ten-fold dilutions were plated on NSA supplemented with 0.2 mg ml^{-1} cycloheximide (Sigma, USA) and 0.08 mg ml^{-1} cephalexin (Sigma, USA), and incubated at 25-27°C for 3 days. The remaining 800 µl were centrifuged at 10,000 rpm for 5 min and the pellet used for DNA extraction (DNeasy Plant Mini Kit, Qiagen, Italy). The detection level was determined with calibrated dilutions of *Psa* cell suspensions in fruit macerates. The contaminated sample was prepared by adding 1 ml of bacterial suspension to the soaking step of the procedure. A ten-fold dilution suspension was inoculated to obtain a theoretical initial bacterial concentration between 10^5 and 1 CFU ml^{-1}. A fruit extract without inoculum served as negative control. All samples were processed as described above for isolation and DNA extraction. Each experiment was repeated at least twice.

Detection and characterization of *Pseudomonas syringae pv. actinidiae.* *Psa* was detected with duplex-PCR and Rees-George *et al.* (2010) PCR either from levan-positive colonies grown in isolation (NSA medium) or from DNA extracted from diseased kiwifruits matrices and artificially contaminated pollen and fruit samples, treated as previously described. Aliquots (5 µl) of a heat-denatured bacterial suspension prepared in sterile double distilled water at about 10^8 CFU ml^{-1} were used in PCR after growing each bacterial colony on NSA medium for 48 h at 27°C. *Psa* strains ISF Act.1, ISPaVe 019 and NCPPB 3740 were used as positive controls. All PCR-duplex positive colonies were compared with reference strains using a repetitive sequence PCR (ERIC-PCR) (Louws *et al.*, 1994) and the Rees-George *et al.* (2010) PCR, using bacterial suspension prepared as described above. Five microliters of DNA extracted with DNeasy Plant Mini Kit (Qiagen, Italy) from symptomatic samples, artificially contaminated

Table 2. Bacterial strains used in this study, their geographic origin and host of origin.

Species/pathovar/Strain	Geographic origin	Host	Year of isolation
Pseudomonas syringae pv. *actinidiae*			
NCPPB 3739 = KW11^T	Japan	*Actinidia chinensis*	1984
NCPPB 3740 = KW30	Japan	*A. chinensis*	1984
ISPaVe 019	Italy	*A. deliciosa*	1992
ISPaVe 020	Italy	*A. deliciosa*	1992
ISF (= CRA-FRU) 8.57	Italy	*A. chinensis*	2009
ISF Act.1	Italy	*A. chinensis*	2008
ISF 8.69	Italy	*A. chinensis*	2009
ISF 5.1	Italy	*A. chinensis*	2009
ISF 10.22	Italy	*A. chinensis*	2008
ISF 10.6	Italy	*A. chinensis*	2009
ISF 10.14	Italy	*A. chinensis*	2009
OMP-BO 1875,1	Italy	*A. chinensis*	2008
OMP-BO 1875,3	Italy	*A. chinensis*	2008
CRA-PAV 1530	Italy	*A. chinensis*	2009
CRA-PAV 1531	Italy	*A. chinensis*	2009
CRA-PAV 1532 - 1535*	Italy	*A. deliciosa*	2010
CRA-PAV 1539 - 1542*	Italy	*A. chinensis*	2010
CRA-PAV 1562 - 1574*	Italy	*A. chinensis*	2010
P. syringae pv. *theae*			
CFBP 4097	Japan	*Camellia sinensis*	NA
NCPPB 2598	Japan	*Camellia sinensis*	1970
P. syringae pv. *tomato*			
NCPPB 2563	UK	*Solanum lycopersicum*	1973
DC3000	UK	*Solanum lycopersicum*	1960
P. syringae pv. *syringae*			
OMP-BO 3909B,1	Italy	*Actinidia chinensis*	2009
OMP-BO 4250,1	Italy	*Actinidia chinensis*	2009
ISPaVe_1231	Italy	*Actinidia deliciosa*	2001
ISPaVe 326	Italy	*Magnolia purpurea*	-----
ISPaVe 087	Italy	*Prunus cerasus*	-----
ISPaVe 1066	Italy	*Pyrus communis*	1994
ISPaVe 1203	Italy	*Prunus domestica*	2000
ISPaVe 1205	Italy	*Pyrus communis*	2000
NCPPB 3969	Italy	*Laurus nobilis*	1992
P. syringae pv. *phaseolicola*			
1449B (R. Jackson)	NA	*Phaseolus vulgaris*	----
ISF 3634	Italy	*Phaseolus vulgaris*	----
P. syringae pv. *coryli*			
DPP 51	Italy	*Corylus avellana*	1999
P. syringae pv. *lachrymans*			
NCPPB 3544	UK	*Cucumis sativus*	1987
P. syringae pv. *morsprunorum*			
ISPaVe 432	Italy	*Prunus cerasus*	1992
P. syringae pv. *marginalis*			
ISPaVe 1184	Italy	*Cichorium intybus*	2000
P. syringae pv. *glycinea*			
ISPaVe 1155	Italy	*Glycine max*	1999
IPV-BO 2116	Italy	*Glycine max*	1985
P. syringae pv. *papulans*			
NCPPB 2848	Canada	*Malus sylvestris*	1975
P. avellanae			
NCPPB 3872	Italy	*Corylus avellana*	1991
ISPaVe 1267	Italy	*Corylus avellana*	2003

P. viridiflava			
OMP-BO 4664,1	Italy	*Actinidia deliciosa*	2009
OMP-BO 4254 A,1	Italy	*Actinidia chinensis*	2009
P. corrugata			
NCPPB 2445	UK	*Solanum lycopersicum*	1973
P. savastanoi			
ISPaVe 1093	Italy	*Oleae europea*	1998
P. putida			
ISPaVe 224	Italy	Soil	1994
Erwinia amylovora			
OMP- BO 1077/7	Italy	*Pyrus communis*	1994
E. billingiae			
NCPPB 661	UK	*Pyrus communis*	1959
E. tasmaniensis			
NCPPB 4357	Australia	*Malus domestica*	2006
Xanthomonas arboricola pv. *juglandis*			
NCPPB 411	New Zealand	*Juglans regia*	1957
X. arboricola pv. *pruni*			
ISPaVe 1224	Italy	*Prunus persica*	2001
X. arboricola pv. *corylina*			
NCPPB 935	USA	*Corylus maxima*	1961
Brenneria nigrifluens			
ISPaVe 1061	Italy	*Juglans regia*	1998
Agrobacterium tumefaciens			
LC.58	USA	*Prunus avium*	----

NA, unknown or not assigned.
* also the strains included among the codes listed were used in this study : NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; ISPaVe/CRAPAV: Culture Collection of Research Centre on Plant Pathology, Rome, Italy; CFBP: Collection Francaise de Bacteries Phytopathogenes, Angers, France; ISF/CRA-FRU: Culture Collection of Fruit Trees Research Centre, Rome, Italy; OMP-BO: Osservatorio delle Malattie delle Piante, Bologna, Italy; IPV-BO: Culture Collection of Istituto di Patologia Vegetale, Università di Bologna; DPP: Culture Collection of Dipartimento Protezione delle Piante, Università di Sassari, Sassari, Italy; R. Jackson, University of Reading Whiteknights, Reading, UK.

symptomless fruits and pollen were used for duplex-PCR and Rees-George *et al.* (2010) PCR amplifications.

All *Psa* isolates recovered from diseased kiwifruits matrices were also characterized by the following biochemical tests as described by Lelliot and Stead (1987) and Schaad (1988): levan production, presence of oxidase, soft rot activity on potato slices, presence of arginine dehydrolase, hypersensitivity reaction in tobacco leaves (LOPAT tests), fluorescence on King's medium B (King *et al*., 1954) and arbutin hydrolysis. Kiwifruit-associated bacteria were purified to verify possible cross-reactions (false-positive) in duplex-PCR, of saprophytic or opportunistic bacteria present in kiwifruit matrices. Stock cultures were lyophilized and regenerated on NSA medium for duplex-PCR assay.

RESULTS

Gene amplification and sequence analysis. Homologues of *avrD1*, *hrpW*, *hrpL, rpoD* and of the 492 bp amplicon of Koh and Nou (2002) were identified by PCR in the majority of the tested strains (Table 3). No amplicons were obtained for *avrD1* from *P. syringae* pv. *theae* CFBP 4097 and for KN-PCR from *P. avellanae* NCPPB 3872. On the other hand, *hopAB1* homologues were amplified only from *P. avellanae* NCPPB 3872 and from *P. syringae* pv. *theae* CFBP 4097, but not from *Psa* strains ISF Act.1, ISPaVe 019, NCPPB 3740. As a consequence, *hopAB1* was not included in the gene sequence analysis for the marker detection of *Psa*. Sequencing of the other above-mentioned genes revealed high identity values (Table 3) between homologous genes of *Psa* strains ISF Act.1 and the representatives of *P. syringae* pv. *tomato* (DC3000 and NCPPB 2563), *P. syringae* pv. *theae* (CFBP 4097), *P. avellanae* (NCPPB 3872) and *Psa* (NCPPB 3740, ISPave 019 and ISF8.53).

The KN-amplicon sequence, analysed by the BLAST2.24 (Zhang *et al.*, 2000) program, showed a 97% identity with a sequence coding for a putative

Table 3. Identity (%) of *P. syringae* pv. *actinidiae* ISF Act.1 gene and the heterologous genes of *P. syringae* pv. *actinidiae* NCPPB 3740 and ISPaVe 019, *P. syringae* pv. *theae* CFBP 4097, *P. syringae* pv. *tomato* DC 3000, and *P. avellanae* NCPPB 3872.

	avrD1	*hrpW*	*hrpL*	*rpoD*	KN-PCR amplicon
P. s. pv. *actinidiae* ISF 8.57	99	100	100	ND	ND
P. s. pv. *actinidiae* NCPPB 3740	99	93	100	100	98
P. s. pv. *actinidiae* ISPaVe 019	99	93	97	ND	98
P. s. pv. *theae* CFBP4097	---	92	97	98	98
P. s. pv. *tomato* DC3000	NF	93	96	97	96
P. s. pv. *tomato* NCPPB 2563	95	93	96	ND	95
P. avellanae NCPPB 3872	98	93	98	91	----

NF = a heterologous gene not found in GenBank; ND =not done.

lipoprotein, (outer membrane protein P1) of *P. syringae* pv. *tomato* DC3000.

Phylogenetic trees were constructed with MEGA version 4.0 using *hrpW, hrpL, avrD1* and KN-amplicon sequences. The tree based on the *hrpW* sequence showed that all *Psa* strains grouped into one main cluster (I) (Fig. 1A). However, *Psa* ISF Act.1 and ISF 8.57, recovered in the 2009 outbreak, branched separately from the *Psa* strains NCPPB 3740 previously recovered from Japan, and from the Italian *Psa* strain ISPaVe 019 isolated in 1992, which clustered in a subgroup with *P. avellanae* NCPPB 3872. Both these subgroups were supported by a high bootstrap value (100%). *P. syringae* pv. *tomato* and *P. syringae* pv. *theae* grouped separately into cluster II (99% bootstrap).

The *avrD1* phylogenetic tree (Fig. 1B) showed that *Psa* strains and *P. avellanae* NCPPB 3872 grouped into one main cluster (I) well separated from *P. syringae* pv. *tomato* NCPPB 2563 (cluster II). Within cluster I, even within the same well supported subgroup (88%), *Psa* ISF Act. 1 and ISF 8.57 branched separately (70% bootstrap) from *Psa* NCPPB 3740 and ISPaVe 019.

A similar branching pattern was found in the KN-amplicon phylogenetic tree (Fig. 1C). Within the main cluster I, which included all *Psa* strains and *P. syringae* pv. *theae* CFBP 4097, *Psa* ISF Act.1 and ISF 8.57 branched separately (94% bootstrap) from *Psa* NCPPB 3740 and ISPaVe 019 (95% bootstrap). Both *P. syringae* pv. *tomato* strains grouped apart in cluster II.

The *hrpL* phylogenetic tree (Fig. 1D) showed that all *Psa* strains were comprised into cluster I (with 66% bootstrap) along with *P. syringae* pv. *theae* CFBP 4097. *P. avellanae* NCPPB 3872, which also belongs to cluster I, branched separately from *Psa* strains. *P. syringae* pv. *tomato* strains grouped in cluster II.

Development of duplex-PCR assay. The alignment of the *avrD1, hrpW, hrpL, rpoD* and KN-amplicon sequences with each other and with known NCBI GenBank sequences did not disclose any useful DNA region for the selection of *Psa*-specific primers. On the other hand, a comparison between the *avrD1* genes of *Psa* ISF Act.1 and other homologous genes, enabled the design of primers AvrDdpx-F and AvrDdpx-R which, when used in combination with KN-PCR primers, amplified, in a single PCR reaction, two bands of 492 and 226 bp, specific for all *Psa* strains tested (see Table 2), but not for any of the strains of the other *P. syringae* pathovars, or species, or genera of plant pathogenic bacteria reported in Table 2. In particular, the duplex-PCR assay was unable to amplify both bands from strains of *P. syringae* pv. *theae*, *P. syringae* pv. *tomato*, *P. avellanae,* which gave only one of the two amplicons, and from strains of *P. syringae* pv. *syringae* and *P. viridiflava* isolated from kiwifruit, that yielded no amplicons (Fig. 2).

The specificity of this method was confirmed by testing several bacteria associated with kiwifruit plants affected by bacterial canker (not shown). The sensitivity threshold of the method corresponded to 2×10 CFU/PCR reaction from bacterial suspension and 0.5 pg/PCR reaction from genomic DNA (Fig. 3). The use of a recombinant *Taq* polymerase for duplex-PCR did not always reproduce the standards of specificity above described. Thus, we recommend using a hot-start *Taq* polymerase for successful analysis.

Detection and characterization of *Psa* from symptomatic kiwifruit matrices. Isolation from *A. chinensis* and *A. deliciosa* yielded *Psa* isolates from different diseased matrices such as twigs, branches, flowers, leaves. All these isolates were: levan-positive, oxidase and potato soft rot negative, induced a hypersensitive reaction on tobacco leaves after 24 h of infiltration, did not produce a fluorescent pigment on medium B of King *et al.* (1954) (KB), were arbutin negative, and showed the same fingerprinting profile with ERIC primers as the reference strains ISF Act.1. Duplex-PCR assays using genomic DNA extracts from diseased plant matrices (twigs, branches, flowers, leaves) (Fig. 4), were always confirmed by the positive result obtained by isolation.

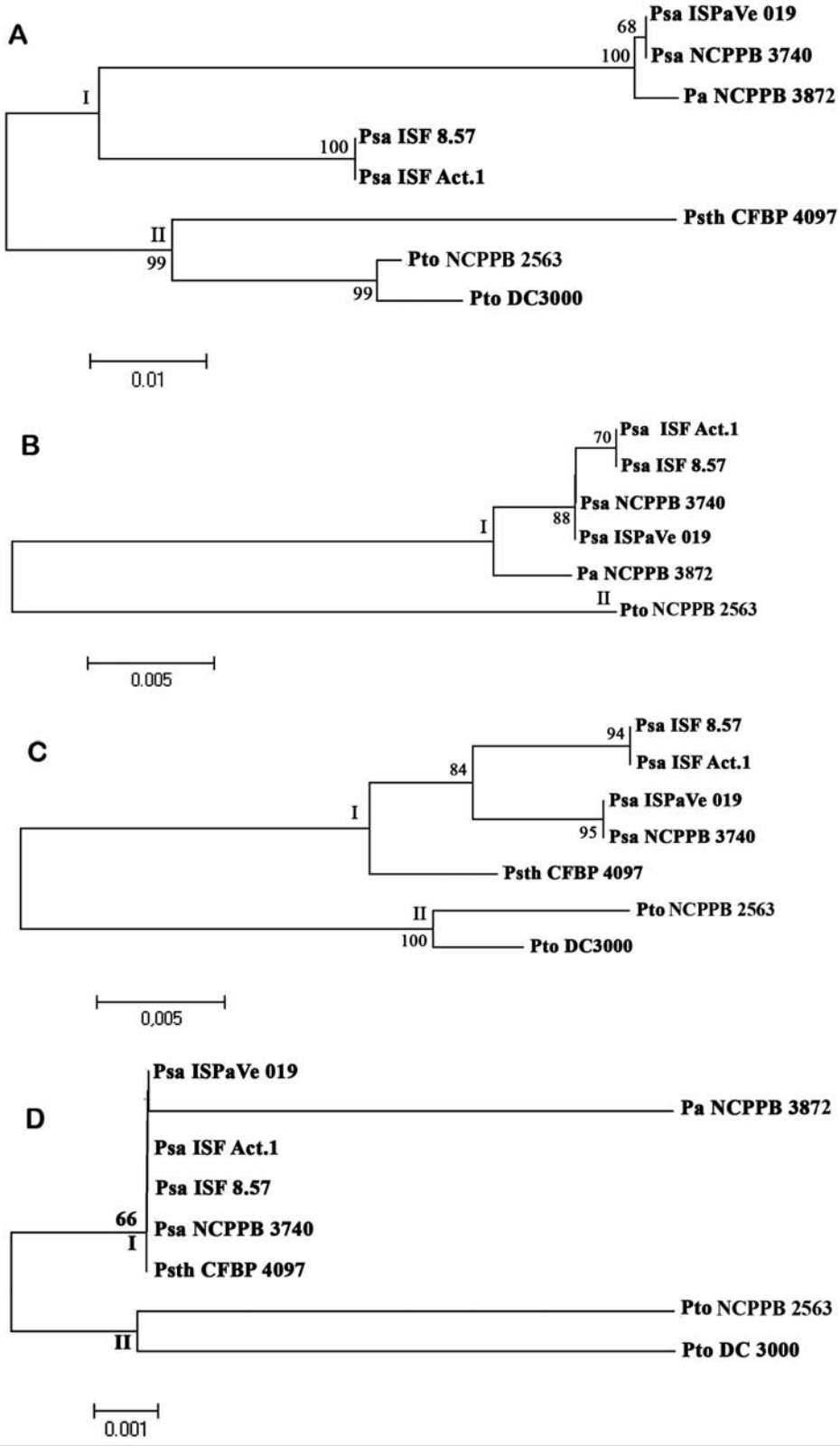

Fig. 1. Neighbour-joining tree based on the ClustalW alignment of partial nucleotide sequences of: A, *hrpW;* B, *avrD1;* C, KN-PCR amplicon; D, *hrpL*. Reference strains are reported as below: *Pseudomonas syringae* pv. *actinidiae* (Psa) ISF Act.1, ISF 8.57, ISPaVe 019 and NCPPB 3740, *P. syringae* pv. *tomato* (Pto) NCPPB 2563 and DC 3000, *P. syringae* pv. *theae* (Psth) CFBP 4097, *P. avellanae* (Pa) NCPPB 3872. Bootstrap values (10,000 replicates) are given for the branches with >50% support. The scale at the bottom indicates a genetic distance proportional to the number of substitutions *per* site. Vertical separations are for clarity only.

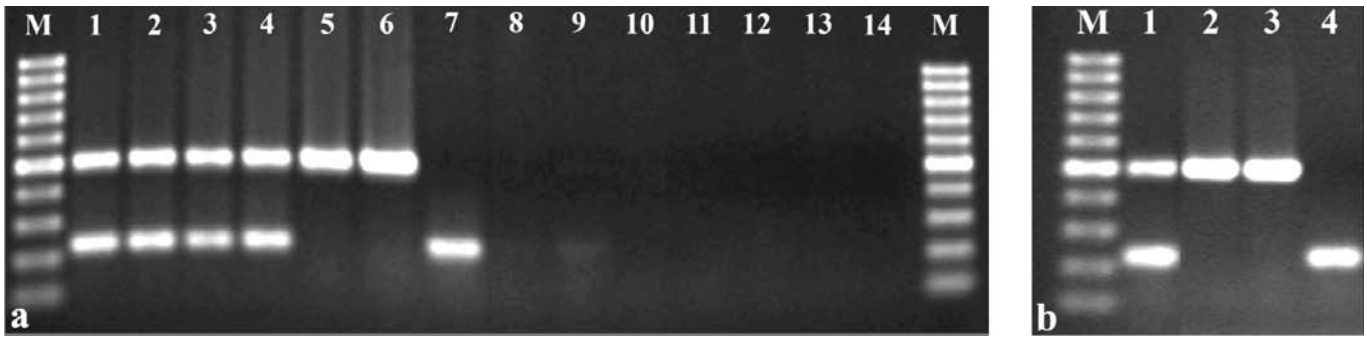

Fig. 2. Duplex-PCR from bacterial suspension (10^8 CFU/ml). a. *Pseudomonas syringae* pv. *actinidiae* ISF Act.1, ISPaVe 019, ISPaVe 020, NCPPB 3740 (lanes 1-4), *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *theae* CFBP 4097 (lanes 5-6), *Pseudomonas avellanae* NCPPB 3872 (lane 7), *P. syringae* pv. *phaseolicola* 1448A (lane 8), *P. syringae* pv. *glycinea* ISPaVe 1155 (lane 9), *P. syringae* pv. *syringae* OMP-BO 4250,1 (lane 10), *P. syringae* pv. *papulans* NCPPB 2848 (lane 11), *P. viridiflava* OMP-BO 4254A,1 (lane 12), *P. syringae* pv. *syringae* OMP-BO3909B,1 (lane 13), water control (lane 14). b. *Pseudomonas syringae* pv. *actinidiae* ISF 8.57 (lane 1), *P. syringae* pv. *tomato* NCPPB 2563 and *P. syringae* pv. *theae* NCPPB 2598 (laned 2-3), *P. avellanae* ISPaVe 1267 (lane 4). M: molecular markers (Gene Ruler^{TM} 100 bp DNA ladder, Fermentas, Lithuania).

Detection of *Psa* from contaminated pollen and fruit samples. *Psa* detection level in artificially contaminated pollen was as little as 10^3 CFU/ml by duplex-PCR, isolation and RG-PCR (Rees-George *et al.*, 2010) (Fig. 5). The detection level from artificially contaminated fruits corresponded to 10^2 CFU/ml with isolation, duplex-PCR, and RG-PCR (Rees-George *et al.*, 2010) (not shown).

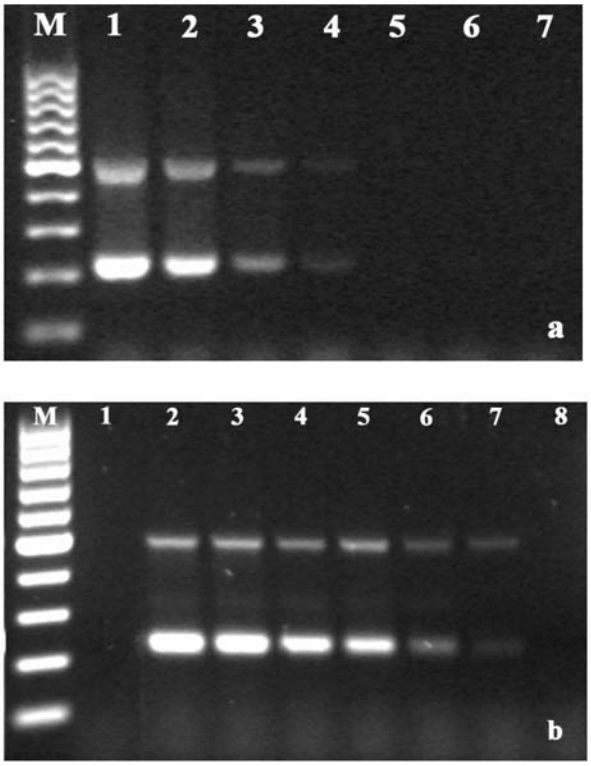

Fig. 3. Sensitivity threshold from: a. ten-fold dilution of bacterial suspension of *Pseudomonas syringae* pv. *actinidiae* ISF Act.1. Lane 1, undiluted sample ($2x10^4$ CFU/PCR reaction); lane 2, 10^{-1}; lane 3, 10^{-2}; lane 4, 10^{-3}; lane 5, 10^{-4}; lane 6, 10^{-5}; lane 7, water control; b. ten-fold dilution of genomic DNA purified from a bacterial culture of *P. syringae* pv. *actinidiae* ISF Act.1. Lane 1, water control; lanes 2-8, 50 ng, 5 ng, 0,5 ng, 50 pg, 5 pg, 0.5 pg, 50 fg. M: molecular markers (Gene Ruler^{TM} 100 bp DNA ladder).

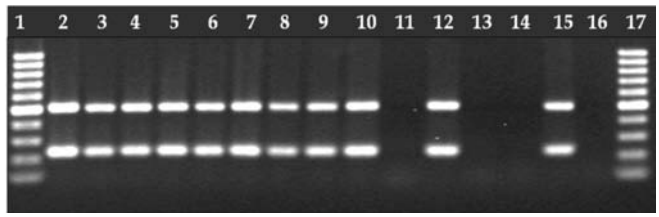

Fig. 4. Duplex-PCR of DNA extracts from: infected wood (lanes 2-4), leaves (lanes 5-7), flowers (lanes 8-10), uninfected wood, leaves, flowers (lanes 11, 13, 14), ISF Act.1 bacterial suspension (10^8 CFU ml^{-1}) (lanes 12, 15), water control (lane 16); lanes 1 and 17, molecular markers (Gene Ruler^{TM} 100 bp DNA ladder).

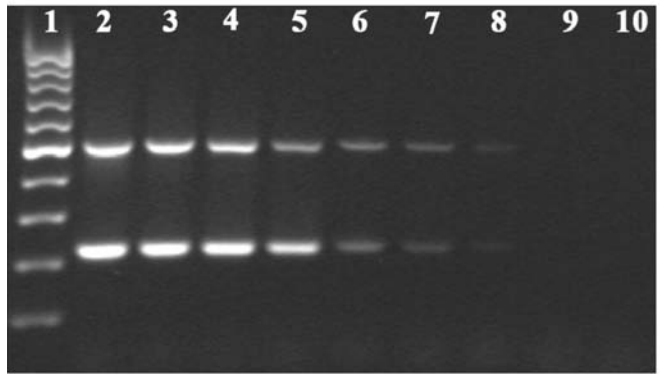

Fig. 5. Detection level of duplex-PCR amplicons obtained from pollen artificially contaminated with *Psa* ISF Act.1 bacterial suspension from 10^8 CFU ml^{-1} to 10 CFU ml^{-1} and extracted with the described procedure (lanes 2-9), water control (lane 10). Lane 1, molecular marker (Gene Ruler^{TM} 100 bp DNA ladder).

DISCUSSION

Because of the severe *Psa* epidemics on kiwifruits in central Italy, a reliable detection system for this bacterium, recently included in the EPPO pest alert list, is badly needed since the currently available molecular assays are reported to lack in specificity (Rees-George *et al.*, 2010).

In this study a gene sequence analysis was done to find new specific DNA markers for a specific PCR-based diagnosis of *Psa*, suitable for detecting it also from symptomless kiwifruit matrices. In view of the strong relationship of *Psa* with other pseudomonads, we focused on several genes involved in the interaction with the host (*hrpW, hrpL, avrD1, hopAB2*), and took in consideration also the housekeeping *rpoD* gene and the KN-PCR amplicon of Koh and Nou (2002).

The branching pattern of the phylogenetic trees based on *hrpW, avrD1*, KN-amplicon sequences clearly showed the genetic diversity of the *Psa* strains isolated from the recent epidemics in central Italy and those previously recovered in Italy and Japan (Scortichini, 1994; Takikawa *et al.*, 1989). The difference between the Italian and the Asian *Psa* populations is now well documented (Ferrante and Scortichini, 2010; Vanneste *et al.*, 2010; Mazzaglia *et al.*, 2011). Our results support also the suggestion of Ferrante and Scortichini (2010), later confirmed by Vanneste *et al.* (2010), that the actual *Psa* population, agent of the recent epidemics in Italy, differs from the previously recorded Italian *Psa* strains. As already suggested by Ferrante and Scortichini (2010), these results seem to exclude that the current outbreak originates from the inoculum infecting *A. deliciosa* plants in 1992.

The *hrpL* gene, that codes for a putative sigma factor, is considered a good candidate for inferring the phylogenetic relationship between phytopathogenic bacteria and has been used to investigate the genetic relationships of *P. syringae* spp. (Cournoyer *et al.*, 1996; Sawada *et al.*, 1999; Scortichini *et al.*, 2005; Loreti *et al.*, 2008). The phylogenetic analysis based on the *hrpL* gene grouped together all the *Psa* strains, but was unable to highlight heterogeneity among them. Probably, the regulation function of this gene makes its sequence more conserved than that of the other genes considered. However, a more in-depth gene sequence analysis on a larger number of *Psa* isolates will better explain the phylogenetic relationship within the *Psa* population and the epidemiological connotations.

Of the genes tested, only *hopAB2* (*avrPtoB*) homologues were not detected in *Psa*, despite their wide distribution among phytopathogenic bacteria. It is known that *hopAB2* homologues from different *P. syringae* pathovars have conserved avirulence and virulence activities (Lin *et al.*, 2006), thus suggesting a similar virulence function for these pathogens. Homologues of these genes were previously found in *P. avellanae* (Loreti *et al.*, 2003) and *P. syringae* pv. *theae* (accession No. AM410896). It is probable that other effectors control the virulence of *Psa*. Nevertheless, it is possible that, despite the genetic correlation among these pseudomonads, *hopAB2* homologs may have sequence variations.

With this study we were able to set up a duplex-PCR based on the use of four primers, selected on *avrD1* and KN-PCR sequences, that identified all *Psa* strains, without detecting other related pseudomonads. This PCR assay provides a means to distinguish *Psa* colonies from those of *P. syringae* pv. *theae, P. syringae* pv. *tomato*, *P. avellanae*, *P. syringae* pv. *syringae* and *P. viridiflava* and other saprophytic or opportunistic bacteria recovered from infected kiwifruits.

The advantages of duplex-PCR are: (i) quick and specific *Psa* detection if used directly on diseased kiwifruit tissues treated with a commercial kit for DNA extraction; (ii) quick monitoring and distinction of *Psa* colonies from those with a similar morphology, if used on pure bacterial cultures. The analysis of symptomatic samples collected in the province of Latina confirmed the reliability of duplex-PCR for *Psa* detection directly from kiwifruit bark, leaves, flowers and petioles. By contrast, following isolation in culture, the occurrence of *Psa*-like colonies (i.e. levan positive colonies, fluorescence negative) complicated the accuracy of diagnosis. Such colonies were clearly distinguished from those of *Psa* by the duplex-PCR analysis. Nevertheless, isolation is a useful technique to confirm positive PCR results, and in some bacterial models is required to comply with phytosanitary regulations.

The sensitivity limit of this method (2x10 CFU/PCR reaction), suggests that it could be used to identify *Psa* also in symptomless materials. This prompted the setting up of protocols for extracting and detecting the bacterium from pollen and fruits. Duplex-PCR showed the same detection limit as isolation from experimentally infected fruit and pollen, but was more rapid than isolation, providing a response within two working days. A similar detection limit was reported by Rees-George *et al.* (2010) when PCR was applied to kiwifruit buds. In our view, because the use of more than one technique is always advisable for bacterial detection, and considering that *Psa* is currently included in the EPPO alert list, a strategy comprising two PCR assays (i.e. duplex-PCR and RG-PCR) with primers sets from different genes should be pursued for a preliminary screening of the samples to be tested, especially if they consist of symptomless material. These protocols may be useful for the inspection of materials that are traded between countries.

A major concern of the nursery industry is the health status of the propagation material produced. Official protocols for obtaining certified nursery productions

are being enforced in Italy for a number of crops which, however, do not include kiwifruit, notwithstanding the fact that this is one of the main fresh fruit products of the country. Now that effective diagnostic protocols are available for defining the presence and spread of *Psa*, the hope is that effective phytosanitary measures could be implemented for the prevention and control of this bacterium also in planting material, possibly in the framework of a national certification scheme. This would lead to economic benefits for the whole kiwifruit production chain from the nursery sector to related industries.

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