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Draft Whole Genome Sequence Analyses on *Pseudomonas syringae* pv. *actinidiae* Hypersensitive Response Negative Strains Detected from Kiwifruit Bleeding Sap Samples

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ABSTRACT

Kiwifruit bleeding sap samples, collected in Italian and Chilean orchards from symptomatic and asymptomatic plants, were evaluated for the presence of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker. The saps were sampled during the spring in both hemispheres, before the bud sprouting, during the optimal time window for the collection of an adequate volume of sample for the early detection of the pathogen, preliminarily by molecular assays, and then through its direct isolation and identification. The results of molecular analyses showed more effectiveness in the *P. syringae* pv. *actinidiae* detection when compared with those of microbiological analyses through the pathogen isolation on the nutritive and semiselective media selected. The bleeding sap analyses allowed the isolation and identification of two hypersensitive response (HR) negative and hypovirulent *P. syringae* pv. *actinidiae* strains from different regions in Italy. Moreover, multilocus sequence analysis (MLSA) and whole genome sequence (WGS) were carried out on selected Italian and Chilean *P. syringae* pv. *actinidiae* virulent strains to verify the presence of genetic variability compared with the HR negative strains and to compare the variability of selected gene clusters between strains isolated in both countries. All the strains showed the lack of *argK* and *coronatine* gene clusters as reported for the biovar 3 *P. syringae* pv. *actinidiae* strains. Despite the biologic differences obtained in the tobacco bioassays and in pathogenicity assays, the MLSA and WGS analyses did not show significant differences between the WGS of the HR negative and HR positive strains; the difference, on the other hand, between PAC_ICE sequences of Italian and Chilean *P. syringae* pv. *actinidiae* strains was confirmed. The inability of the hypovirulent strains IPV-BO 8893 and IPV-BO 9286 to provoke HR in tobacco and the low virulence shown in this host could not be associated with mutations or recombinations in T3SS island.

Pseudomonas syringae pv. *actinidiae*, the etiological agent of bacterial canker of *Actinidia* species, is up to date classified in four biovars according to phenotypic and pathogenic and molecular characteristics (Cunty et al. 2015b; Sawada et al. 2014; Vanneste et al. 2013). The biovar 1 was first isolated in Japan in 1984 (Serizawa et al. 1989; Takikawa et al. 1989) and Korea (Ciarroni et al. 2015); a few years later, in 1992, a strain belonging to biovar 1 was isolated also in Italy (Scortichini 1994). Biovar 2 was detected in Korea during the 1990s (Koh et al. 1994), while biovar 3 was responsible for the pandemic bacterial canker spread that occurred since 2008 in Europe (Abelleira et al. 2011; Balestra et al. 2008, 2011; NPPO of Switzerland 2011; Vanneste et al. 2010), Asia (Mazzaglia et al. 2012), Oceania (Anonymous 2011a; Everett et al. 2011), and South America (Anonymous 2011b; SAG 2011).

During the last years, the bacteria of the virulent biovar 3 has been characterized through biochemical, physiological, and different molecular assays; in particular, studies were conducted using multilocus sequence analysis (MLSA) (Butler et al. 2013; Chapman et al. 2012) and whole genome sequence (WGS) (Marcelletti et al. 2011; Mazzaglia et al. 2012; Butler et al. 2013; McCann et al. 2013). Biovar 3 was first discriminated from biovars 1 and 2 by its inability to produce phaseolotoxin and coronatine, respectively, where the lack of these virulence factors did not affect the bacterial aggressiveness. The spreading of this virulent biovar, coupled with the increasing cultivation rate of highly susceptible varieties, mostly belonging to *A. chinensis* genotype, was the principal cause of the worldwide outbreaks (EPPO 2014). Susceptibility studies, carried out in several *Actinidia* species, experimentally inoculated with a *P. syringae* pv. *actinidiae* biovar 3 strain, confirmed the highest susceptibility of *A. chinensis* genotypes compared with those of *A. deliciosa*, *A. arguta*, *A. eriantha*, and *A. hemsleyana* (Biondi et al. 2015). Further recent studies, carried out using variable-number of tandem repeats multilocus analysis on the core genome of a large number of bacterial strains, allowed a deeper discrimination of haplotypes in *P. syringae* pv. *actinidiae* biovar 3 strains. Moreover, it was demonstrated that, since these strains are monophyletic, the haplotypes isolated in Italy and France originated in China (Butler et al. 2013; Mazzaglia et al. 2012) and are genetically distinguishable from those isolated in Chile and New Zealand (Butler et al. 2013; Cunty et al. 2015b).

Biovar 4, a low virulent group of fluorescent strains isolated in Australia, New Zealand, and France, was thereafter separated from the *P. syringae* pv. *actinidiae* biovars and assigned to pathovar *actinidifoliorum* (Cunty et al. 2015a); while biovar 5, recently isolated in Japan and characterized by biochemical, physiological,

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*The *e-Xtra* logo stands for "electronic extra" and indicates that one supplementary table is published online.

and molecular assays, was clearly formed by different strains and it was proposed as an independent MLSA group (Fujikawa and Sawada 2016; Sawada et al. 2014).

Basically, the control of *P. syringae* pv. *actinidiae* relies on preventive treatments with copper compounds and use of resistance inducers (Cellini et al. 2014). In the perspective of an integrated pest control management, the production of plants *P. syringae* pv. *actinidiae*-free and the continuous monitoring of kiwifruit orchards are considered efficient methods to reduce the bacterial inoculum sources (EPPO 2014). *P. syringae* pv. *actinidiae* detection at the early disease stages or before the appearance of symptoms is indeed necessary, in particular, the detection of latently infected kiwifruit plants is one of the principal needs, especially taking into account that symptoms do not appear during the first 2 or 3 years of infection (Koh and Nou 2002). Several studies concerning *P. syringae* pv. *actinidiae* detection using molecular methods such as PCR (Balestra et al. 2013; Biondi et al. 2013; Gallelli et al. 2011; Koh et al. 2014; Rees-George et al. 2010), qPCR (Gallelli et al. 2014), and loop-mediated isothermal amplification (Buriani et al. 2015; Ruinelli et al. 2017) have been proposed.

In this study, several bleeding sap samples were collected during Italian and Chilean vegetative seasons in kiwifruit orchards located in different regions of the two countries. The sampling and the testing aimed to find a time window before bud sprouting, in which to carry out the bleeding sap collection to detect the pathogen early in both hemispheres. Moreover, MLSA and WGS were carried out on Italian and Chilean *P. syringae* pv. *actinidiae* strains, and some Italian strains were also assayed for their virulence on yellow kiwifruit plants.

MATERIALS AND METHODS

Bacterial reference strains. *P. syringae* pv. *actinidiae* strains NCPPB 3739 (from National Collection of Plant Pathogenic Bacteria, York, UK), CFBP 7286 (from Collection Française de Bactéries Phytopathogènes, INRA Angers, France), CRA-FRU 3.1 (from Culture Collection of C.R.E.A.-Research Centre for Fruit, Roma, Italy), and *P. syringae* pv. *actinidifoliorum* strain ICMP 19074 (from International Collection of Micro-organisms from Plants, Manaaki Whenua Landcare Research, Auckland, New Zealand) isolated from *A. chinensis*- and *A. deliciosa*-infected plants were used as reference strains in microbiological, molecular, and pathogenicity assays. Strains were routinely grown on King's medium B agar (KB) (King et al. 1954) at 27°C for 48 h.

Bleeding sap sampling. Bleeding saps were collected, until bud sprouting, during the Italian and Chilean vegetative seasons in 2013. Italian samples were collected in three orchards located in Veneto, Emilia Romagna, and Lazio regions, while in Chile the bleeding saps were collected in three orchards in Maule and Biobío regions. In particular, in Italy, the samples collected in Emilia Romagna and Veneto were from green-fleshed symptomatic and nonsymptomatic kiwifruit fields, respectively, while in Lazio the samples were collected from asymptomatic yellow-fleshed species. In Chile, the sap samples were all collected from *A. deliciosa* 'Hayward' asymptomatic plants.

Saps were collected from three marked 1-year-old shoots on different branches of 10 to 12 randomly selected kiwifruit trees (Table 1). The marked shoots were externally sterilized with 70% ethanol in their 10 to 15 cm distal part, and then ca. 2 cm was removed and 15-ml Falcon tubes were fixed to the just open wound to collect the dripping saps. After approximately 2 h, the three samples from each kiwifruit plant were collected, mixed in one 50-ml Falcon tube, and brought to the laboratory. All samples were centrifuged at 10,000 × *g* for 20 min at 4°C and the pellets were resuspended in 1 ml of sterile distilled water (SDW). Three hundred microliters of the resuspension was directly used for DNA extraction and PCR assays and stored at −20°C. The remaining 700 µl was used for direct isolations, amended with 20% glycerol, and stored at −80°C.

Microbiological analyses. The resuspended bacterial pellets, obtained from bleeding saps, were 10-fold diluted and plated (50 µl) onto nutrient sucrose agar (NSA) (Crosse 1959) and onto semiselective NSA (SsNSA) amended with 1.5% boric acid, 80 ppm cephalexin sulfate, and 120 ppm cycloheximide (EPPO 2014; Gallelli et al. 2011; Stefani and Giovanardi 2011). The plates were incubated at 27°C for 72 to 96 h. The *P. syringae* pv. *actinidiae*-like colonies, isolated from bleeding saps, were characterized by the following biochemical tests: levan production, oxidase presence, soft rot activity on potato disks, presence of arginine dehydrolase, hypersensitive response (HR) in tobacco leaves (LOPAT tests) (Lelliot and Stead 1987; Schaad et al. 2001), and fluorescence on KB medium.

The sensitivity threshold of NSA and SsNSA media was assessed by contaminating nine Eppendorf tubes containing 900 µl of *P. syringae* pv. *actinidiae*-free bleeding sap by adding 100 µl of 10-fold dilutions (from approximately 10^8 to 1 CFU ml^{-1}) of *P. syringae* pv. *actinidiae* CRA-FRU 3.1 suspension. The last sample was not contaminated and was used as negative control. One hundred microliters of each dilution was inoculated and spread on NSA and SsNSA plates and incubated at 27°C for 72 to 96 h. The grown colonies were then counted to calculate the *P. syringae* pv. *actinidiae* CFU/ml bleeding sap. The experiment was replicated three times.

DNA extraction and molecular assays. DNA was extracted from processed bleeding saps and from aqueous suspensions (ca. 10^8 CFU/ml) of *P. syringae* pv. *actinidiae*-like colonies by heating the samples at 100°C for 15 min. The heat-shocked lysates were then immediately cooled in ice.

On bleeding sap samples collected both in Italy and Chile, PCR and nested-PCR assays were performed in 25-µl reaction mixtures,

TABLE 1. Sampling collections carried out in Italy and in Chile; the kiwifruit species from which samples were collected are also shown

Italian regions selected for bleeding sap sampling[z]	Number of sampling	Date of sampling	Number of samples collected/number of marked plants	Average of sap volume (ml)	*Actinidia* sp. (plant age)
Veneto (a)	I	21/03/13	12/12	40.8	*A. deliciosa* (6 years)
	II	28/03/13	12/12	27.6	
	III	11/04/13	12/12	19.8	
Emilia Romagna (b)	I	12/03/13	5/12	9.2	*A. deliciosa* (3 years)
	II	19/03/13	9/12	6.9	
	III	04/04/13	11/12	15.7	
Lazio (ab)	I	21/03/13	10/12	17.2	*A. chinensis* (3 years)
	II	28/03/13	12/12	30.3	
Maule (Curicó) (a)	I	03/10/13	7/10	14.0	*A. deliciosa* (6 years)
Biobío (San Carlos) (a)	I	11/10/13	10/10	21.0	*A. deliciosa* (6 years)
Biobío (Chillán) (a)	I	11/10/13	8/10	24.0	*A. deliciosa* (6 years)

[z] Letters indicate different statistical categories, related to the mean volume collected in each region, in Italy and Chile.

following the protocol proposed by Biondi et al. (2013) by adding 2% BLOTTO (10% skim milk in 0.2% NaN_3) (de Boer et al. 1995) to the chemical profile. Heat-shocked lysates of *P. syringae* pv. *actinidiae*-like colonies were assayed using different PCR methods (Biondi et al. 2013; Gallelli et al. 2011). SDW was used in all molecular assays as negative control. All amplification products were analyzed on 1.5% (wt/vol) agarose gel in TAE buffer (0.04 M Tris, 0.001 M NaEDTA, and 0.02 M glacial acetic acid), stained in 0.03% ethidium bromide, and visualized under UV light (312 nm).

MLSA and WGS. Single colonies of five *P. syringae* pv. *actinidiae* strains (three isolated in Italy and two in Chile) were grown in LB broth for 24 h at 27°C. The 24-h suspensions were centrifuged for 20 min at 10,000 × *g* at 4°C, and the DNA was extracted using GeneJet genomic DNA purification kit (Thermo Fisher Scientific, Lithuania). The strains were assayed by MLSA according to previous studies (Sarkar and Guttman 2004). Seven housekeeping genes were amplified and sequenced by the Sanger method in Macrogen USA Corp. (Rockville, MD). The obtained sequences of *acnB*, *cts*, *gapA*, *gyrB*, *pfk*, *pgi*, and *rpoD* were concatenated and aligned together with reference sequences of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *actinidifoliorum*, using ClustalW2. Maximum likelihood (ML) and maximum parsimony analysis were performed using MEGA v 7.0 (Kumar et al. 2016).

For WGS, genomic DNA (gDNA) was extracted using GeneJet genomic DNA purification kit (Thermo Fisher Scientific, Lithuania). DNA integrity was evaluated using capillary electrophoresis (AATI Fragment Analyzer, HS Large Fragment analysis kit, DNF493, AATI, IA). For library construction, sonication of 200 ng of DNA was made on Covaris (model M220), according to Illumina instructions, in a total volume of 50 µl of TE buffer. After checking fragmented sizes on Fragment Analyzer, sonicated gDNA was size-selected by Agencourt AMPure XP Beads (Beckman Coulter). The 400 to 500 bp of gDNA was treated with TruSeq Nano DNA Library Prep kit reagents, following Illumina's kit protocol. The final size of the libraries was determined by Fragment Analyzer, concentration was assessed using fluorometric method (Qubit 2.0, BR DNA kit) and finally sequenced using Illumina Miseq system using 2×300 paired ends system.

Total readings were trimmed and de novo assembled using CLC genomics workbench v7.0. Selected quality trimming limit value was 0.02, and de novo assembly was performed with a length fraction of 0.6, and identity value of 0.9. Assembled contigs were annotated using the Rapid Annotation Subsystem Technologies (RAST) server. Whole genome plasmids and PAC_integrative conjugative element (PAC_ICE) alignments were performed using progressive MAUVE; *argK* (phaseolotoxin) and coronatine gene cluster analyses were performed using CLC genomics workbench. After a WGS, another MLSA analysis was performed on 58 genes coding type three secretion system-related proteins (T3SS) structural (Supplementary Table S1) and effector proteins. Analyzed genes were selected from annotation available for ICMP 18884 strain and compared with Italian and Chilean strain contigs by using BLASTn tool in CLC genomics workbench and by selecting each strain contigs set as a local database. Retrieved sequences were concatenated and aligned with reference strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *actinidifoliorum*. Phylogenetic analysis was performed with ML method, using CLC genomics workbench.

Pathogenicity assay and virulence evaluation of selected *P. syringae* pv. *actinidiae* strains. One-year-old kiwifruit plants of cultivar Belèn were grown in 7.0-liter pots, containing a peat and vermiculite soil mix, and pruned to stimulate the emergence of new shoots and young leaves. All the tested plants were inoculated at the same phenological stage. Two plants were then distributed in four randomized blocks and experimentally inoculated by spraying a calibrated water suspension on young leaves containing the selected *P. syringae* pv. *actinidiae* strains (0.01 $OD_{600\ nm}$, ca. 10^7 CFU/ml). The plants were sealed in polyethylene bags for 48 h to favor the water congestion and allow the pathogen inoculation. The controlled conditions, hold until disease assessment, were 16 h of day light and 8 h of darkness, 23 and 17°C during day and night, respectively. The relative humidity was maintained at approximately 90%. Plants sprayed with SDW and noninoculated ones were used as negative control. Water suspensions of reference *P. syringae* pv. *actinidiae* biovar 3 strains CFBP 7286 and CRA-FRU 3.1 were used as positive control. The hypovirulent strain ICMP 19074 of *P. syringae* pv. *actinidifoliorum* was also assayed as additional control. The disease severity was evaluated 21 days after *P. syringae* pv. *actinidiae* inoculation by counting the number of leaf spots on 10 leaves/plant (80 leaves per treatment). The data were analyzed by analysis of variance (Duncan's test, $P \leq 0.05$) using software SPSS 15.0 for Windows. The pathogen strains of *P. syringae* pv. *actinidiae* and *P. syringae* pv. *actinidifoliorum* were re-isolated onto NSA plates from selected leaf spots, and the isolates were identified through LOPAT tests and molecular assays (Biondi et al. 2013).

RESULTS

Molecular and microbiological analyses on bleeding sap samples. In the Veneto region (Italy), bleeding saps were collected from all 12 plants selected at each collection time with average volumes of approximately 41, 28, and 20 ml from March to April. In Emilia Romagna, the frequency of collected saps was 5, 9, and 11 out of the 12 plants, with average volumes of approximately 9, 7, and 16 ml from the first to the last collection time. In Lazio, the sap frequency in the two collection dates (in March) was 10 and 12 out of the 12 selected trees, with average volumes of approximately 17 and 30 ml in the first and second collection time, respectively (Table 1).

In Curicó, Chillán, and San Carlos (Chile), the sole collection was carried out at the beginning of October 2013, and different sap frequency and volume, according to the locality, was observed: 7, 8, and 10 out of 10 selected plants were collected, with average volumes of approximately 14, 21, and 24 ml, respectively (Table 1).

The direct and nested PCR on the Italian bleeding saps was 100% positive for all the samples collected in Emilia Romagna; while in Lazio, the direct PCR was positive in 30 and 40% in the first and last collected saps, respectively, and the nested PCR raised the detection of 80 and 100%, respectively. In Veneto, the direct PCR was positive in 25, 92, and 25% of the saps collected from the first to the last sampling, and when the nested PCR was applied the percentages of detection were 75, 100, and 58%, respectively (Table 2). Molecular results showing a similar behavior were obtained in samples from Chile, where the sap samples collected in Curicó, Chillán, and San Carlos were positive to the direct PCR in 14, 25, and 30%, respectively, and when the nested PCR was applied, the positive samples were 71, 87, and 90%, respectively (Table 2).

In Italy, the direct isolation of the bleeding sap samples collected in Emilia Romagna at the first and second sampling time detected *P. syringae* pv. *actinidiae* in 43 and 89% of them, respectively, on both NSA and SsNSA media. From the sap samples collected at the last collection time, *P. syringae* pv. *actinidiae* was isolated on NSA and on SsNSA in 9 and 46% of the samples, respectively. *P. syringae* pv. *actinidiae* was not isolated on the nutritive medium from the sap samples collected in Lazio at the first sampling time, while it was isolated from 8% of the samples on the semiselective medium. At the last collection time, the pathogen was isolated from 10 and 20% of the samples on NSA and SsNSA, respectively. *P. syringae* pv. *actinidiae* was isolated from sap samples collected in Veneto on both nutritive and semiselective media for 17% at the first sampling, and for 8% on both NSA and SsNSA at the other times of collection (Table 2). The sensitivity threshold of both NSA and SsNSA for the isolation of the pathogen was approximately 10^2 CFU/ml.

All isolated *P. syringae* pv. *actinidiae* strains belonged to the LOPAT group Ia and were levan positive, oxidase negative, potato

rot negative, arginine dehydrolase negative, and HR positive, with the exception of two strains, IPV-BO 8893 and IPV-BO 9286, isolated in Emilia Romagna (from symptomatic kiwifruit plant) and Veneto (from asymptomatic kiwifruit plant), respectively, which were HR negative. The semiselective NSA medium was not employed for the direct isolations from Chilean samples and *P. syringae* pv. *actinidiae* was isolated on NSA from bleeding saps collected in Curicó, Chillán, and San Carlos in the 20, 32, and 24% of the samples, respectively.

MLSA and WGS. MLSA clustered Chilean and Italian isolates with *P. syringae* pv. *actinidiae* biovar 3 reference strains. Identical topology was obtained using ML and MP. In particular, HR negative samples showed a differentiation from biovar 3 reference strains only by a single nucleotide polymorphism in *pgi* gene (Fig. 1). Whole genome de novo assembly and genome annotation data for *P. syringae* pv. *actinidiae* strains are shown in Table 3. Genome sizes of the sequenced strains ranged between 6.1 and 6.2 Mb corresponding to a genome fold coverage that ranged from 207.6 to 270.8, all of them having a G+C content of 58.5%. Local BLASTx analysis showed the absence of coronatine and *argK* gene clusters that are associated with the synthesis of coronatine and phaseolotoxin, respectively. Phylogenetic analysis of concatenated T3SS genes showed no differences among Chilean and Italian isolates that clustered together in the group of biovar 3 strains. Sequence comparison of T3SS genes coding sequences did not show mutations or recombinations that could be associated with a loss of function of the coded proteins (Fig. 2). PAC_ICEs sequences obtained from HR negative isolates did not show differences with PAC_ICE2_It, kept as *P. syringae* pv. *actinidiae* Italian reference sequence (GenBank accession number KC148186). The same situation was observed in

TABLE 2. Results of molecular analyses and isolations from bleeding saps collected in Italy and Chile in springs

Region of bleeding sap collection	Number of sampling	Positive bleeding sap samples (%)			
		PCR B1/B2	PCR-nested KNF/KNR	Direct isolation on NSA	Direct isolation on SsNSA
Veneto, Italy	I	25	75	17	17
	II	92	100	8	8
	III	25	58	8	8
Emilia-Romagna, Italy	I	100	100	43	89
	II	100	100	43	89
	III	100	100	9	46
Lazio, Italy	I	30	80	0	8
	II	40	100	10	20
Curicó – Maule, Chile	I	14	71	20	–
Chillán – Biobío, Chile	I	25	87	32	–
San Carlos – Biobío, Chile	I	30	90	24	–

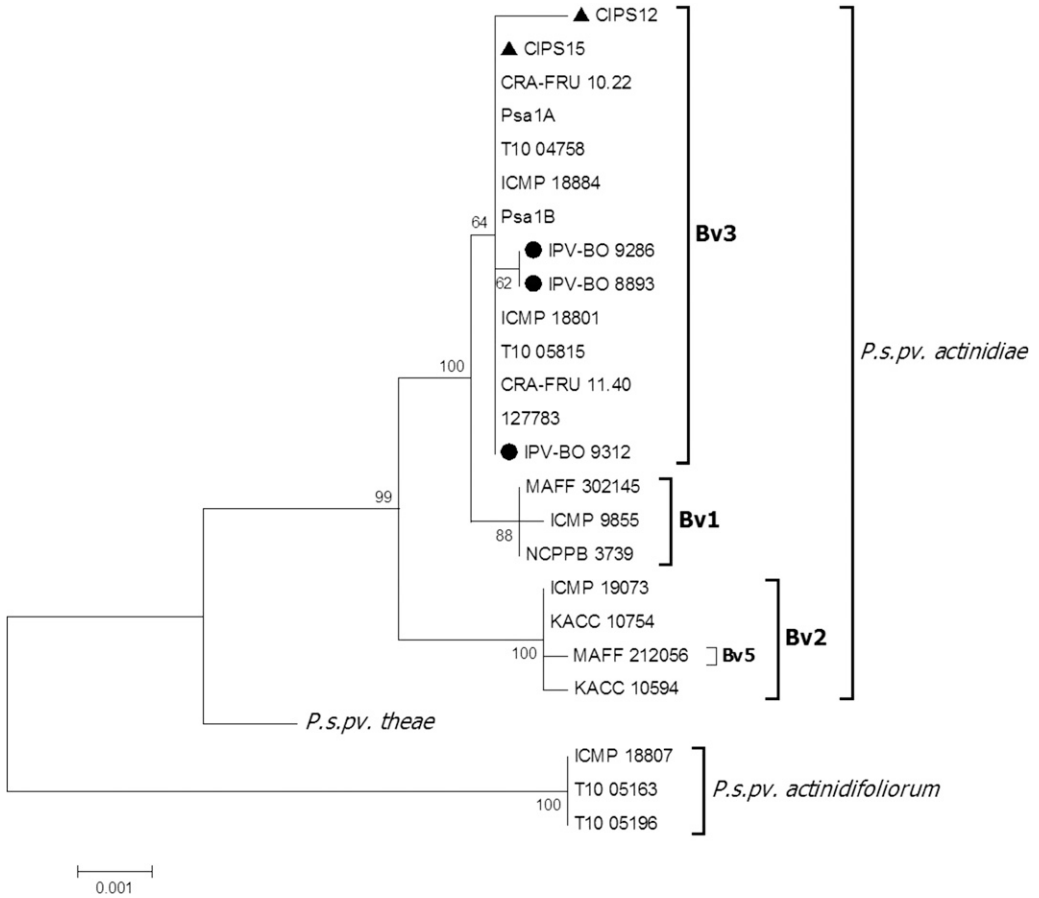

Fig. 1. Maximum likelihood phylogenetic tree of *Pseudomonas syringae* pv. *actinidiae* isolates, inferred from the concatenation of seven house-keeping genes (*acn*, *cts*, *gapA*, *gyrB*, *pfk*, *pgi*, and *rpoB*). Labels indicate the isolates obtained in this work (● Italian isolates; ▲ Chilean isolates). Numbers in nodes represent bootstrap values obtained from 500 replicates.

Chilean isolates, showing identical nucleotide sequences to that of PAC_ICE3_cl, used as *P. syringae* pv. *actinidiae* Chilean reference sequence (GenBank accession number KC148188) (Fig. 3). Plasmid comparison showed 99.9% identity of the HR negative isolate IPV-BO 9286 with the plasmid of Chilean HR positive isolates and 99.8% identity of the HR negative isolate IPV-BO 8893 with the Italian HR positive isolates IPV-BO 9312 and CRA-FRU 14.08 used as reference (GenBank accession number CP019733). The *argK* and coronatine gene clusters were not present in all Italian and Chilean strains of *P. syringae* pv. *actinidiae* biovar 3, both HR negative and positive strains (Fig. 4).

Pathogenicity assay and virulence evaluation of selected *P. syringae* pv. *actinidiae* strains. The disease severity of *P. syringae* pv. *actinidiae* strain IPV-BO 9312 (isolated in Veneto at the second sampling time) was the highest (approximately 14 spots/leaf) compared with CFBP 7286 (reference strain, approximately 12 spots/leaf), IPV-BO 9266 (isolated in Emilia Romagna at the second sampling time, approximately 11 spots/leaf), CRA-FRU 3.1 (reference strain, approximately 10 spots/leaf), and IPV-BO 9294 (isolated in Lazio at the second sampling time, approximately 7 spots/leaf). The HR negative *P. syringae* pv. *actinidiae* strains IPV-BO 9286 and IPV-BO 8893 resulted in low virulence, showing disease severities of approximately 0.3 and 0.5 spots/leaf, respectively, significantly similar to that caused by *P. syringae* pv. *actinidifoliorum* strain ICMP 19074 (reference strain, approximately 0.9 spots/leaf). Control plants inoculated with SDW did not show any symptoms (Fig. 5).

DISCUSSION

The bleeding sap analyses on samples collected in the Italian and Chilean fields were confirmed to be a useful and fast method to early detection of *P. syringae* pv. *actinidiae* latent infections (Biondi et al. 2013). In addition, this study evaluated the time windows, in both hemispheres, suggesting optimal timing to collect sap samples directly from the kiwifruit plants to be before bud sprouts.

In Italy, the frequency of bleeding, in samples collected from the 12 selected plants in Veneto and Lazio, was high from the first collection time to the last one, ranging between approximately 83 and 100%. Moreover, the average volume of bleeding saps collected in these two regions, from the third week of March (first collection time) to the second week of April (third collection time), was approximately 30 and 24 ml, respectively, and it was adequate for the subsequent microbiological and molecular analyses. In particular, in Veneto, the average bleeding volume showed a consistent decrease until the last collection time, where the mean volume was less than 20 ml, due to the beginning of bud sprouting. The average temperature during this time window reached approximately 9 and 11°C in Veneto and in Lazio, respectively (data not shown). On the contrary, the frequency of saps collected in Emilia Romagna was lower with respect to that calculated in Veneto and Lazio, and also the mean sap volume collected during the three collection times was significantly lower (approximately 10 ml). Since the mean temperature during all collection times in Emilia Romagna was approximately 9°C, the reason for the lower frequency and the lower mean volume of sap samples collected might be due to the severity of the bacterial cankers in almost the entire kiwifruit field under observation. Several plants, in fact, showed abundant reddish exudates on the trunks branches before bud sprouting, and wilted shoots and leaves during the season, thus indicating the presence of high pathogen concentration in the xylem vessels (Balestra et al. 2009). The percentages of *P. syringae* pv. *actinidiae* isolations in fact was evaluated as approximately

TABLE 3. Genome de novo assembly and genome annotation data for the studied *Pseudomonas syringae* pv. *actinidiae* strains

	GenBank accession number	Number of contigs	Maximum contig size (bp)	N_{50} (bp)	Total length (bp)	Average coverage	Predicted CDS	Predicted RNAs
IPV-BO[y] 8893	PEIN00000000	297	141.708	42.770	6.175.775	160×	5.529	61
IPV-BO 9286	PEIO00000000	296	141.707	43.968	6.171.732	165×	5.533	60
IPV-BO 9312	PEIP00000000	310	141.790	43.897	6.133.400	180×	5.490	62
PS[z] 12	PEHG00000000	317	141.707	43.856	6.142.385	170×	5.524	66
PS 15	PEHP00000000	300	141.707	43.602	6.120.148	168×	5.486	62

[y] IPV-BO: strains of the bacterial collection of Department of Agricultural Sciences, University of Bologna.
[z] PS: strains of the bacterial collection of Departamento de Sanidad Vegetal, Facultad de Ciencias Agronómicas, Universidad de Chile.

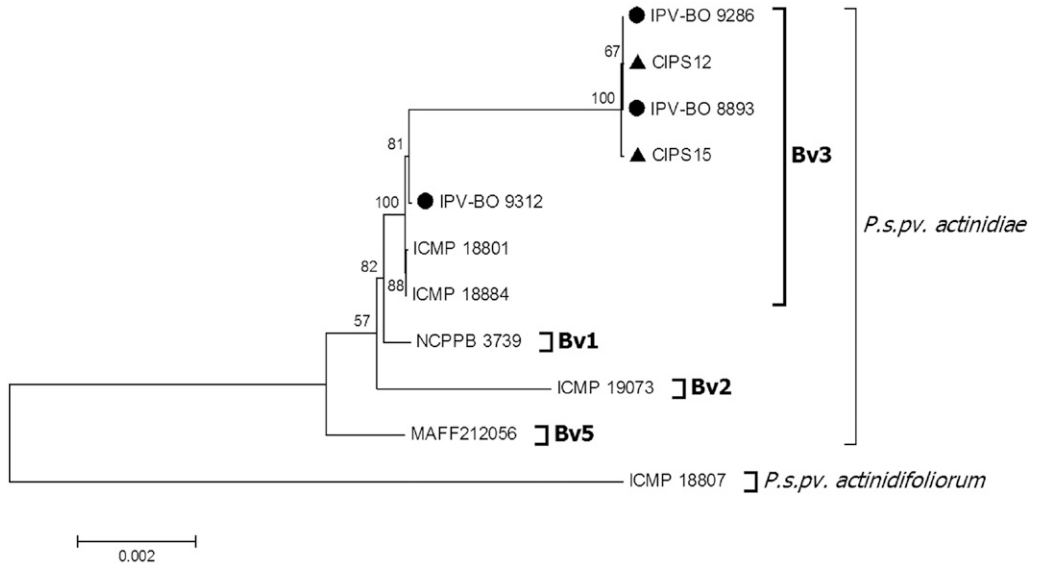

Fig. 2. Maximum likelihood phylogenetic tree of *Pseudomonas syringae* pv. *actinidiae* isolates, inferred from the concatenation of 58 T3SS genes. Labels indicate the isolates obtained in this work (● Italian isolates; ▲ Chilean isolates). Numbers in nodes represent bootstrap values obtained from 250 replicates.

89% in the first two series (times I and II) of collection samples. Moreover, during the previous year (2012), bleeding sap samples from the same field were collected with a frequency of 14/14 with a mean sap volume of approximately 10 ml, and *P. syringae* pv. *actinidiae* was detected with PCR and nested-PCR at 21 and 28%, respectively (Biondi et al. 2013), thus demonstrating the increase of *P. syringae* pv. *actinidiae* population in the xylem vessels.

In Chile, all sap samples were collected during the first 10 days of October (spring in the southern hemisphere), and therefore in parallel with the last period (approximately 15 to 20 days) of

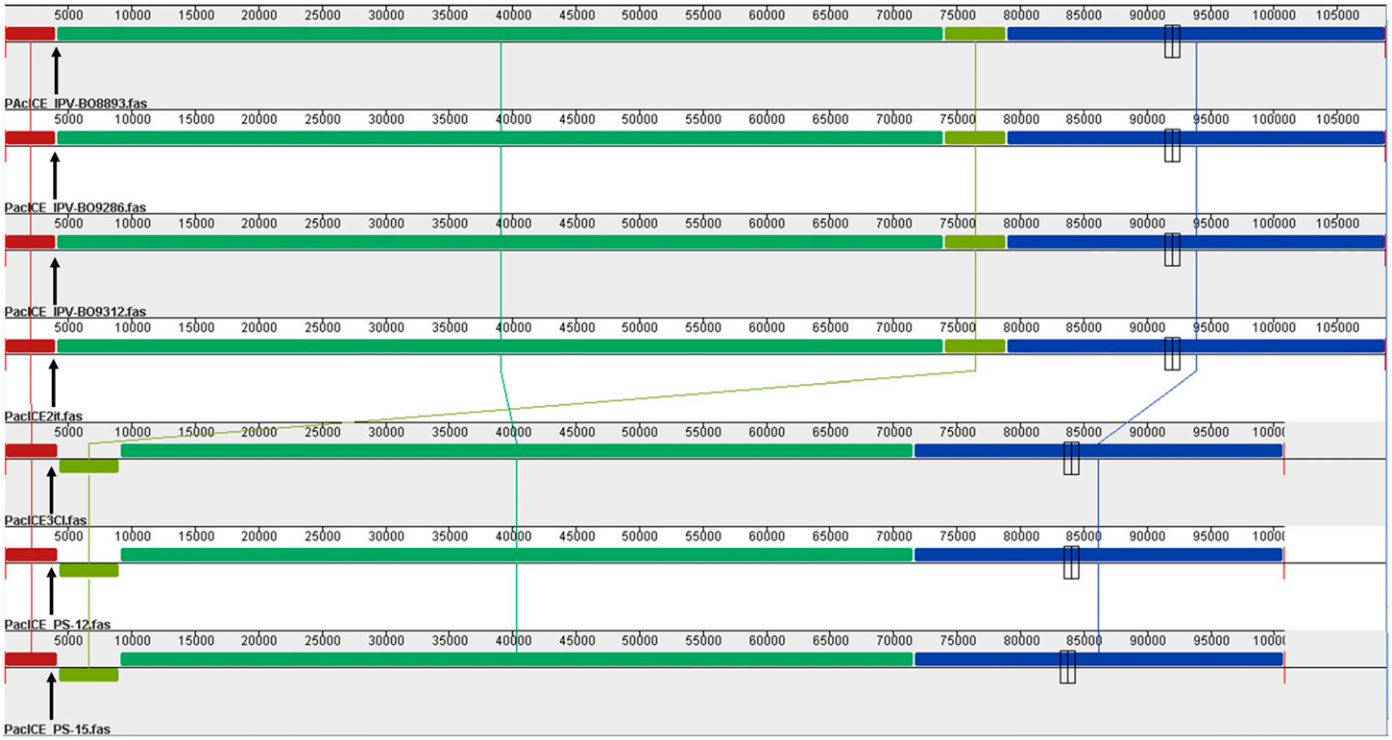

Fig. 3. MAUVE alignment diagram of PAC_ICE sequences of Italian (IPV-BO 8893, IPV-BO 9286, and IPV-BO 9312) and Chilean (PS 12 and PS 15) *Pseudomonas syringae* pv. *actinidiae* strains aligned to Italian and Chilean reference sequences PacICE2it (KC148186) and PacICE3Cl (KC148188).

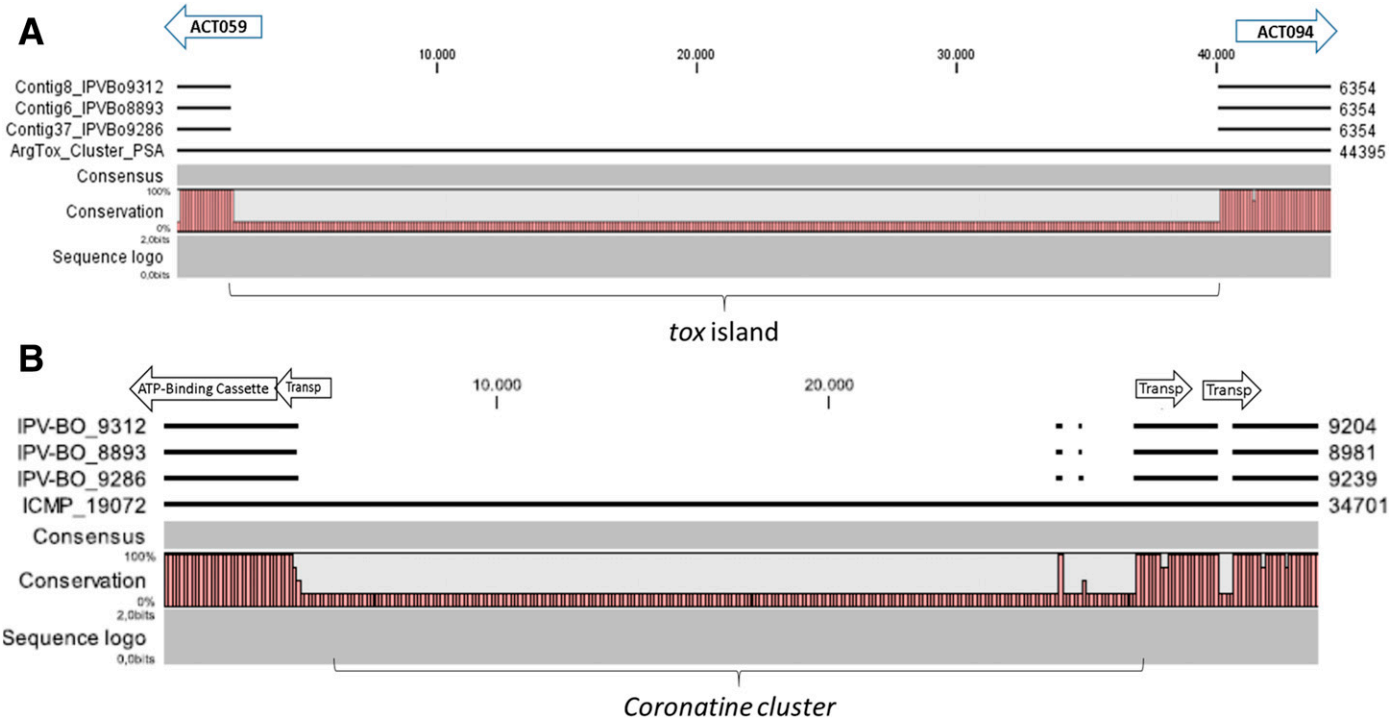

Fig. 4. Comparative analysis of biovar 3 strains from Italy enclosing both HR positive (IPV-BO 9312) and HR negative (IPV-BO 8893, IPV-BO 9286) isolates. Results, obtained by CLC genomics workbench alignment tool, are as follows: **A,** the lack of the genomic island containing *argK* cluster (phaseolotoxin synthesis gene cluster) of three Italian strains compared with reference strain NCPPB 3739 (biovar 1) and in **B,** the lack of island containing the coronatine cluster of the three strains compared with reference strain ICMP 19072 (biovar2).

collection times carried out in Italy during the vegetative season of the north hemisphere. The mean temperatures during such period were evaluated at approximately 14°C. The frequency of collected bleeding saps was high, ranging from 70 to 100%, and the sap volumes reached a mean of approximately 20 ml, not as high as those collected in Italy, in particular in Veneto and Lazio, but statistically similar among the three fields employed for the trials.

The molecular analyses carried out in Italy showed an overestimation of the *P. syringae* pv. *actinidiae* detection percentage when compared with results of microbiological analyses through the pathogen isolation on nutritive and semiselective media. This difference was observed in all sap samples collected in the three regions during the whole collection time window. The bleeding saps collected in Emilia Romagna infected fields during the three collection times were found to be 100% positive for *P. syringae* pv. *actinidiae* by PCR and nested-PCR. The microbiological analyses allowed the detection of the pathogen in 89 and 46% of the samples collected during the first two and the third collection times, respectively, by using the semiselective medium. Also, the samples collected from asymptomatic plants in Veneto and Lazio fields were found to be 60 to 100% positive for *P. syringae* pv. *actinidiae* by molecular analyses, while isolation of the pathogen was only possible in 8 to 20% of the saps. As expected, considering the additional antibiotics, SsNSA was always more effective in pathogen isolation compared with NSA medium, through the reduction of bacterial and fungal contamination.

Approximately 4 weeks before bud sprouting, from the end of March to the beginning of April 2013, the pathogen concentration increased, allowing its isolation from the saps originated by the most severely infected/symptomatic plants. However, the population of bacterial endophytes increased as well and it interfered with the pathogen isolation when the nutritive medium was used. The absence of antibiotic in NSA allowed the growth of faster bacterial contaminants inhibiting the *P. syringae* pv. *actinidiae* growth (EPPO 2014). In Chile as well, similar results were obtained from all bleeding sap samples; thus, molecular analyses were 70 to 90% *P. syringae* pv. *actinidiae*-positive, while the pathogen was isolated on nutritive medium only in 20 to 30% of the cases.

Similar studies were carried out in the 1980s (Burr and Katz 1983) and later in the 2000s (Flamini et al. 2006; Szegedi and Bottka 2002) on *Agrobacterium vitis*, the causal agent of the monocyclic disease grapevine crown gall, aimed to the detection of tumorigenic *A. vitis* strains from sap samples collected from asymptomatic plants during the grapevine bleeding at the beginning of the vegetative growth. Such studies revealed the efficacy of the method in the detection of *A. vitis* latent infections. In the case of *P. syringae* pv. *actinidiae*, which is a polycyclic pathogen, the early detection through bleeding sap analysis and immediate removal of infected plants might raise the possibility to reduce the numerous inoculum sources.

The discrepancy between molecular and microbiological analyses could be explained by the low sensitivity of the media used for the isolation. Both media in fact, NSA and SsNSA, showed a sensitivity threshold of approximately 30 to 100 CFU/ml. In several cases, the pathogen present at low population level was detected by the molecular assay, but it cannot be isolated. Moreover, the sensitivity threshold on NSA was reduced by the presence of several bacterial contaminant species that affected the colony recognition, or in the worst cases, the growth of the culture. The antibiotics contained in SsNSA enhanced the effectiveness of *P. syringae* pv. *actinidiae* isolation; however, the sensitivity threshold was not improved. The lack of sensitivity of an isolation medium considerably limits the diagnostic analysis and it might also affect alternative molecular methods, which are usually combined with isolation methods to increase the sensitivity threshold for the pathogen detection: e.g., bio-PCR for *Acidovorax citrulli* and *A. vitis* detection (Bini et al. 2008; Feng et al. 2013). The medium KBC (Mohan and Schaad 1987) is considered more effective in the *P. syringae*

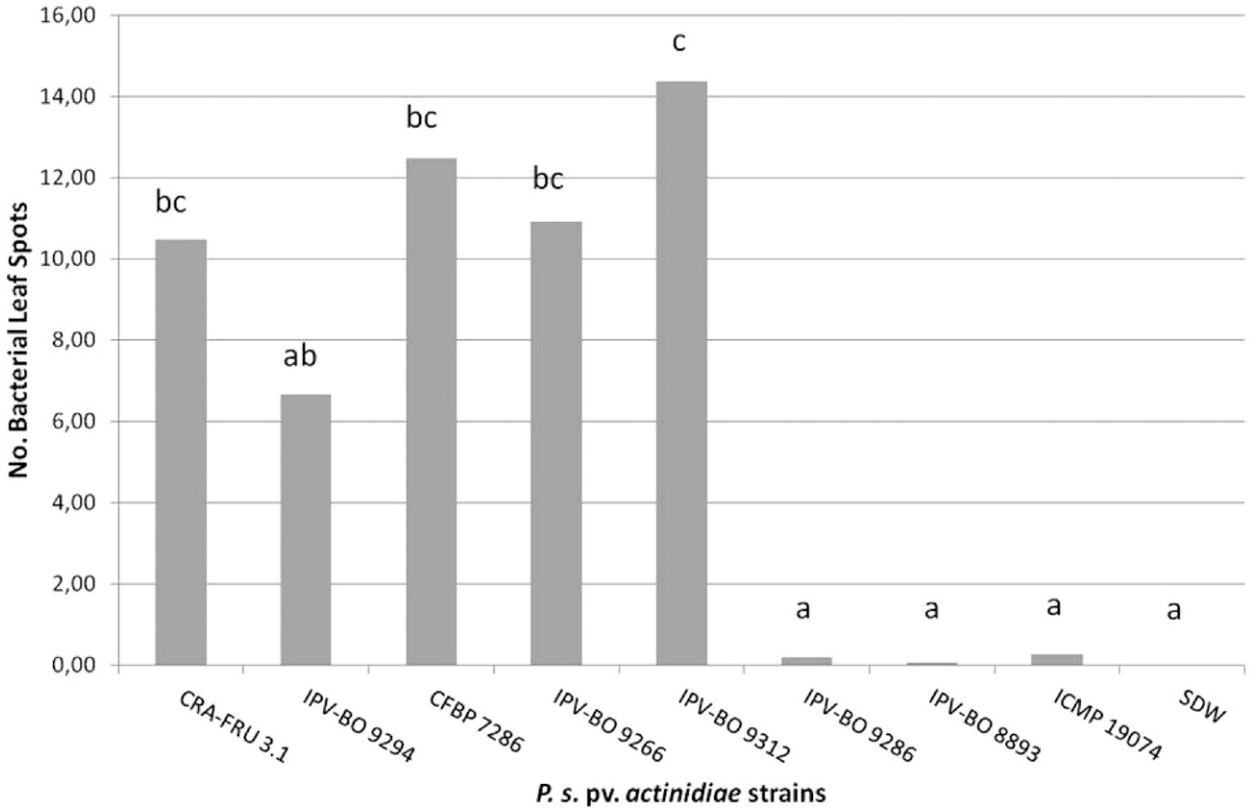

Fig. 5. The histogram shows the disease severity expressed as number of leaf spots related to the virulence of several Italian *Pseudomonas syringae* pv. *actinidiae* strains (IPV-BO) isolated from bleeding saps, compared with *P. syringae* pv. *actinidiae* reference strains (CRA-FRU 3.1 and CFBP 7286) and to the strain ICMP 19074 of *P. syringae* pv. *actinidifoliorum.* SDW: Sterile distilled water. Different letters indicate different statistical categories (Duncan's test, $P \leq 0.05$).

pv. *actinidiae* isolation. However, it does not show colonies with differential characteristics in the colony morphology; *P. syringae* pv. *actinidiae*, in fact, does not produce fluorescent pigments (Balestra et al. 2009; Scortichini 1994). Therefore, NSA was selected for this study. *P. syringae* pv. *actinidiae* colonies grown on NSA were levan positive, allowing for easier recognition among the saprophytes.

Despite the biologic differences obtained in the pathogenicity assays, the MLSA and WGS analyses did not show significant differences between the WGS of the HR negative and HR positive strains. The Italian strain IPV-BO 9312, which resulted in the most virulent strain, did not show significant differences in the T3SS genes among all Chilean and Italian strains of *P. syringae* pv. *actinidiae* biovar 3; in particular, the strain IPV-BO 9312 showed the T3SS gene cluster significantly similar to that of HR negative IPV-BO 8893 and IPV-BO 9286, hypovirulent *P. syringae* pv. *actinidiae* strains. According to previous studies, PAC_ICE sequences included open reading frames associated with adaptability of the strains with the environment and also possible characteristics related to pathogenicity. The almost-identical sequences obtained for HR positive and negative Italian strains, and also identical to Italian reference PAC_ICE sequence, clearly showed that the genetic explanation of the distinct pathogenic phenotype is located in other genomic regions. As expected, Italian and Chilean strains showed different PAC_ICE sequences; however, they were identical to each country reference: PAC_ICE2 from Italy and PAC_ICE3 from Chile (Butler et al. 2013). In addition, as for the *P. syringae* pv. *actinidiae* strains belonging to biovar 3 (Sawada et al. 1995), no *argK* and coronatine gene clusters were detected in hypovirulent strains IPV-BO 8893 and IPV-BO 9286. As T3SS associated genes showed no differences in nucleotide sequences, the low pathogenicity observed in HR negative strains IPV-BO 8893 and IPV-BO 9286 could not be associated with mutations that could alter the protein structure as previously reported in *P. syringae* isolates (Mohr et al. 2008). Results reported by Diallo et al. (2012) indicated that differences in hypersensitivity tests lay in the absence of at least one of the genes contained in the *hrp/hrc* locus. On the contrary, in this study the structure of the *hrp/hrc* locus was the same in all the Italian strains, even though their virulence varied. Also, the gene *hopA1*, which differentiated the *P. syringae* pv. *actinidiae* strains biovar 1 from those belonging to biovar 3 (Vanneste et al. 2011), were present without polymorphisms in HR negative *P. syringae* pv. *actinidiae* strains as well. According to Diallo et al. (2012), HR negative strains of *P. syringae* pv. *syringae* have been frequently found in different nonagricultural matrices and their association with lack of pathogenicity was not as strict as it appeared, showing that some HR negative strains caused similar severe symptoms in cantaloupe to those observed by HR positive strains. These observations lead to the hypothesis that the hypovirulence observed could be not associated with T3SS genes, but to genes associated with environmental response and adaptation, or in response to the host gene transcripts, whose increase was reported a few hours after pathogen inoculation (Michelotti et al. 2015). Thus, regulatory regions of different pathogenicity genes have to be more deeply explored in order to associate a possible alteration in gene expression to the lack of HR.

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