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Short Notes

Widespread distribution of kiwifruit bacterial canker caused by the European *Pseudomonas syringae* pv. *actinidiae* genotype in the main production areas of Portugal

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Summary. In Portugal, in 2010, after the first recorded occurrence of *Pseudomonas syringae* pv. *actinidiae* (Psa) in a few kiwifruit orchards, and following subsequent records of suspected symptoms at the beginning of 2011, an extensive survey concerning Psa was carried out. One hundred and sixty-two samples (trunks, twigs, leaves, flowers, buds) presenting symptoms were collected from the main Portuguese kiwifruit areas and submitted to phyto-bacteriological analyses to verify possible relationships between the symptoms/damage observed and the presence of Psa in kiwifruit orchards. The results confirmed the widespread presence of the pathogen in the main production areas of Portugal, where different *Actinidia* spp. cultivars are grown. Genetic investigations revealed that all the Psa strains detected belong to the European genotype. Preliminary considerations concerning pathogen dissemination are made.

Key words: bacterial disease, epidemiology, *Actinidia deliciosa*, *Actinidia chinensis*.

Introduction

The cultivation of kiwifruit is an increasing sector of the fruit market worldwide. Italy is the world leader for the production and exportation of kiwifruit, but, after its main non- EU competitors (New Zealand, Chile), other European countries like Portugal, Greece, Spain and France have greatly increased their efforts in the area of kiwifruit production over the past few years. Several parasites can affect kiwifruit plants but, at present, the *Pseudomonas syringae* pv. *actinidiae* (Psa), a causal agent of bacterial canker, is by far the most dangerous one (Balestra *et al*., 2009, 2010a). Psa was first isolated and described during the 1980's in Asia (China, Japan, Korea) and then reported in the most important kiwifruit cultivation areas of the world (Italy, France, New Zealand, Chile), where it was found on the most frequently cultivated species (*A. deliciosa* and *A. chinensis*) and cultivars of kiwifruit plants (green and yellow fleshed fruits). In 2010, Psa was also recorded in Portugal (Balestra *et al*., 2010b). After that report, the situation in the main kiwifruit areas of Portugal was monitored again in 2011, to evaluate the spread of this pathogen. This paper aims at describing the current state of the Psa epidemic in Portugal and to assess genetic relationships existing both between the Portuguese isolates among themselves and between them and other Psa strains from different geographical areas.

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Materials and methods

Sampling was carried out during the 2011 vegetative season, throughout the whole of the main Por-

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tuguese kiwifruit area in the Northern part of the country; the survey involved twenty-one orchards that were between 5 to 24 years old and located in the following areas: Santa Maria da Feira, Vila boa de Quires, Quinta dos Bocas, Felgueiras, Lago - Braga, Valença. One hundred and sixty two samples from *Actinidia deliciosa* plants, 'Hayward', 'Summer' and 'Bo Erica' cultivars and from *Actinidia chinensis*, 'Soreli' and 'Belen' cultivars affected by symptoms referable to Psa damage, such as brown spots with yellow haloes on leaves, reddish oozing cankers on trunks and twigs, the collapse of shoots, the discolouring of flowers and buds, were collected and then immediately conveyed to the laboratory for analysis (Figure 1).

To isolate the pathogen, small pieces of diseased tissues were ground in sterile mortars containing sterile distilled water. The suspensions were diluted and sprinkled onto nutrient-sucrose agar plates (NSA) and incubated for 24–48 h at 28°C, after which, single colonies were transferred to King's B (KB) medium (King *et al*., 1954) at 25–28°C. The biological characterization and identification of the bacteria isolated from this plant material was carried out according to the morphological, cultural, physiological and biochemical characteristics described by Takikawa *et al*. (1989).

Twenty isolates, here labelled as Port1 to Port20, in representation of all the areas investigated (see Table 1), were randomly chosen for molecular characterization in order to confirm their identification, and to assess their genetic relationships with other known Psa strains. Genomic DNA was extracted from each isolate and used for the following molecular investigations.

Amplifications of the 16S rDNA using fD1 and rP1 primers, as described by Weisburg *et al.* (1991), and of the 16S-23S rDNA inter-genic spacer using sD21 and D22 primer, as described by Manceau and Horvais (1997), were performed. The amplification products were purified, sequenced and the sequences compared to those in the databases available at the National Centre for Biotechnology Information (NCBI) using their Blast search software (Altschul

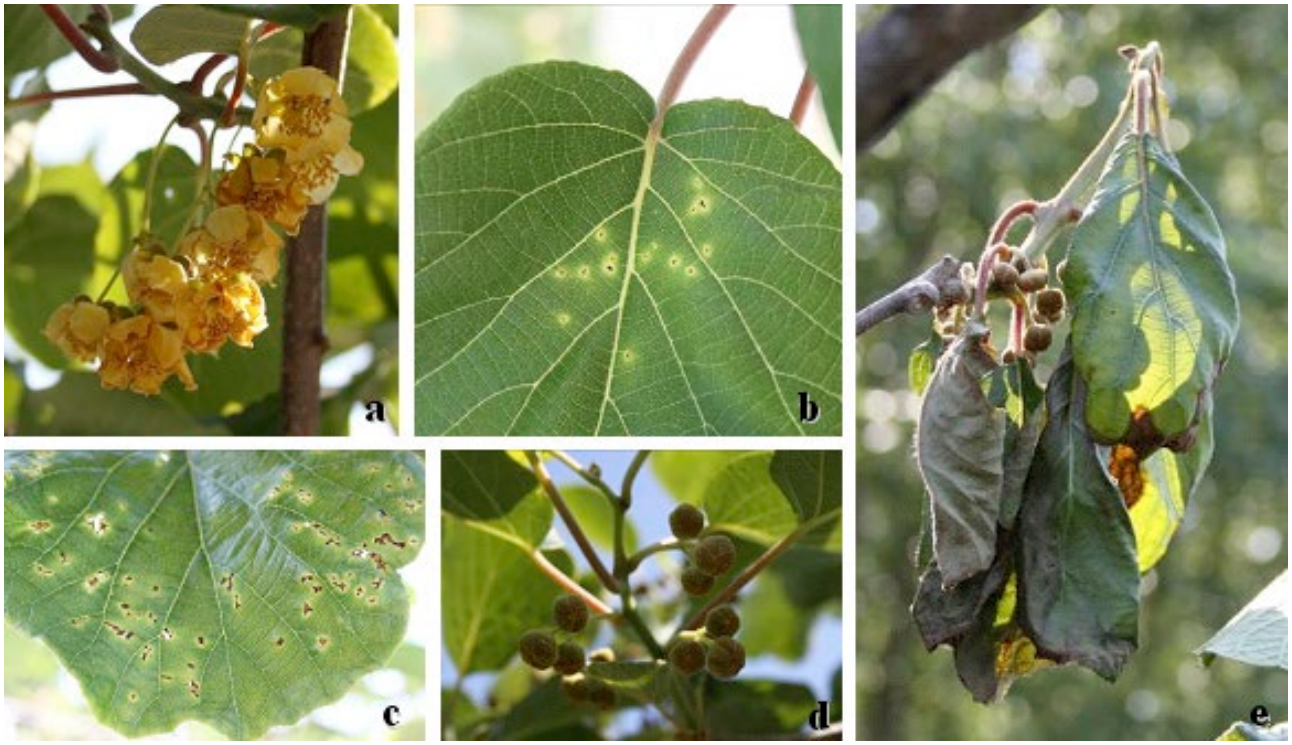

Figure 1. Natural symptoms caused by *Pseudomonas syringae* pv. *actinidiae* on flowers of *Actinidia chinensis* cv. Soreli (a) and leaf of cv. Belen (b), and on a leaf of *A. deliciosa* cv. Hayward (c), buds of cv. Bo Erica (d) and a twig of cv. Summer (e).

Table 1. *Pseudomonas syringae* strains included in the molecular investigations.

Strain	Species and pathovar	Geographic origin (date of isolation)	Host species (cultivar)	Source
4284	*P. syringae*	France (1985)	*Actinidia* sp.	CFBP
2598	*P. syringae* pv. *theae*	Japan (1970)	*Thea sinensis*	NCPPB
KW11	*P. syringae* pv. *actinidiae*	Japan (1984) *	*A. deliciosa*	Y. Takikawa
KW1	*P. syringae* pv. *actinidiae*	Japan (1984)	*A. deliciosa*	Y. Takikawa
Kn2	*P. syringae* pv. *actinidiae*	Korea (-)	*A. chinensis*	Y.J. Koh
7285	*P. syringae* pv. *actinidiae*	Italy (2008)	*A. chinensis* (Jin Tao)	CFBP
7286	*P. syringae* pv. *actinidiae*	Italy (2009)	*A. chinensis* (Hort16A)	CFBP
7287	*P. syringae* pv. *actinidiae*	Italy (2008)	*A. deliciosa* (Hayward)	CFBP
Port1	*P. syringae* pv. *actinidiae*	Valença, Portugal (2011)	*A. deliciosa* (Bo Erica)	This study
Port2	*P. syringae* pv. *actinidiae*	Valença, Portugal (2011)	*A. deliciosa* (Bo Erica)	This study
Port3	*P. syringae* pv. *actinidiae*	Valença, Portugal (2011)	*A. deliciosa* (Summer)	This study
Port4	*P. syringae* pv. *actinidiae*	Valença, Portugal (2011)	*A. deliciosa* (Summer)	This study
Port5	*P. syringae* pv. *actinidiae*	Vila Boa de Quires, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port6	*P. syringae* pv. *actinidiae*	Vila Boa de Quires, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port7	*P. syringae* pv. *actinidiae*	Vila Boa de Quires, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port8	*P. syringae* pv. *actinidiae*	Quinta dos Bocas, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port9	*P. syringae* pv. *actinidiae*	Quinta dos Bocas, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port10	*P. syringae* pv. *actinidiae*	Quinta dos Bocas, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port11	*P. syringae* pv. *actinidiae*	Felgueiras, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port12	*P. syringae* pv. *actinidiae*	Felgueiras, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port13	*P. syringae* pv. *actinidiae*	Felgueiras, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port14	*P. syringae* pv. *actinidiae*	S. Maria da Feira, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port15	*P. syringae* pv. *actinidiae*	S. Maria da Feira, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port16	*P. syringae* pv. *actinidiae*	S. Maria da Feira, Portugal (2011)	*A. deliciosa* (Hayward)	This study
Port17	*P. syringae* pv. *actinidiae*	Lago - Braga, Portugal (2011)	*A. chinensis* (Soreli)	This study
Port18	*P. syringae* pv. *actinidiae*	Lago - Braga, Portugal (2011)	*A. chinensis* (Soreli)	This study
Port19	*P. syringae* pv. *actinidiae*	Lago - Braga, Portugal (2011)	*A. chinensis* (Belen)	This study
Port20	*P. syringae* pv. *actinidiae*	Lago - Braga, Portugal (2011)	*A. chinensis* (Belen)	This study

* Pathotype strain.

et al. 1990). Sequences of 16S and 16S-23S ITS rDNA obtained from the four PSA825 (Port4), PSA835 (Port7), PSA838 (Port 10), PSA853 (Port18) strains isolated respectively, from the flowers, trunks, twigs and leaves of the *Actinidia* species, were deposited in GenBank with accession numbers JN378727 (16S) and JN378731 (16S-23S ITS); JN378726 (16S) and JN378730 (16S-23S ITS); JN378729 (16S) and 16S-23S ITS JN378733 (16S-23S ITS); JN378728 (16S) and JN378732 (16S-23S ITS), respectively. To further confirm their identity, the presence of sequences specific to pathovar *actinidiae*, was tested through PCR amplification using two pairs of pathovar-specific primers (Koh and Nou, 2002; Rees-George *et al*., 2010).

Also, some additional PCR-based fingerprinting approaches were used to evaluate possible genetic relationships between the Portuguese isolates, representative Psa strains from other worldwide diseased areas and strains of related pathovars (Table 1). In detail, the rep-PCR's were conducted availing of four primer sets, as described by Weingart and Völksch (1997) and Louws and colleagues (1994); the RAPD's were obtained using three random primers, selected for producing informative amplimers on Psa (Mazzaglia *et al*., 2011). Finally, IS50-PCR reactions were performed using a single 20 bp oligonucleotide primer as described by Sundin and Murillo (1999). The electrophoretic profiles which emerged were compared by evaluating the presence or absence of each band and converted into binary data; their analysis, separately and jointly, generated genetic similarity matrices and, finally, dendrograms, availing of unweighted pair-group method analysis (UPGMA), were produced.

Also, MLST analysis (Sarkar and Guttman, 2004) was performed using four genes (*gyrB*, *rpoD*, *gap1* and *gltA*) from the core genome of the *Pseudomonas syringae*, using primers for both amplification and sequencing, and following the methodology described by Hwang and colleagues (2005). The sequences were then concatenated, aligned with those of PSA strains from other geographical regions, while a neighbour-joining analysis, with 500 bootstrap replicates, was performed.

Pathogenicity experiments were carried out according to the Balestra *et al*. (2010b) procedure and the sample plants were recorded daily for symptom development; re-isolation was attempted for all the plants presenting symptoms typical of Psa. Groups of five healthy plants (two-years-old *A. chinensis* cv. Soreli in single pots) were artificially contaminated with four strains, identified as Psa (PSA825, PSA835, PSA838, PSA853) according to the Serizawa and Ichikawa (1993) method. Control kiwifruit plants were similarly treated with sterile distilled water. Reference strains of *P. s.* pv. *actinidiae* (NCPPB 3739, CFBP 7825, CFBP 7826, CFBP 7827) were used as controls during these tests.

Results

The pathogen was isolated within the different sampling sites: Santa Maria da Feira, Vila boa de Quires, Quinta dos Bocas, Felgueiras, Lago – Braga and Valença. These areas had already been surveyed during the 2010 season but, except for Valença (Balestra *et al*., 2010b), they had all been found free of Psa. Bacterial colonies with a typically Psa morphology were isolated from 98% (158/162) of the trunk, twig, leaf, bud and flower samples processed. The colonies appeared whitish, convex, with smooth to undulate margins on the nutrient agar plates; they were also Gram negative, did not produce fluorescent pigments on King's medium B (King *et al*., 1954), caused hypersensitivity on tobacco leaves (var. Virginia Bright), were levan and urease positive and hydrolysed Tween 80. Moreover, they produced acid from sorbitol and were negative for oxidase, potato soft rot, arginine dihydrolase, tyrosinase, and nitrate reduction (Lelliott and Stead, 1988; Takikawa *et al*., 1989).

The rDNA sequences, obtained from the molecularly characterised strains, shared a 99.9–100% (16S rDNA) and 100% (16S-23S ITS rDNA) identity with the corresponding *P. syringae* pv. *actinidiae* sequences in the NCBI database. Their identity was also confirmed by positive PCR amplification using both of the two pairs of pathovar-specific primers (Koh and Nou, 2002; Rees *et al*., 2010), which led, in each case, to amplification of single specific DNA fragments of the expected size.

The molecular genotyping of the Portuguese Psa strains, carried out according to our recent paper (Mazzaglia *et al*., 2011), resulted in the production of several amplification profiles, always identical to each other and to reference strains from Italy, and presenting very few differences when compared to the Asiatic reference strains. On the other hand, the *Pseudomonas syringae* CFBP 4284 and the *Pseudomonas syringae* pv. *theae* NCPPB 2598 strains showed remarkably different and distinguishable patterns. The dendrogram for genetic similarity is provided in Figure 2 (right).

The sequences for each of the four genes of the core genome are all of the same length in all the Portuguese strains, and respectively 854 bp (*gyrB*), 807 bp (*rpoD*), 709 bp (*gap1*) and 572 bp (*gltA*). Consequently, entire concatenated sequence total 2942 bp. They proved identical both to each other and to those from Italian strains, and presented only infinitesimal differences compared to the Asiatic ones. Again, the *Pseudomonas syringae* CFBP 4284 and the *Pseudomonas syringae* pv. *theae* NCPPB 2598 strains revealed significant differences when compared

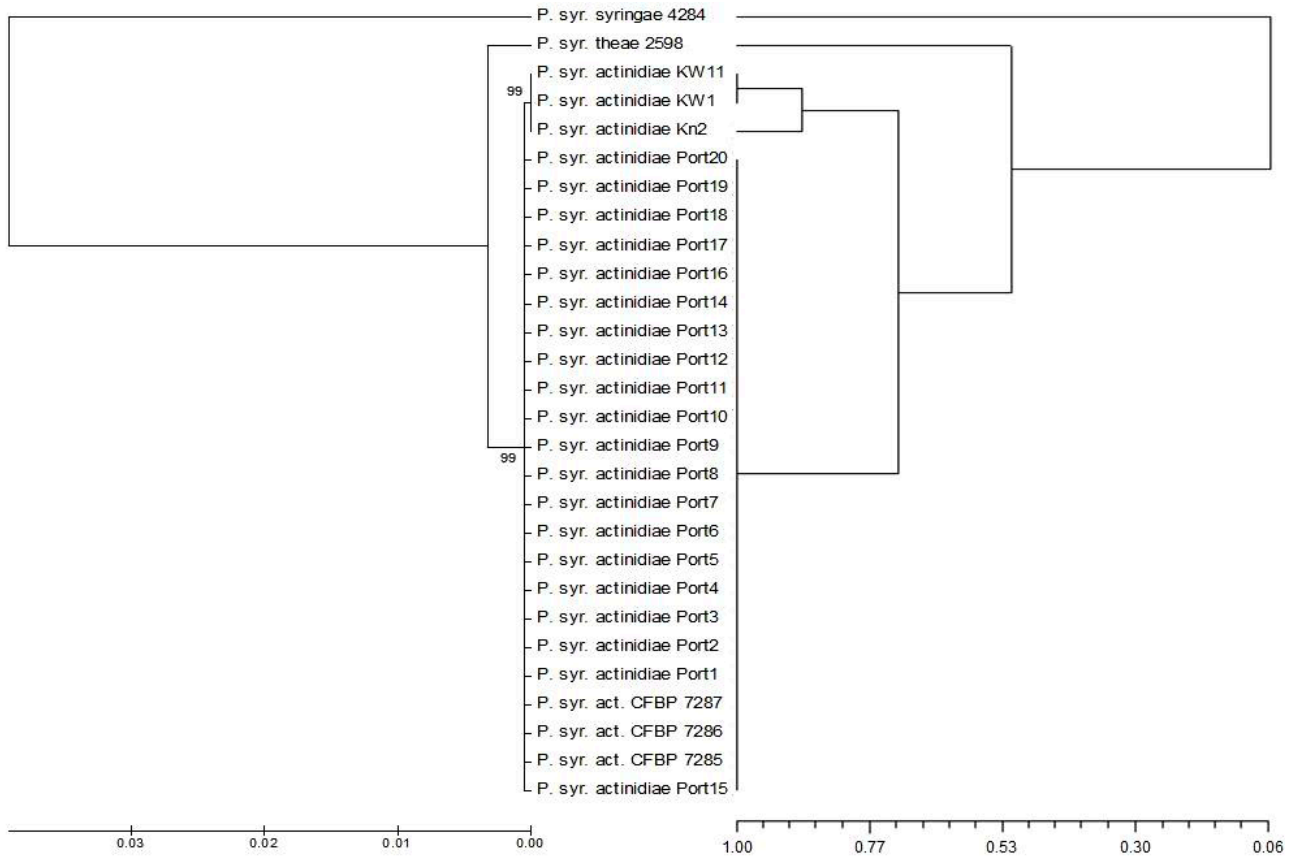

Figure 2. Comparison of results obtained by MLST (left) and fingerprint (right) analyses on 20 Portuguese Psa strains selected for molecular investigation and on reference strains (see Table 1).

MLST (left): dendrogram of phylogenetic relationships inferred using the Neighbour-Joining method. The optimal tree with a branch length sum= 0.0874 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5.
Fingerprintings (right): dendrogram of genetic similarity resulting from the combined data set from rep-PCR, RAPD and IS50-PCR amplifications, using UPGMA analysis and Dice's coefficient. The analyses were conducted in NTSYSpc 2.02.

with those of Psa. The neighbour-joining consensus tree is also shown in Figure 2 (left).

The dendrogram clusterings in both the MLST and fingerprinting analyses are perfectly comparable.

Pathogenicity on the kiwifruit was confirmed for the PSA825, PSA835, PSA838, PSA853 strains on all the artificially contaminated plants. Symptoms on leaves and twigs were observed within two-three weeks after artificial contamination. No symptoms were observed on the control plants contaminated with water. The results of these tests confirmed that all the bacterial isolates collected during the field surveys in Portugal were *P. s.* pv. *actinidae*.

Discussion

During the past ten years in Portugal, the cultivation of kiwifruit has increased greatly, almost doubling its cultivation area (from 700 to more than 1.300 hectares; Associação Portuguesa de Kiwicultores, *personal communication*). Meanwhile, recent records of damage caused by bacterial plant pathogens, such as *P. s.* pv. *syringae* (Balestra *et al*., 2009) and Psa (Balestra *et al*., 2010a; Balestra *et al*., 2010b), have prompted cooperatives and kiwifruit producers to request a thorough investigation of these phytopathological problems. The need to clarify the current distribution of these pathogens and understand

the most important issues related to them (genetic origin/similarity, control strategies, abiotic and biotic factor that help their spread, etc.) is a keenly felt priority at the moment.

As to the epidemiological investigation discussed here and which focused on Psa in Portugal, the results obtained indicate that this pathogen affects, at present, kiwifruit orchards in all the main areas where kiwifruit is cultivated. This contrasts with the situation of 2010, when only one of the locations sampled (Valença) was affected by kiwifruit bacterial canker disease (Fig. 3).

Independently of the kiwifruit cultivar, the disease incidence ranged from 30% to 85% and, in some cases, due to the extremely high percentage of damage observed, the eradication of the whole orchard was strongly advocated. At present, the areas with the highest incidence of the disease were Lago - Braga, Quinta dos Bocas, and Valença.

Considering the North of Portugal, where the production of kiwifruits is concentrated, the rather swift spread of Psa in only one year should not be underestimated. Additional investigations are under way also in Central Portugal where, some areas are starting to play a significant role in kiwifruit cultivation.

The genetic investigation carried out, availing both of molecular fingerprint and MLST analyses on a representative sample of the isolates obtained from diseased kiwifruit plants in Portugal, revealed a very high degree of genetic uniformity among all the samples; only few differences were recorded when comparing them to reference strains from the Asiatic area. This agrees with other results reported in several recent papers. Ferrante and Scortichini (2010), using both MLST and repetitive-sequence PCR, reported that strains from the bacterial canker outbreak in Italy 2008–2009 are almost identical, with some differences compared to the Italian strains isolated in 1994 and the Japanese and Korean ones. Vanneste and colleagues (2011a) also report that strains from both Italy and France share the same BOX-PCR electrophoretic pattern and the same haplotype based on the *cts* gene sequence. Even the most recent observations on the outbreak of the disease in New Zealand seem to link, genetically, the most aggressive types of strains from New Zealand to those isolated all over the Europe during the past three years (Vanneste, *personal communications*).

The results reported here, therefore, seem to confirm the fact that Portugal is simply one of the last

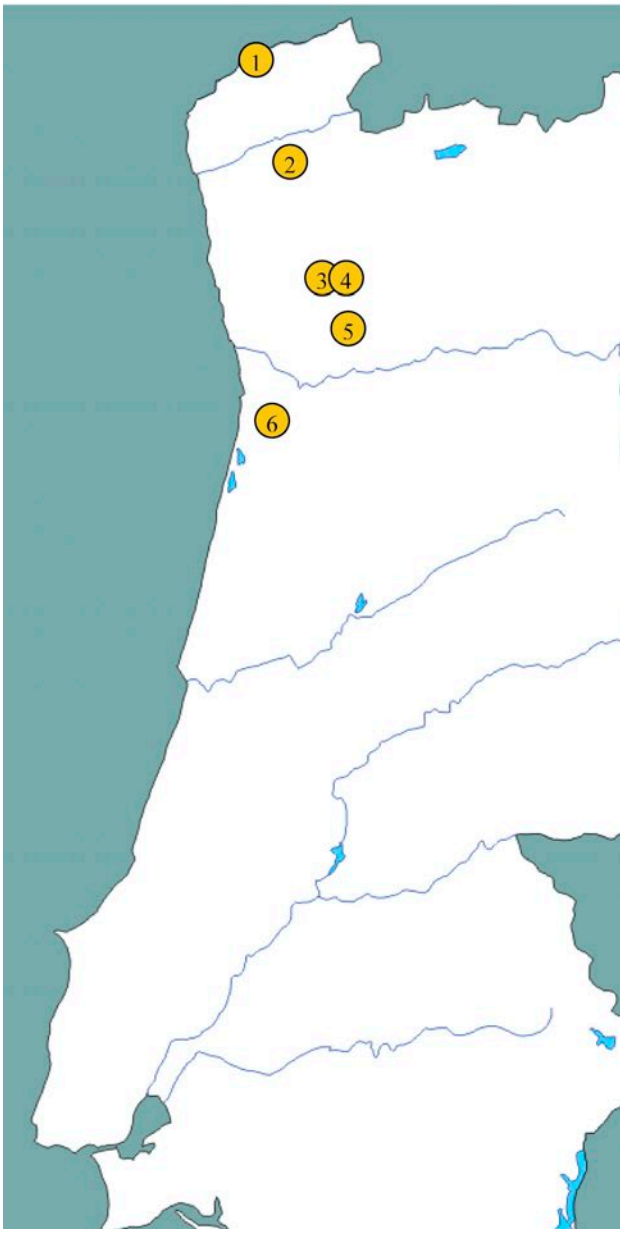

Figure 3. A map of Portugal showing the kiwifruit areas investigated and recordings of *Pseudomonas syringae* pv. *actinidiae* in order of time for the main production areas: 1) Valença (2010, 2011); 2) Lago Braga, 3) Quinta dos Bocas, 4) Felgueiras, 5) Vila boa de Quires, and 6) Santa Maria Da Feira (2011).

frontiers reached by the current dramatic European outbreak of Psa; in a little more than a couple of years after the outbreak of bacterial canker reported in Italy in 2008 (Balestra *et al*, 2008), Psa has spread

to almost all the major kiwifruit cultivation areas: the whole of the Italian peninsula, Spain (Balestra *et al*., 2009, 2011), France (Vanneste *et al*., 2011b), and also Switzerland (EPPO, 2011).

The genetic homogeneity of the European strains, despite the wide-ranging nature of the geographical areas they were found in, suggests that the disease, at least as far as the latest epidemic is concerned, has a common origin, even if it what it might be remains unclear. What is clear, however, is that the rapidity of the spread (associated with the above-described uniformity of the strains) is probably due to the dissemination of infected plant material. Thus, the results obtained during the above-mentioned surveys, seem to suggest the need to avail only of propagation material which has been adequately certified as being free of Psa.

As a matter of fact, infected plant material and inadequate pruning practices, possibly in association with unfavourable environmental conditions (late frost, hail, strong winds, etc.), have probably played a central role in the outbreak of the pathogen. In addition, a lack of knowledge regarding Psa symptoms and the best strategies to avail of to prevent them, has, most probably facilitated the spread of this pathogen even in areas far apart from each other, and permitted Psa to colonise several kiwifruit orchards.

Different aspects of the question still remain to be studied in depth (e.g. the molecular characterization of Psa isolates, the management of kiwifruit nurseries, field control trials) in order to overcome this serious phytosanitary situation and permit the Portuguese kiwifruit industry to continue its positive trend.

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