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RESEARCH PAPERS

The Italian inter-laboratory study on the detection of *Pseudomonas syringae* pv. *actinidiae*

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Summary. A severe form of bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), has been detected in all the main areas of cultivation of kiwifruit (*Actinidia deliciosa* and *A. chinensis*). Since 2010 several research groups have been assessing methods and procedures to detect and identify Psa, both from symptomatic and symptomless host material. In 2011, a study to compare Psa diagnostic methods was performed with reference to Psa strains and related pathovars, and with plant extracts or DNA obtained from healthy and naturally infected leaves, pollen or wood. The study revealed the strengths and the weaknesses of the assessed methods. The procedure included screening tests for Psa detection and for identification of Psa colonies. The methods assessed were bacterial isolation on generic and semi-selective media, PCR analysis (single, duplex and rep-PCR assay, the latter for identification only). The results highlighted the best performance of semi-selective with respect the generic media; the usefulness of the direct-PCR as screening tests for Psa detection; and the greater specificity of duplex-PCR and sensitivity of simple-PCR. The use of semi-selective medium for isolation and of two PCR-based methods - in parallel - for Psa detection are suggested. Both rep-PCR and duplex-PCR, were found to be specific, and are recommended as an identification test for this pathogen.

Key words: kiwifruit bacterial canker, symptomatic/symptomless host material, dilution plating, qualititative PCR analysis.

Introduction

Until now, there has been no formal guidance on procedures for the detection of *Pseudomonas syringae* pv. *actinidiae* (Psa), the bacterium that is causing serious damage to *Actinidia* spp. world-wide. The European Plant Protection Organization (EPPO) Secretariat added this pathogen to the EPPO A2 List of quarantine pests (version September 2012, Panel review date March 2013). One of the main problems for the control of the disease is the availability of standardized and validated diagnostic methods to be used for Psa detection, either from symptomatic (i.e. in cases of financial reimbursement for the eradication of infected plants) or symptomless kiwifruit (i.e. for designating 'certified' Psa-free nursery plant material).

At the time of the first outbreak of this new severe form of the disease (Balestra *et al*., 2008), the detection of the causal agent was mainly based on the isolation, purification and identification of pure cultures of Psa by rep-PCR (Ferrante and Scortichini, 2009) or sequencing of 16S rDNA (Balestra

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et al., 2009). Molecular identification of bacterial cultures by PCR analysis has been reported using single PCRs: KN-PCR (Koh and Nou, 2002) and RG-PCR (Rees-George *et al.*, 2010), and by duplex-PCR (Gallelli *et al*., 2011a). The KN-PCR is known to give false positive results with *P. s.* pv. *syringae* (Pss), *P. s*. pv. *theae* (Psth), and *P. s.* pv. *tomato* (Pto), whereas RG-PCR cross-hybridizes with Psth (Rees-George *et al.*, 2010). The duplex-PCR method was developed for a specific detection of Psa (Gallelli *et al*., 2011a). Moreover, the use of the integrated approach based on DNA extraction followed by the two qualitative PCR assays (direct-PCRs) (Rees-George *et al.*, 2010; Gallelli *et al.*, 2011a) was used to detect Psa in symptomless host fruit, twigs and pollen (Gallelli *et al*., 2011b).

As several diagnostic methods were available, laboratories like the Plant Protection Services which were frequently required to carry out official analyses, experienced problems determining the most appropriate methodologies to be used in different cases. Because there had been no external standardisation of procedures for the detection of Psa, the 'Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per la Patologia Vegetale' (CRA-PAV) decided to organize an inter-laboratory test, for Psa detection and identification in June 2011. This study was performed with seven Italian laboratories, including two Universities (Alma Mater Studiorum - University of Bologna and 'La Tuscia' University of Viterbo) and five Plant Protection Services (Regions: Piedmont, Emilia-Romagna, Friuli Venezia Giulia, Sardinia, Trentino Alto Adige). The main objectives of the study were: (i) to compare molecular diagnostic methods, described up to 2011, either for the detection or for the identification of Psa; (ii) to evaluate the morphological identification methods for Psa using naturally infected host samples; and (iii) to complete preliminarily standardization, to support the development of the EPPO protocol, in case the methods were found to produce consistent results.

Materials and methods

Experimental procedures

A complete protocol was established from CRA-PAV. This included i) isolation of bacteria on King's B medium (KB) (King *et al*., 1954), or on NSA (Oxoid nutrient agar supplemented of 5% w/v sucrose) (generic media) and on KB and NSA each modified by adding antibiotics and boric acid (KBC and NSA-AB respectively) following Mohan and Schaad (1987) (semi-selective media); ii) identification of Psa-like colonies by single PCR, (primers PsaF1/R2) (Rees-George *et al*., 2010), duplex PCR (Gallelli *et al.*, 2011a), rep-PCR (ERIC primer) (Louws *et al.*, 1994); and iii) direct-PCR from DNA purified from plant tissue (Gallelli *et al.*, 2011a, b) by single PCR (primers PsaF1/R2) (Rees-George *et al*., 2010) and d-PCR (Gallelli *et al*., 2011a). PCR analysis conditions were carried out as described by Rees-George *et al*. (2010) and Gallelli *et al*. (2011a). The primers PsaF1/R2 were selected instead of primers PsaF3/R4, due to better reliability of previously obtained results (data not shown).

Constitution of samples and sets

Sets of twenty samples were prepared from CRA-PAV, either including bacterial suspension of reference cultures and of contaminants isolated from infected kiwifruit, plant extract from infected and healthy host leaves or pollen, or DNA purified from infected or healthy wood of *A. chinensis* (Table 1). The samples were randomized within each set and the sets were randomly assigned to the participants. Although the order of the samples was subject to randomization, the preparation and constitution of the samples within each set was identical, thus maximizing the sample homogeneity. After the randomization process, each sample was labeled with a code consisting of the set number and the sample number. Each set also contained all the occurrences required to perform the whole procedure. In order to guarantee uniform sample conditions, all the material was sent stored on dry ice, and each laboratory checked if the samples and material were in suitable condition after receipt (i.e. all samples were frozen).

Materials and reagents

Symptomatic plant extracts were obtained from portions of about 0.5–1 g aseptically removed at the margins of the necrotic host tissues and crushed in 1–2 mL of sterile physiological saline solution (SPS) (0.85% NaCl in distilled water). After incubation for 10 min at room temperature, the suspension was centrifuged at 8000 *g* for 1 min and the supernatant was collected to constitute the crude plant extract.

Table 1. Constitution of the 20 samples used within each set of samples assayed by different laboratories for bacterial pathogens.

Sample	Type of sample	Material	Origin
1	Plant extract[a]	Infected leaf	*Actinidia chinensis* (leaf spots)
2	Plant extract	Healthy leaf	*A. chinensis* (healthy leaf)
3	Plant extract	Infected pollen	*A. deliciosa*
4	Plant extract	Healthy pollen	*A. deliciosa*
5	Bacterial suspension[b]	Psa CRA-FRU 8.43	*A. chinensis* (leaf)
6	Bacterial suspension	Psa CFBP 7287	*A. deliciosa*
7	Bacterial suspension	Psa CRA-PAV 1583	*A. chinensis* (fruit)
8	Bacterial suspension	Psa NCPPB 3740	*A. chinensis*
9	Bacterial suspension	*P. viridiflava* OMP-BO 4254A,1	*A. chinensis* (leaf)
10	Bacterial suspension	*P. syringae* pv. *syringae* OMP-BO 3909B,1	*A. chinensis* (twig)
11	Bacterial suspension	*P. syringae* pv. *tomato* NCPPB 2563	*Lycopersicon esculentum*
12	Bacterial suspension	*P. syringae* pv. *theae* CFBP 4097	*Camellia sinensis*
13	Bacterial suspension	*P. avellanae* NCPPB 3873	*Corylus avellana*
14	Bacterial suspension	CRA-PAV 1686	NC *A. chinensis* (fruit)
15	Bacterial suspension	CRA-PAV 1687	NC *A. chinensis* (pollen)
16	Bacterial suspension	CRA-PAV 1688	NC *A. chinensis* (pollen)
17	Bacterial suspension	CRA-PAV 1689	NC *A. chinensis* (wood)
18	DNA[c]	Infected wood	*A. chinensis* (branch canker)
19	DNA	Healthy wood	*A. chinensis* (healthy branch)
20	Water	---	---

[a] The procedure to obtain the plant extract was as described by Gallelli *et al.* (2011).
[b] Bacterial suspensions at a concentration of 2×10^7 CFU mL^{-1} were used.
[c] DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen).
NC. not classified contaminants levan-positive bacteria isolated from infected kiwifruit.

The crude extract was supplemented with 20–30% glycerol and stored at -80°C before dispatch.

The turbidity of the suspension was measured spectrophotometrically as absorbance at 660 nm. The concentration of bacterial suspension was determined by spectrophotometry (A_{660} = 0.1 OD corresponded to about $5 \times 10^{-10\text{^}8}$ cfu mL^{-1}) taking a loopful from a 24–48 h bacterial culture in a 0.5 mL volume of distilled sterile water. Each participant denatured a 100 μL aliquot of bacterial suspension at 95°C for 10 min, cooled it at 4°C on ice, and after centrifugation at 6000 *g* for 1 min, used the lysate (5 μL) as template in the PCR assays.

The following reagents were used by all the participants in the standardisation assays to perform the analyses described above: DNA was extracted from plant extract samples using the DNeasy® Plant Mini Kit (Qiagen S.r.l.). Single (Rees-George *et al.*, 2010) and duplex PCR (Gallelli *et al.*, 2011a) were performed, following the published protocols, using Platinum Taq DNA polymerase (Invitrogen, Life Technologies). Rep-PCR (ERIC primer) was performed, using GoTaq® Flexi DNA Polymerase (Promega), following the EPPO procedure (PM 7/100: Rep-PCR tests for identification of bacteria).

Performance criteria (adapted from EPPO procedures PM 7/98, EPPO PM 7/ 76 and ISO 16140)

For the purposes of this study the performance criteria were the following:
Inclusivity: the ability of a test to detect the target from a collection of target strains (synonymous of sensitivity).
Exclusivity: the lack of interference of a test from a range of non-target strains. Inclusivity and exclusivity were evaluated on pure cultures.
Relative Accuracy (A): the closeness of agreement between a test result and the accepted reference value (or the expected response from reference material).
Diagnostic Sensitivity (SE): the ability of a test to detect the target in the contaminated or infected reference samples.
Diagnostic Specificity (SP): the ability of a test not to detect the target in the non-contaminated reference samples.
Concordance: or qualitative reproducibility, was the percentage of chance of obtaining identical results (i.e. both positive or both negative) for two identical samples analyzed in different laboratories. We here refer to this parameter as reproducibility. A high proportion for this parameter supported the reliability of a protocol.
Analytical specificity was detected for molecular methods on a set of target and non-target bacterial strains either phylogenetically related or associated to the host material. This parameter indicated the performance of a test with regards to cross-reactions with non-target organisms.
Analytical sensitivity determined the lowest cell concentration giving a positive result. This parameter indicated the smallest amount of target that can be reliably detected.

Processing and validation of the data

The validation procedure of the parameters described above was as reported in OEPP/EPPO PM7/98 (2010); in particular:

$A = 100\% \times [(PA+NA)/(PA+PD+ND+NA)]$; $SP = 100\% \times [NA/(NA+PD)]$;

$SE = 100\% \times [PA/(PA+ND)]$. The following terms and definitions were used:
Positive agreement (PA): number of positive results obtained with a test on contaminated or infected reference samples.
Negative agreement (NA): number of negative results obtained with a test on non-contaminated reference samples.
Positive deviation (PD): number of positive results obtained with a test on non-contaminated reference samples; these can be considered as false positive results.
Negative deviation (ND): number of negative results obtained with a test on contaminated or infected reference samples; these can be considered as false negative results.

Results

Bacterial isolation

The isolation of Psa from generic (KB or NSA) and semi-selective KBC and NSA-AB media resulted in the performance criteria reported in Table 2. The greatest values of diagnostic sensitivity were obtained with semi-selective KBC medium (86%) that gave better results than NSA-AB (79%). The use of generic media KB or NSA resulted in the lower values of 71%. These results reflected the problems in isolating Psa from pollen samples, because all the laboratories were able to isolate Psa from infected leaves, giving, for leaves, 100% of diagnostic sensitivity for all the media. Observing the analytical data, the false positive results obtained from the different media were the following: KB/NSA, four out of seven; NSA-AB, three out of seven; and KBC, two out of seven. The high specificity (100%) showed that no false negative results were obtained from the samples tested.

The accuracy, that showed the correlation between the results obtained by the test and the expected response of reference material assessed, reflected the results of diagnostic sensitivity, resulting in the

Table 2. Performance criteria obtained by isolation on generic (KB/NSA) and semi-selective media (KBC and NSA-AB). All values were expressed in percentages (%).

Performance criteria	KB/NSA	KBC	NSA-AB
Diagnostic sensitivity	71	86	79
Diagnostic specificity	100	100	100
Relative accuracy	86	93	89
Reproducibility	89	93	89

greatest value for KBC (93%), followed by NSA-AB (89%) and, with the lowest value, NSA/KB (86%). A better degree of reproducibility (concordance) among different laboratories was obtained by using KBC (93%), rather than with the generic media and NSA-AB (89%).

Psa morphology description

All laboratories reported descriptions of each bacterial culture. For Psa strains the following descriptions were reported; NSA/NSA-AB media: mucous, with colonies of circular shape, convex, dome or umbonate elevation, entire or wavy margins; KB or KBC media: translucent colonies, with circular shape, high or convex elevation, corrugated margins, not fluorescent.

Direct-specific PCR assay

The results obtained from DNA extracted from infected and healthy host wood, leaf and pollen samples and subsequent PCR analyses showed greater values of diagnostic sensitivity (95%) compared with those obtained by isolation (Table 3).

By direct-PCR analysis, all laboratories were able to isolate Psa DNA from infected leaves and wood, giving 100% of diagnostic sensitivity; also in this case, the least value of diagnostic sensitivity (95%) was due to difficulty of detecting Psa from pollen samples (one false negative out of seven was detected with each PCR method). If compared with isolation, the direct PCR showed the greatest percentage of both accuracy and reproducibility (concordance) (98%). The high specificity (100%) showed that no false negative results were obtained from the samples tested.

Molecular methods for Psa identification from bacterial cultures

The results obtained for the molecular identification of bacterial cultures of target and non target bacteria are shown in Table 4. In this case the inclusivity was greater when RG-PCR was applied to detect the cultures (96%) than for d-PCR (93%) and rep-PCR (89%). However, the exclusivity value was greater for d-PCR (100%) and rep-PCR (97%) whereas RG-PCR gave a lower value (74%). The value of accuracy was greater for d-PCR (96%) followed by rep-PCR (92%) and RG-PCR (79%). Reproducibility showed similar values for the three PCR-based methods (from 94% for RG-PCR to 95.5% for d-PCR).

Analytical specificity

When analytical specificity was taken into account (Table 5), fewer false negative results were obtained by RG-PCR (only one out of 28), with d-PCR (two out of 28) and with rep-PCR (three out of 28). False negative results were obtained, with Psa strains CRA-FRU 8.43 (by d-PCR and rep-PCR) and CFBP 7287 (by all the PCR-based methods). Both these strains were genome-sequenced (Marcelletti *et al*., 2011; Mazzaglia *et al*., 2012), and were recovered in the recent kiwifruit bacterial canker outbreak, and belonged to the highly virulent Psa (Psa-V) population. Another false negative, out of 28 tested, was

Table 3. Performance criteria for detection of *Pseudomonas syring*e pv. *actinidiae* obtained by specific PCR (RG-PCR: Rees-George *et al*., 2010; d-PCR: Gallelli *et al*., 2011a) on DNA extracted from pollen, leaf and wood samples by DNeasy plant mini kit (Qiagen) (direct-PCR). All values were expressed in percentages (%).

Performance criteria	RG-PCR	d-PCR
Diagnostic sensitivity	95	95
Diagnostic specificity	100	100
Relative accuracy	98	98
Reproducibility	98	98

Table 4. Performance criteria for detection of *Pseudomonas syringe* pv. *actinidiae* obtained by specific PCR (RG-PCR: Rees-George *et al*., 2010; d-PCR: Gallelli *et al*., 2011) and rep-PCR (Louws *et al*., 1994) on bacterial cultures reported in Table 1. All values were expressed in percentage (%).

Performance criteria	RG-PCR	d-PCR	rep-PCR
Inclusivity (syn. sensitivity)	96	93	89
Exclusivity (syn. specificity)	74	100	97
Relative accuracy	79	96	92
Reproducibility	94	95.5	95

Table 5. Amplification results obtained by RG-PCR (Rees-George *et al*., 2010), d-PCR (Gallelli *et al*., 2011) and rep-PCR (Louws *et al*., 1994) on target and non-target bacterial cultures reported in Table 1 from the seven laboratories participants in the comparative test. For target strains (1–4) the expected results for each method were: 7/7 (seven positive results obtained from each laboratory); for non-target strains (5–13) the expect results for each method were: 0/7 (null positive results obtained from the seven laboratories). Psa = *Pseudomonas syringae* pv. *actinidiae*; Pv = *P. viridiflava*; Pss = *P. s.* pv. *syringae*; Pto = *P. s.* pv. *tomato*; Psth = *P. s.* pv. *theae*; Pa = *P. avellanae*.

		Strains	PCR assays		
			RG-PCR	d-PCR	rep-PCR
Target	1.	Psa CRA-FRU 8.43	7/7	6/7	6/7
	2.	Psa CFBP 7287	6/7	6/7	6/7
	3.	Psa CRA-PAV 1583	7/7	7/7	7/7
	4.	Psa NCPPB 3740	7/7	7/7	6/7
Expected results			27/28	26/28	25/28
False negative results			1/28	2/28	3/28
Non-target	5.	Pv OMP-BO 4254A,1	0/7	0/7	0/7
	6.	Pss OMP-BO 3909B,1	0/7	0/7	0/7
	7.	Pto NCPPB 2563	3/7	0/7	0/7
	8.	Psth CFBP 4097	7/7	0/7	1/7
	9.	Pa NCPPB 3872	6/7	0/7	1/7
	10.	CRA-PAV 1686	0/7	0/7	0/7
	11.	CRA-PAV 1687	0/7	0/7	0/7
	12.	CRA-PAV 1688	0/7	0/7	0/7
	13.	CRA-PAV 1689	0/7	0/7	0/7
Expected results			0/62	0/62	0/62
False positive results			16/62	0/62	2/62

obtained with the strain NCPB 3740 (by rep-PCR) recovered in Japan in 1989. This strain belongs to different genetic lineage from the Psa strains responsible of the recent outbreaks (Psa-V) (Ferrante and Scortichini, 2010; Gallelli *et al*., 2011a; Marcelletti *et al*., 2011; Mazzaglia *et al*., 2012). The majority of false positive results were obtained from RG-PCR (16 out of 62), and only two out of 60 were from rep-PCR (ERIC primer). No false positive results were obtained from d-PCR. In particular, using RG-PCR, false positive results were produced by all the laboratories (seven out of seven) with Psth CFBP 4097, six out of seven with *P. avellanae* (Pa) NCPPB 3872, three out of seven with Pto NCPPB 2563 (Table 4).

Discussion

The availability of suitable methods for the diagnosis of phytopathogenic microorganisms allows several objectives to be pursued: to support the monitoring of the disease in a territory, to allow the control of the sanitary status of propagation or commercial plant material, and to support epidemiological studies. Laboratories that are required to perform official analyses need to adopt validated and standardized procedures for the diagnosis they carry out. However, the sudden outbreak of bacterial canker of kiwifruit and the lack of official protocols at that time, meant that laboratories that should carry out official analyses were unprepared. To overcome

this problem, an inter-laboratory test was organized in June 2011, in which seven Italian laboratories participated. This initiative was intended to disseminate and verify the reliability of the methods set out in the diagnostic protocols here described. The analysis of comparitive data provided objective values for the performance criteria. Determining analytical sensitivity was not the objective of this study, but was reported, for PCR methods, in Gallelli *et al*. (2011a) and Rees-George *et al*. (2010).

In the present collaborative study the experimental protocols did not take into account the DNA extraction step; however, as previously reported by other authors (Ioos and Iancu, 2008), this step was generally standardized in the quarantine laboratories, as many of them use commercial extraction kits. In this study, all the laboratories used the same commercial kit and the analyses of undiluted and ten decimal dilution of the DNA extracted samples was recommended in the case of negative results.

For bacterial isolation, the semi-selective media gave better performance criteria than the generic media NSA/KB. The KBC medium (Mohan and Schaad,1987) provided the best results, even when compared to the modified NSA-AB. However, the choice of the culture medium can be influenced by the skill of the operator to recognize Psa-*like* colonies on a culture medium, rather than on another (selecting levan-positive colonies on NSA or non-fluorescent colonies on KB). A description of the Psa colonies on both media was reported. It should also be emphasized that the use of a semi-selective medium facilitates Psa isolation from plant tissues containing large number of contaminating bacteria. On the contrary, for Psa isolation from symptomatic plant material, it may be advisable to use a generic medium (NSA or KB), as these media reduced the time required for bacterial growth (3 d) compared to that on the semi-selective (5–6 d). However, the choice of semi-selective media is desirable in the case of compromised symptomatic samples (e.g. tissues in advanced stages of necrosis), because these are likely to contain contaminants or antagonists that can inhibit Psa growth. This was probably the cause of the problems in isolating Psa from pollen compared with isolation from infected leaves. As reported here, the performance criteria of the isolation procedure were negatively influenced by the inability to recover Psa from pollen samples.

On the contrary, the application of direct-PCR analysis facilitated Psa detection. All values of the performance criteria were greater than those obtained by bacterial isolation. The direct-PCR assay was therefore very useful as a preliminary screening test performed in parallel to bacterial isolation, which was more time consuming. The two PCR methods gave the same performance. However, differences among the two PCR-based methods were revealed when processing bacterial cultures of different strains. Better inclusivity (synonymous with sensitivity) of simplex-PCR (Rees-George *et al*., 2010) was demonstrated, followed by duplex-PCR (Gallelli *et al*., 2011a) and by the rep-PCR. Conversely, the exclusivity (synonymous with specificity) was greater for duplex-PCR (Gallelli *et al*., 2011a), followed by ERIC-PCR and by simplex-PCR (Rees-George *et al*., 2010).

These results emphasize that the two PCR methods were complementary: one was more sensitive and the other was more specific. Both molecular methods have been used individually for the diagnosis of plant material (Gallelli *et al*., 2011b; Mucini *et al.,* 2011; Stefani and Giovanardi, 2011; Vanneste *et al*., 2011; Biondi *et al*., 2013) or for the study of some peculiar epidemiological aspects (Ferrante and Scortichini, 2013). However, the evidence obtained in the present study leads to the recommendation that both PCR methods are used in parallel, in particular for the diagnostic analysis of symptomless material. In this way it is possible to ensure both specificity and sensitivity. The results indicate that it would be inadvisable the use of the simplex-PCR assay (Rees-George *et al*., 2010) as the sole method for the analysis of plant material by direct-PCR, or for the identification of Psa-like bacterial colonies, due to the likelihood of false positive results. Vanneste *et al.* (2011) have reported that several strains of *P. syringae* belonging to pathovars other than *actinidiae* have been found to give the 280 bp amplicon using the simplex-PCR of Rees-George *et al.* (2010). Moreover, Gallelli *et al.* (2014) recently observed that two low virulent strains isolated from kiwifruit, assigned to Psa biovar 4 (Vanneste *et al*., 2013), but referred as PsD population (Butler *et al*., 2013), because apparently non-pathogenic strains genetically quite distinct from Psa, yielded false positive results by simplex-PCR (Rees-George *et al*., 2010). This evidence indicated that positive results could be obtained by the simplex-PCR protocol of Rees-George *et al*. (2010), also from bacteria that colonize kiwifruit. This aspect did not emerge from the analysis of leaves and pollen plant extracts and the DNA from wood; so the

specificity was always high. Further analyses are required, however, on a large number of samples to better exploit this parameter from plant extracts.

The high number of false positives obtained from bacterial cultures of phylogenetically related *Pseudomonas* spp. used in this study has confirmed the specificity problems of the simplex-PCR. The three related *Pseudomonas* spp. (Pto, Psth, Pa) that gave false positive results were included in this study, although they are non-pathogenic on kiwifruit. Mechanisms of genomic evolution of bacterial species, can include horizontal transfer of genomic islands among related bacteria, and these transfers can contribute to the alteration of the host ranges or confer resistance or adaption to environmental factors (Juhas *et al.,* 2009). This has also been supported by evidence of the transmission of three integrative conjugative elements from *P. syringae* strains to Psa (Butler *et al*., 2013).

To overcome the described limitation, in addition to the duplex-PCR, new methods for the specific identification of Psa strains causing sudden and re-emerging outbreaks of bacterial canker have been recently developed (Balestra *et al*., 2013; Biondi *et al.,* 2013; Gallelli *et al.,* 2014). The method of Balestra *et al.* (2013) consisted of a multiplex-PCR able to differentially detect genetically diverse Psa populations from different geographic origins. Biondi *et al*. (2013) developed a nested PCR/RFLP assay capable of specifically detecting virulent Psa strains using a nested PCR followed by *BclI* restriction analysis that digest Psa strains but not the aspecific amplicon of Pto and Psth strains. Gallelli *et al*. (2014) developed specific detection of virulent Psa, either using a new conventional PCR or a real time-PCR method.

Further research is required to compare these three new detection methods and with the previously used assays, in order to accurately assess their respective performance. Only through a validation procedure will it be possible to highlight the advantages and disadvantages of each method in order conclude which is the most appropriate for practical use. Moreover, due to the fact that assessing the complete range of non-target organisms is not possible, this validation confirmation will help to minimize the risk of false positive and false negative results.

It is important to emphasize that if PCR methods are useful to obtain rapid results, they do not allow verification of the viability of bacterial cells. Viability assessments must include the isolation or at least a BIO-PCR step. The inclusion of an enrichment step before performing the molecular assay has been used to screen symptomless kiwifruit material and pollen (Gallelli *et al*., 2011b; Minardi *et al*., 2011; Vanneste *et al*., 2011). Moreover, for valid Psa diagnosis, pathogenicity tests are essential to determine the ability of isolates to induce disease symptoms in host plants. Further studies are currently underway to improve the Psa detection from symptomless kiwifruit material in order to support kiwifruit production industries and to improve the sanitary status of plant propagation.

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