Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

TaqMan qPCR assays improve *Pseudomonas syringae* pv. actinidiae biovar 3 and *P. viridiflava* (PG07) detection within the *Pseudomonas* sp. community of kiwifruit

Sara CAMPIGLI[1,*], Simone LUTI[2], Tommaso MARTELLINI[1], Domenico RIZZO[3], Linda BARTOLINI[3], Claudio CARRAI[3], Jeyaseelan BASKARATHEVAN[4], Luisa GHELARDINI[1], Francesca PEDUTO HAND[5], Guido MARCHI[1]

[1] *Department of Agriculture, Food, Environment and Forestry, University of Florence, P.le delle Cascine 18, Florence, 50144, Italy*
[2] *Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Morgagni 50, Florence, 50134, Italy*
[3] *Regione Toscana, Servizio Fitosanitario Regionale e di Vigilanza e Controllo Agroforestale, Via A. Manzoni 16, Florence, 50121, Italy*
[4] *Plant Health & Environment Laboratory, Diagnostic and Surveillance Services, Biosecurity New Zealand, Ministry for Primary Industries, PO Box 2095, Auckland, 1140, New Zealand*
[5] *Department of Plant Pathology, The Ohio State University, Columbus, OH 43220, U.S.A.*
*Corresponding author. E-mail: sara.campigli@unifi.it

Summary. Kiwifruit is inhabited by a heterogeneous community of bacteria belonging to the *Pseudomonas syringae* species complex (Pssc). Only a few of its members, such as the specialist *Pseudomonas syringae* pv. actinidiae biovar 3 (Psa3), are known as pathogens, but for most of the species, such as *P. viridiflava* (Pv), a generalist with high intraspecific variation, the nature of their relationship with kiwifruit is unclear. Currently, no culture independent molecular diagnostic assay is available for Pv. In this study we validated two TaqMan qPCR diagnostic assays adopting a strategy that for the first time widely focuses on the *Pseudomonas* sp. community associated to kiwifruit in Tuscany (Italy). Primers and probes were designed based on the sequence of the *lscy* gene of Psa3 (qPCRPsa3) and the *rpoD* gene of Pv phylogroup 7 (qPCRPv7). Both qPCR assays have a LOD of 60 fg of DNA. By using reference strains along with 240 strains isolated from kiwifruit and characterized *ad hoc* as *Pseudomonas* sp., specificity was proven for members of six of the 13 Pssc phylogroups. Moreover, to evaluate the possible effects of seasonal variations in the *Pseudomonas* sp. community composition on assay specificity, the assays were tested on naturally infected leaves and canes sampled from different orchards throughout a growing season. At last, by proving qPCR's capacity to detect latent infections in artificially inoculated leaves, their potential usefulness in surveillance programs and for epidemiological studies was verified.

Keywords: *Actinidia* sp., orchard variability, *Pseudomonas syringae* species complex, specificity.

OPEN ACCESS

Citation: S. Campigli, S. Luti, T. Martellini, D. Rizzo, L. Bartolini, C. Carrai, J. Baskarathevan, L. Ghelardini, F. Peduto Hand, G. Marchi (2023) TaqMan qPCR assays improve *Pseudomonas syringae* pv. actinidiae biovar 3 and *P. viridiflava* (PG07) detection within the *Pseudomonas* sp. community of kiwifruit. *Phytopathologia Mediterranea* 62(1): 95-114. doi: 10.36253/phyto-14400

Accepted: April 20, 2023

Published: May 8, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Roberto Buonaurio, University of Perugia, Italy.

ORCID:
SC: 0000-0001-7231-535X
SL: 0000-0001-7050-6590
DR: 0000-0003-2834-4684
JB: 0000-0002-5389-2570
LG: 0000-0002-3180-4226
FPH: 0000-0002-5553-0370
GM: 0000-0002-2937-3500

INTRODUCTION

It has long been assumed that the bacterium *Pseudomonas syringae* (Ps) has evolved in association with plants, on which it forms populations dominated by a few pathogenic strains with narrow host ranges (Dye *et al*., 1980; Hirano and Upper, 2000; Morris *et al*., 2010). In the last two decades however, compelling evidence was gained that members of what is currently termed as the *Pseudomonas syringae* species complex (Pssc, Gardan *et al*., 1999), are ubiquitous bacteria, widespread in all continents both in agricultural and non-agricultural settings, which may live in association with substrates other than plants and take advantage of the water cycle for dissemination (Morris *et al*., 2007; 2008; 2010; Berge *et al*., 2014). Primary studies aimed at establishing the phylogenetic structure of the Pssc, used a MLST (Multi Locus Sequence Typing) approach based on seven core genome genes. This allowed delineation of four main phylogroups (PGs). More recently, the existence of seven and then 13 PGs was proposed based on the polymorphisms observed in the sole *rpoD* and *cts* gene sequences, respectively (Sarkar and Guttman, 2004; Parkinson *et al.,* 2011; Berge *et al.,* 2014; Baltrus *et al*., 2017). However, the relationship between these phylogenetic patterns and the traits required to occupy different ecological niches are still unknown. Many members of the Pssc isolated from water or healthy plant tissues have typical plant pathogenic traits, such as a canonical Type Three Secretion System (TTSS), ability to induce Hypersensitive Response (HR) on tobacco, to produce phytotoxins and effectors, and, most of all, to cause disease in many different hosts at least under experimental conditions (Morris *et al*., 2019). At the same time, strains that cluster in different PGs and cause disease to the same host plants are known to exist (Morris *et al*., 2007; Mohr *et al*., 2008; Bartoli *et al*, 2014; Dillon *et al*., 2019).

The first reports of damage to kiwifruit (*Actinidia* spp.) plants caused by members of the Pssc were recorded in New Zealand (Wilkie *et al*., 1973) followed by Japan, Korea, Italy, and China between the mid 1980s and early 1990s. To date, reports have concerned Ps (unknown PG), Ps pv. syringae (Psyr; PG02), *P. savastanoi* (Psav; PG03), Ps pv. actinidifoliorum (Pfm, PG01), Ps pv. actinidiae (Psa, PG01/b) and *P. viridiflava* (Pv). Pssc strains belonging to either PG7 or PG08 are currently, referred to as *P. viridiflava* nomenspecies (Bull and Koike, 2015). While Ps, Psyr, Pv, Psav, and Pfm have been mostly, but not exclusively, reported as leaf spot and bud rot agents in kiwifruit orchards, Psa also causes shoot dieback and cane and trunk cankers (Vanneste, 2017). Sufficient variability was found in Psa to justify its further differentiation into five biovars, also distinguishable based on virulence traits. A specific lineage of biovar-3 (Psa3) is currently considered the most aggressive and responsible for the bacterial canker pandemic that is threatening kiwifruit production in all countries where this host is grown (McCann *et al*., 2017; Vanneste, 2017; Sawada and Fujikawa, 2019). Recent studies, however, have pointed out that the phylloplane of kiwifruit hosts a much more variegated Pssc community than previously thought (Bartoli *et al*., 2015; Straub *et al*., 2018). In southeastern France, PG01 and PG13 phylotypes are the most widespread, but PG02, PG03, PG07, PG08, PG09, and PG10 also occur, although at much lower frequencies (Borschinger *et al*., 2016). In just two New Zealand orchards, Straub *et al*. (2018) reported that kiwifruit leaves were commonly inhabited by multiple phylotypes of PG01, PG02, and PG03, the latter being by far the most abundant, but rarely inhabitated by PG05 and never by PG07. It was also observed that genetic diversity of the Pssc community was strongly affected by the presence or absence of disease, host genotype (cultivar), as well as environmental factors, including humidity, nutrient availability, UV radiation and orchard management practices (Straub *et al*., 2018).

Given the importance of kiwifruit production worldwide (approx. 4.4M tons produced in 2020; FAOSTAT, 2020), great emphasis has been given to understanding the life cycle of Psa on this host, and to developing reliable and selective assays for rapid routine diagnoses (Vanneste, 2017; Donati *et al*., 2020). Most assays are PCR based (simplex, nested, multiplex, and Real-Time) and their pros and cons have been assessed through an interlaboratory comparison (Loreti *et al*., 2018). Among major findings were: i) isolation on semi-selective media followed by bacterial characterization, although having limited sensitivity, had very reliable specificity compared to several PCR methods; and ii) high risks of false positives or inconclusive PCR results came from testing bacterial cultures of phylogenetically closely related *Pseudomonas* sp. or kiwifruit-associated bacteria. In view of these findings, the EPPO international standard recommends that both PCR and isolation are performed for "critical" symptomatic samples and for all asymptomatic samples (EPPO PM 7/120 [2], 2021). To date, no culture independent molecular diagnostic assay is available for Pv.

The present study outlines two novel TaqMan Real-Time PCR assays (qPCR) for specific detection of Psa3 and Pv belonging to PG07 (Pv7) on kiwifruit, and the strategy implemented to verify their specificity against the *Pseudomonas* sp. community that was associated with kiwifruit during a growing season in Tuscany, Italy.

MATERIAL AND METHODS

Bacterial strains

Fifty-seven Pssc reference strains and 21 plant associated bacteria from national and international collections, were used for initial testing of qPCR assays' specificity (Table 1). The phylogenetic position of reference strains within the Pssc complex was determined based on partial *cts* (citrate synthase) and *rpoD* (RNA polymerase sigma factor) genes sequence analysis according to Berge *et al.* (2014) and Parkinson *et al.* (2011), respectively.

All strains were grown on Nutrient Agar (NA) at 27°C and were preserved in 30% glycerol at -80°C.

Plant material and bacteria isolation procedures

To verify the specificity of the qPCR assays against the cultivable *Pseudomonas* sp. kiwifruit-associated microflora, bacteria were isolated from *A. chinensis* var. *deliciosa* cv. Hayward (AcdH) plants from Tuscany (Italy) from 2014 to 2020. With the exception of 2016, when plant samples were collected from one orchard in the province of Lucca and one in Pistoia, all other tested samples were collected from six orchards in Lutirano (province of Florence), the only kiwifruit growing area of Tuscany where the presence of Psa3 has been officially confirmed (DDR n.512/2013). All orchards were planted in the 1990s, except for orchard No. 6 in Lutirano, which was 3 years old in 2018 (Supplementary Figure S1). Most isolates were obtained from fully expanded kiwifruit leaf blades, but isolations from host sap (2017) and canes showing dieback symptoms (2014 and 2018) were also carried out (Figure 1). In 2016 and 2020, bacteria were also isolated from leaves collected from nursery potted plants.

All samples were transported to the laboratory on ice and were processed within 24 h. For bacterial isolation from leaves, approx. 1 g of fully expanded leaf blade was crushed in a universal extraction bag (480100, BIOREBA, Reinach, Switzerland) using a hammer, and was then homogenized under a laminar flow hood by adding 7 mL of sterile saline solution (0.85% NaCl) and incubated for 5 min at room temperature. Twenty cm dieback cane sections were thoroughly washed under tap water and air dried. After soaking in 70% ethanol for 5 min, segments were each decorticated, superficially disinfested by wiping with a sterilized paper towel soaked with 50% ethanol, and then air dried inside a laminar flow hood. After removing 5 cm of tissue from each end using flame disinfested shears, approx. 0.5 g of wood chips were aseptically excised with a sterile scalpel and were then macerated in 7 mL of saline solution for 1 h with gentle shaking at room temperature. Five mL of xylem sap were collected according to Biondi *et al.* (2013), diluted 1:2 (v:v) with saline solution and thoroughly vortexed. Isolations from leaf and cane macerates or from sap were carried out by dilution-plating on modified King's B semi-selective agar medium (KBCA), as outlined by the EPPO standard for Psa3 (King *et al.*, 1954; Mohan and Schaad, 1987; EPPO PM7\120[2], 2021). For bacterial isolation from artificially inoculated nursery potted plants, Nutrient Sucrose Agar supplemented with 5% sucrose (NSA) amended with 60 mg L^{-1} cycloheximide was also used (Figure 1). All isolation plates were incubated for 96 h at 27°C, checked daily, and the most represented bacterial morphotypes were selected and streaked twice on NSA to ensure purity. All isolates were stored in 30% glycerol at -80°C until further use.

Nucleic acid extraction procedures from plant tissues and purified bacteria

Leaf tissues

Total nucleic acids were extracted from naturally and artificially infected as well as non-infected kiwifruit (AcdH) and tobacco (*Nicotiana tabacum* cv. Virginia Bright) leaves, or from a cell suspension of the culturable microbial community growing on NSA plates (Figure 1), using CTAB buffer [100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2.5% (w:v) CTAB, 2.5% (w:v) polyvinylpyrrolidone (PVP-40)]. For non-infected or naturally infected leaves, approx. 1 g of each leaf blade was crushed using a hammer, in a universal extraction bag. Seven mL of CTAB buffer were directly added to the bag and, after 5 min incubation, 500 µL were transferred to a 1.5 mL capacity microcentrifuge tube. For artificially inoculated leaves, 1 mL of leaves macerates (1 g) in 7 mL of saline solution, or of a cell suspension of the culturable microbial community growing on NSA plates obtained by washing plates with 3 mL of the same solution, were centrifuged for 5 min at 17089 × *g* in a 1.5 mL microcentrifuge tubes. After removing the supernatant, the resulting pellet was re-suspended in 500 µL of CTAB buffer. In all cases, after incubating for 30 min at 65°C, purification was carried out according to Angelini *et al.* (2001). Nucleic acids were re-suspended in 100 µL of Tris HCl (10mM, pH 8) and stored at -20°C until further use.

Cane tissues

Total nucleic acids were extracted from non-infected or naturally infected kiwifruit canes (Figure 1), or from

Table 1. Bacterial reference strains used in qPCR assays deployed in this study, along with their geographic origins and hosts of isolation as indicated by the collection curators. For strains that were included in phylogenetic analyses, their classification phylogroups (PG) and subclades are also indicated.

Bacterial species[a]	Strain[b]	Host plant/matrix	Geographic origin	Year of isolation	Phylogroup/subclade	
					cts[c]	*rpoD*[d]
Agrobacterium radiobacter	C58	*Prunus avium*	USA	1958	-[e]	-
Allorhizobium vitis	ICMP 11960	*Vitis vinifera*	France	1985	-	-
A. vitis	CG 628	*V. vinifera*	USA	1983	-	-
A. vitis	CG 634	*V. vinifera*	USA	1983	-	-
Pantoea agglomerans	PVFi FL1	*Olea europaea*	Italy	2002	-	-
P. agglomerans	NCPPB 653	*Pyrus communis*	United Kingdom	1958	-	-
P. agglomerans	NCCPB 656	*Malus sylvestris*	United Kingdom	1959	-	-
P. agglomerans pv. gypsophilae	824/1	*Gypsophila paniculata*	Israel	1991	-	-
Pectobacterium carotovorum	PVFi Pcc7	*Zantedeschia aethiopica*	Italy	2012	-	-
P. carotovorum	PVFi PCC23	*Z. aethiopica*	Italy	2013	-	-
Pseudomonas amygdali	NCPPB 2607	*Prunus dulcis*	Greece	1967	-	-
P.amygdali	NCPPB 2608	*P. dulcis*	Greece	1967	-	-
P.amygdali	NCPPB 2610	*P. dulcis*	Greece	1967	-	-
P. cichorii	ICMP 5707	*Cichorium endivia*	Germany	1929	-	-
P. corrugata	C2P1	*Chrysanthemum morifolium*	Italy	1990	-	-
P. mediterranea	C5P1-rad1	*C. morifolium*	Italy	1990	-	-
P. savastanoi pv. nerii	ITM 510	*Nerium oleander*	Italy	1983	-	-
P. savastanoi pv. phaseolicola	CFBP 1390	*Phaseolus vulgaris*	Canada	1949	-	-
P. savastanoi pv. phaseolicola	NCPPB 2571	*P. vulgaris*	United Kingdom	1974	-	-
P. savastanoi pv. phaseolicola	RW60	*Lablab purpureus*	Ethiopia	1985	-	-
P. savastanoi pv. savastanoi	PVFi MLLI2	*O. europaea*	Italy	2001	-	-
P. savastanoi pv. savastanoi	PVBa 229	*O. europaea*	Italy	1968	-	-
P. syringae	ICMP 11292	*Actinidia deliciosa*	New Zealand	1991	PG01/a	PG01
P. syringae	CFBP 8517	Lake water	France	2006	PG09/a	und[f]
P. syringae	CFBP 8514	Stream water	France	2007	PG09/b	und
P. syringae	CFBP 8512	River water	France	2011	PG09/c	und
P. syringae pv. aceris	CFBP 2339	*Acer* sp.	USA	1970	-	-
P. syringae pv. actinidiae- Biovar 1	ICMP 9617	*A. deliciosa* cv. Hayward	Japan	1984	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 1	ICMP 9853	*A. deliciosa* cv. Hayward	Japan	1984	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 1	ICMP 19069	*A. deliciosa*	Japan	1984	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 2	ICMP 19072	*A. chinensis*	Korea	1997	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	ICMP 18708	*A. chinensis* var. chinensis	New Zealand	2010	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	ICMP 18884	*A. deliciosa*	New Zealand	2010	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	ICMP 19076	*A. deliciosa*	New Zealand	2011	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	CFBP 7286	*A. chinensis* cv. HORT16A	Italy	2008	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	CFBP 7906	*A. deliciosa* cv. Summer	France	2011	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 3	CFBP 8302	*A. deliciosa*	Chile	2011	PG01/b	PG01
P. syringae pv. actinidiae- Biovar 5	CFBP 8414	*A. chinensis*	Japan	2012	PG01/b	PG01
P. syringae pv. actinidifoliorum	ICMP 18804	*A. chinensis*	New Zealand	2010	PG01/a	PG01
P. syringae pv. maculicula	NCPPB 1777	*Brassica oleracea*	United Kingdom	1965	-	-
P. syringae pv. miricae	CFBP 2897	*Myrica rubra*	Japan	1978	-	-
P. syringae pv. miricae	MAFF 302457	*M. rubra*	Japan	1984	-	-
P. syringae pv. photinae	CFBP 2899	*Photinia glabra*	Japan	1976	-	-
P. syringae pv. primulae	CFBP 1660	*Primula* sp.	USA	1939	PG07/a	PG07
P. syringae pv. ribicola	CFBP 2348	*Ribes aureum*	unknown	1946	PG07/a	PG07

(Continued)

Table 1. (Continued).

Bacterial species[a]	Strain[b]	Host plant/matrix	Geographic origin	Year of isolation	Phylogroup/subclade	
					cts[c]	*rpoD*[d]
P. syringae pv. tabaci	GSPB 1209	*Nicotiana tabacum*	Germany	unknown	-	-
P. syringae pv. theae	CFBP 2353	*Thea sinensis*	Japan	1970	PG01/b	PG01
P. syringae pv. tomato	IPV-BO 1544	*Solanum lycopersicum*	Italy	1989	-	-
P. syringae pv. viburni	CFBP 1702	*Viburnum* sp.	USA	1931	PG01/b	PG01
P. viridiflava	ICMP 9274	*A. deliciosa* cv. Hayward	New Zealand	1985	PG07/a	PG07
P. viridiflava	ICMP 11289	*A. deliciosa* cv. Hayward	New Zealand	1991	PG07/a	PG07
P. viridiflava	ICMP 13105	*A. deliciosa*	France	1985	PG02/d	PG02
P. viridiflava	ICMP 13110	*A. deliciosa*	France	1985	PG02/d	PG02
P. viridiflava	ICMP 13302	*A. chinensis* cv. Earligold	New Zealand	1996	und	PG03
P. viridiflava	ICMP 13303	*A. chinensis* cv. Earligold	New Zealand	1996	PG03	PG03
P. viridiflava	CFBP 8506	Stream water	France	2007	PG07/a	PG07
P. viridiflava	CFBP 8511	Channel water	France	2011	PG08	und
P. viridiflava	CFBP 1590	*Prunus cerasus*	France	1974	PG07/a	PG07
P. viridiflava	CFBP 2107	*Phaseolus* sp.	Switzerland	1927	PG07/a	PG07
P. viridiflava	CFBP 8559	Biofilm on stone	France	2006	PG07/a	PG07
P. viridiflava	CFBP 8508	*A. deliciosa*	Italy	2012	PG07/a	PG07
P. viridiflava	CFBP 8509	*Primula* sp.	France	2007	PG07/a	PG07
P. viridiflava	CFBP 6890	*Raphanus sativus*	France	2004	PG07/a	PG07
P. viridiflava	CFBP 4476	*A. deliciosa*	New Zealand	1984	PG02	PG02
P. viridiflava	UCR-1	*Vitis* sp.	USA	2002	PG07/a	PG07
P. viridiflava	TOMA 3-02	*S. lycopersicum*	USA	2002	PG01/a	PG01
P. viridiflava	TOME 9-02	*S. lycopersicum*	USA	2002	PG01/a	PG01
P. viridiflava	TOMP 2-02	*S. lycopersicum*	USA	2002	PG07/b	und
P. viridiflava	TOMU 4-02	*S. lycopersicum*	USA	2002	PG01/a	PG01
P. viridiflava	OrSU-MM	unknown	USA	unknown	PG03	PG03
Xanthomonas arboricola pv. pruni	PVFi KVPT2A	*A. deliciosa*	Italy	2016	-	-
X. arboricola pv. pruni	PVFi L1	*Prunus laurocerasus*	Italy	2010	-	-
X. citri pv. citri	CFBP 3369	*Citrus aurantifolia*	USA	1989	-	-
X. euvesicatoria	PVFi Xe1	*Capsicum annum*	Italy	2017	-	-
X. euvesicatoria	PVFi 49	unknown	unknown	unknown	-	-
X. phaseoli	CFBP 8462	*P. vulgaris*	USA	unknown	-	-
Xylella fastidiosa subsp. *multiplex*	PVFi Ma29	*P. dulcis*	Italy	2019	-	-
X. fastidiosa subsp. *pauca*	Salento2	*O. europaea*	Italy	2015	-	-

[a] Bacterial nomenclature is according to the Comprehensive List of Names of Plant Pathogenic Bacteria, 1980-2007 published by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (https://www.isppweb.org/about_tppb_names.asp).

[b] ICMP: International Collection of Micro-organisms from Plants, Auckland, New Zealand.
CFBP: Collection Francaise de Bacteries Phytopathogenes, Angers, France.
NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom.
IPV-BO: Culture Collection of Istituto di Patologia Vegetale, Università di Bologna, Italy.
ITM: Culture collection of Istituto Tossine e Micotossine da Parassiti vegetali, C.N.R., Bari, Italy.
PVBa: Culture collection of Dipartimento di Patologia vegetale, Università degli Studi, Bari, Italy.
PVFi: Culture collection of Dipartimento di Biotecnologie Agrarie-Patologia vegetale, Università degli Studi, Firenze, Italy.
GSPB : Göttinger SammLung Phytopathogener Bakterien, University of Göttingen, Germany.
MAFF: Genetic Resources Center, National Agriculture and Food Research Organization (NARO), Japan.
The whole genomic DNA of *Xylella fastidiosa* subsp. *pauca* Salento2 was provided by Dr. Gianluca Bleve.

[c] According to Berge *et al.*, 2014.

[d] According to Parkinson *et al.*, 2011.

[e] Not tested.

[f] Undetermined.

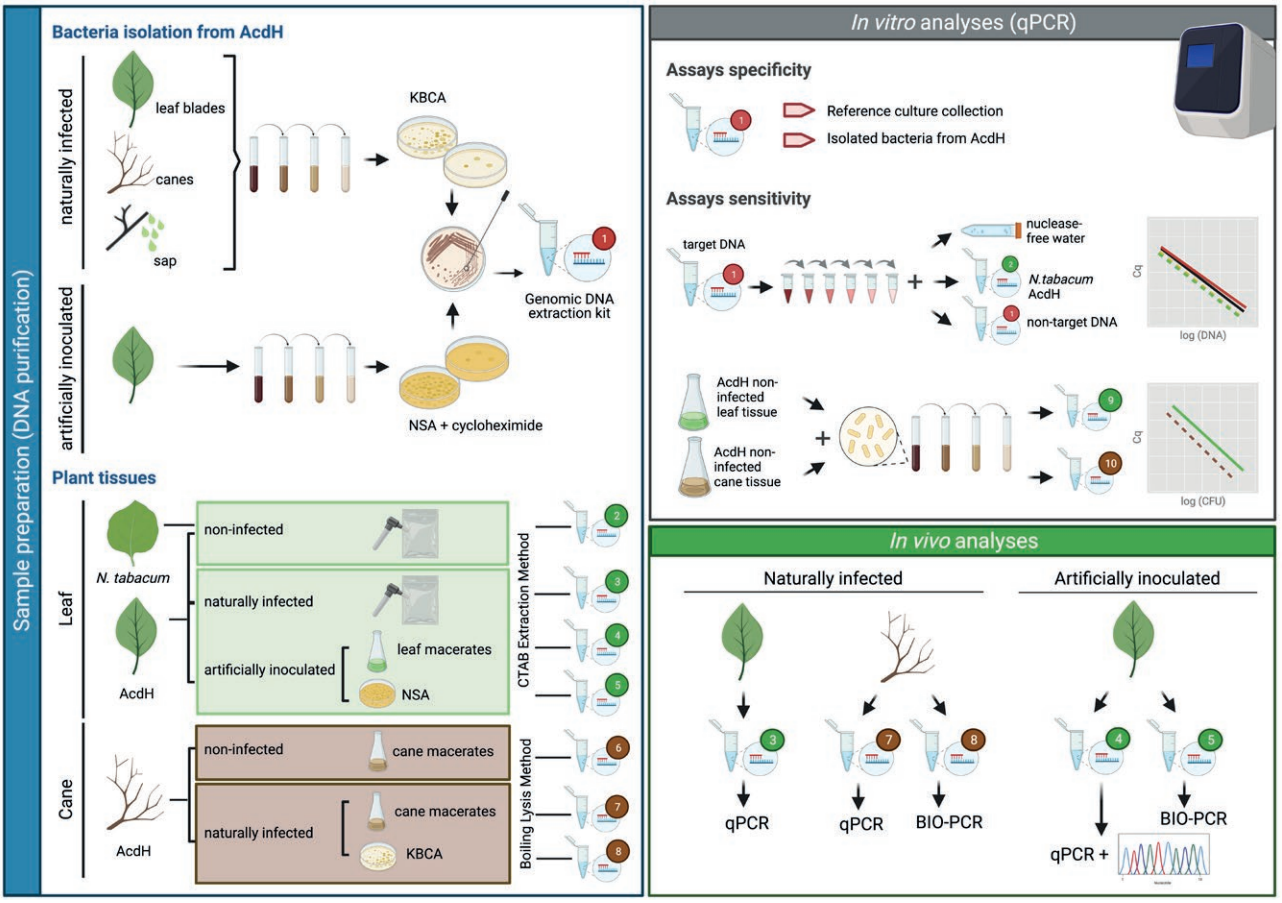

Figure 1. Summary of DNA extraction procedures and *in vitro* and *in vivo* analyses carried out to validate the qPCR assays developed in this study. Sample preparation (DNA purification): PCR templates included total nucleic acids extracted from (1) purified bacteria isolated from different *Actinidia chinesis* var. *deliciosa* cv. Hayward (AcdH) tissues and reference strains from national and international culture collections; (2) non-infected tobacco and kiwifruit leaves; (3) naturally infected or (4) artificially inoculated kiwifruit leaves; (5) culturable bacterial community from artificially inoculated kiwifruit leaves growing on NSA plates; (6) non-infected or (7) naturally infected kiwifruit canes; and (8) culturable bacterial community on KBCA from naturally infected kiwifruit canes. *In vitro* analyses (qPCR): Assays specificity and sensitivity (qPCRPsa3 and qPCRPv) were assessed using DNA extracted from reference bacterial strains and purified isolates from AcdH (1). Assays sensitivity was assessed using serial dilutions of target genomic DNA (1) in either nuclease-free water, plant DNA extracted from non-infected tobacco or kiwifruit leaf tissues (2), or DNA extracted from non-target bacteria (1). Additionally, non-infected kiwifruit leaf or cane tissues were spiked with 10-fold serial dilutions of Psa3KL103 and PvKL5 cells prior to DNA extraction using CTAB (9, leaf) or boiling lysis (10, cane) methods. *In vivo* analyses: Assays specificity (qPCRPsa3 and qPCRPv) was evaluated on total nucleic acids extracted from kiwifruit leaf (3, 4) or cane (7) tissues. BIO-PCR assays (BIO-PCRPsa3 and BIO-PCRPv) were used on the total nucleic acids extracted from the culturable fraction of the microorganisms that were infecting kiwifruit canes or artificially inoculated leaves and that were able to grow on KBCA (8) or NSA (5) plates. To verify the *in vivo* specificity of the two qPCRs, a selection of the amplicons produced from nucleic acids extracted from artificially inoculated leaves (4) was Sanger sequenced. Image created with BioRender.com.

the microbial community growing on KBCA plates, following the boiling lysis procedure (Moore *et al.*, 2004; Tyson *et al.*, 2012) with some modifications. Briefly, 1.5 mL of the suspension obtained by macerating 0.5 g of wood chips in 7 mL of saline for 1 h or of KBCA plates washings with 3 mL of the same solution, were centrifuged (as above) in 2 mL microcentrifuge tubes. The supernatant was removed, and the resulting pellet resuspended in 70 µL of 1 mM EDTA, boiled for 5 min, cooled at room temperature on ice and immediately used in PCR experiments.

Bacterial strains and isolates

Total nucleic acids were extracted from 1 mL of Nutrient Broth cultures of reference bacterial strains or purified isolates grown for 48 h at 27°C, using the Bac-

terial Genomic DNA Isolation Kit (Norgen Biotek Corporation, Thorold, Canada) according to manufacturer's instructions (Figure 1).

Bacterial isolates characterization

Identification and phylogenetic affiliation of all bacterial isolates obtained from kiwifruit between 2014 and 2020 was tentatively determined based on partial *cts* gene sequence analysis according to Berge *et al.* (2014) (Supplementary Table S1). PCR reagents' mix composition was as described herein: 1 × PCR Green Buffer (Thermo Scientific, Waltham, MA, USA), 0.2 mM of each dNTP (Thermo Scientific), 1.25 U of DreamTaq (Thermo Scientific), 0.2 µM of each primer (Table 2) and approx. 5 ng of genomic DNA. PCR reactions were carried out in a T professional trio thermocycler (Biometra, Göttingen, Germany). Thermal cycling consisted of 3 min at 95°C for initial denaturation, 35 cycles of denaturation at 95°C for 30 s, annealing at 57 °C for 30 s, extension at 72°C for 30 s, followed by a final extension of 10 min at 72°C. All PCR products were visualized after electrophoresis in 1.5 % agarose gels (Genaxxon bioscience GmbH, Ulm, Germany) in 1 × Tris–acetate–EDTA buffer (Invitrogen, Waltham, MA, USA) and staining with Midori Green (0.06 µL mL^{-1}; Nippon Genetics Europe GmbH, Düren, Germany). Amplicons were purified using FastAP and Exonuclease I (Thermo Scientific), and Sanger sequenced using primer cts-Rp (Table 2). Nucleotide sequences were visualized and checked for quality using CHROMAS LITE 2.01 (Technelysium, South Brisbane, QLD, Australia), trimmed to 409 bp length and aligned using MUSCLE as implemented in MEGAX (Kumar *et al.*, 2018). Each *cts* sequence that differed from the rest of the sequences by one or more nucleotides was assigned a different allele number. To classify the bacterial isolates according to their phylogroup (PG) of affiliation within the Pssc, a cladogram using the Neighbor Joining (NJ) method and the Maximum Composite Likelihood model of evolution, was constructed using MEGAX. The 64 homologous nucleotide sequences representative of the 13 recognized phylogroups of the Pssc according to Berge *et al.* (2014) and those of 41 Pssc strains used in this study were included in the analysis for comparative purposes.

To confirm identifications based on *cts* sequence analysis, bacteria ascribed to Pssc PG01/b and PG07 were subjected to further molecular analyses (Table 2). The DNA of all isolates allocated within PG01/b, was tested using the Psa specific protocol developed by Gallelli *et al.* (2011), and by analyzing the sequence of a fragment of the *pfk* gene reported as useful to discriminate between biovars 1, 2 and 3 of Psa, and Pfm (Chapman *et al.*, 2012). Bacteria ascribed to PG07 were investigated using the

Table 2. List of PCR primers and probes used or developed in this study, along with their nucleotide sequences.

Primers/Probe	Sequence (5'-3')	Genomic target	Specificity	Expected product size (bp)	Reference
Plasm L1	TCGGCGCCATTTCGATTG	*lscγ* (levansucrase γ)	Psa3	231	Luti *et al.*, 2021
Plasm R1	ATAACCGACCAGCCGTTGA				
Plasm P1	FAM-GCCGCTACATCTAGCAGGTA-TAMRA				
Pvir 1L	GAAGAATCGGCAGACGCTTC	*rpoD* (RNA polymerase sigma 70 factor)	Pv7	81	this study
Pvir 4RD	GCCGAAGCGCTGTGCA				
Pvir 2P	FAM-AGAAGACGAAGTCGAAAGCG-TAMRA				
cts-Fp	AGTTGATCATCGAGGGCGCWGCC	*cts* (citrate synthase)	*Pseudomonas* sp.	480	Sarkar and Guttman, 2004
cts-Rp	TGATCGGTTTGATCTCGCACGG				
PsrpoD FNP1	TGAAGGCGARATCGAAATCGCCAA	*rpoD* (RNA polymerase sigma 70 factor)	*Pseudomonas* sp.	700	Parkinson *et al.*, 2011
PsrpoDnprpcr1	YGCMGWCAGCTTYTGCTGGCA				
pfk-Fp	ACCMTGAACCCKGCGCTGGA	*pfk* (phosphofructokinase)	*P. syringae*	850	Sarkar and Guttman, 2004
pfk-Rp	ATRCCGAAVCCGAHCTGGGT				
KN-F	CACGATACATGGGCTTATGC	*omp*P1 (outer membrane protein P1)	Psa	492	Koh and Nou, 2002
KN-R	CTTTTCATCCACACACTCCG				
AvrDdpx-F	TTTCGGTGGTAACGTTGGCA	*avrD1* (avirulence gene D1)	Psa	226	Gallelli *et al.*, 2011
AvrDdpx-R	TTCCGCTAGGTGAAAAATGGG				
ORF1/2-Fw	CGACCTGCTTTCGATCA	T-PAI	Pssc PG07/08	900	Bartoli *et al.*, 2014
ORF1/2-Rv	TCAATACTCTGGAGATCAG				

rpoD based phylogenetic scheme developed by Parkinson *et al.* (2011) and by sequencing a fragment of a Pv7 characteristic pathogenicity island (T-PAI) described by Araki *et al.* (2006) and Bartoli *et al.* (2014). At last, isolates that according to molecular analyses could be identified as Psa3 and Pv7 were further characterized using the LOPAT scheme (Lelliott and Stead, 1987) and additional biochemical tests according to Berge *et al.* (2014) and Schaad *et al.,* (2001): aesculin degradation, acidification of sucrose, and utilization of D(-) tartrate, mannitol or glucose as sole carbon sources.

The gene sequences analyzed in this study are available in GenBank under the following accession numbers: MW701434 to MW701442, MW716007-15, MW716028-39, MW716040, MW727267, MW814986-92, MW826360 and MW826361.

Primers and TaqMan probes design, PCR approaches and amplification conditions

Primers and probe for Psa3 were previously used by Luti *et al.*, (2021) to study *in vitro* expression of a functional levansucrase coding gene, *lscy*, is located on a non-self-transmissible low copy plasmid (NZ_CP012180.1) in the Psa3 ICMP 18708 genome. In the present study, the identification of a specific genome sequence used to design a PCR assay for Pv7, was obtained by a close evaluation of nucleotide variability existing among Pssc members in the *rpoD* housekeeping gene (HK) (Table 2 and Supplementary Figure S2a and b). All primers and probes were synthetized by Eurofins GmbH. Probes were labeled with 6-carboxyfluorescein (FAM) as the fluorescent reporter dye and 5-Carboxytetramethylrhodamine (TAMRA) as the quencher dye.

Primers/probe sets were used in two types of TaqMan based PCR approaches: qPCR and BIO-PCR (i.e., "biological amplification of PCR targets prior to their enzymatic amplification"; Schaad *et al.*, 1999). TaqMan qPCR was used to index for Psa3 (qPCRPsa3) and Pv7 members (qPCRPv7) in different AcdH tissues. The assays were run from total nucleic acids extracted from kiwifruit leaf and cane tissues, and total genomic DNA from bacterial reference strains and purified isolates from this study (Figure 1). TaqMan BIO-PCR was used to verify the presence living cells of Psa3 (BIO-PCRPsa3) and Pv7 (BIO-PCRPv7) within the culturable bacteria growing on isolation plates. The assays were run on total nucleic acids extracted from the cultivable fraction of the microorganisms that were infecting/contaminating canes or artificially inoculated leaves and that were able to grow on KBCA (canes) or NSA (leaves) plates within 96 h at 27°C.

qPCRs and BIO-PCRs reactions each contained 2 × GoTaq® Probe qPCR Master Mix (Promega Corporation, Madison, Wisconsin, USA), 0.5 µM of each primer and 0.3 µM of probe. One µL of total nucleic acids from isolation plates (BIO-PCR) or of bacterial genomic DNA, or 3 µL of total nucleic acids extracted from kiwifruit leaves or canes washings, were used as template, respectively. Nuclease-free water was added to a final volume of 15 µL. All reactions were carried out in the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA).

The thermal cycling conditions included a denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 62°C for 1 min. Detection and quantification of fluorescence were read after every cycle and data were assembled using the CFX Manager Software (Bio-Rad Laboratories). In all PCR experiments, a positive control (Psa3KL103 or KL318; Pv7aKL5 or KL317) and three non-template controls (NTC) were included to test PCR performance: water, nucleic acids extracted from non-infected plant tissues (leaf or cane), and a non-target bacterial strain (Psa3 or Pv7a). Signal threshold levels were set automatically by the system.

In vitro *determination of qPCR assays specificity and sensitivity*

Specificity of the qPCR assays was assessed using reference bacterial strains and purified isolates listed in Table 1 and Supplementary Table S1.

DNA concentration was measured using a picodrop (Picodrop Ltd, Cambridge, England). A serial dilution of each Pv7aKL5 and Psa3KL103 genomic nucleic acids ranging from 60 ng to 6 fg μL^{-1} was used to evaluate the sensitivity and linearity of the qPCR assays in: i) nuclease-free water, ii) 4 ng of DNA extracted from strains Pv7aKL5 (qPCRPsa3) or Psa3KL103 (qPCRPv7), or iii) 4 ng of DNA extracted from non-infected AcdH (host) or tobacco (non-host) leaves (qPCRPsa3 and qPCRPv7) (Figure 1). Each dilution series always included plant, bacterial DNA, and no-DNA negative controls. Additionally, 10-fold serial dilutions of Psa3KL103 and Pv7aKL5 cells, ranging from 10^8 to 10^2 CFU mL^{-1} of saline (as determined by colony counting on NA plates) were added to AcdH leaf or cane tissues prior to DNA extraction. Briefly, 1 mL of each bacterial suspension was added to 1 g of healthy AcdH crushed leaf blade homogenized in 4 mL of saline solution, or to 0.5 g of wood chips in 6 mL saline (Figure 1). Nucleic acids extraction and qPCR testing were performed as previously described.

In all experiments, qPCR reactions were carried out in quadruplicate, and the limit of detection (LOD) for

each substrate was defined as the lowest target amount giving a positive qPCR result in at least three of the four reactions. The mean values of the quantification cycle (Cq) obtained were plotted against the logarithm of each the DNA or CFU concentration in the reactions, to calculate the slopes (k) and intercepts (q) of the standard regression lines using Excel (Microsoft). Relationships between Cq and target copy numbers were evaluated by calculating the coefficient of determination (R^2). The slopes of the standard regression lines were used to calculate amplification efficiency (E) using the equation $E = 10^{-1/slope} - 1$ (Bustin *et al.*, 2009; Bustin and Huggett, 2017). All experiments were conducted at least twice.

In vivo *assessment of specificity and sensitivity of qPCR assays*

Naturally infected leaves and canes

Leaf samples were collected during the 2018 vegetative season from six AcdH orchards in Lutirano, at 21 (May), 57 (June), 92 (July), 162 (September) and 190 (October) days after bud break (dabb), which occurred on 16 April 2018 in orchard No. 1. In each orchard, seven contiguous plants along a row were arbitrarily chosen 2 weeks prior to bud break, and one leaf was collected from each plant at each sampling time. At each sampling, plants were carefully inspected for presence of leaf symptoms (ts) previously described as typical of kiwifruit bacterial canker (Vanneste *et al.*, 2011) or of bacterial blight (Young *et al.*, 1988), i.e., brown angular lesions surrounded by a distinct yellow halo that becomes narrow and faded on aged leaves. If no leaves showing ts could be found, plants were screened again for presence of generic bacterial canker spots (gs) on leaves, i.e., necrotic spots brown to dark brown in color, size varying from pinpoint to large, and elliptical, angular, or irregular shaped without yellow halos (Serizawa *et al.*, 1989; Vanneste *et al.*, 2011). If no symptoms were present, an asymptomatic leaf (al) was collected (Supplementary Figure S3). Total nucleic acids were directly extracted and indexed using qPCRPsa3 and qPCRPv7, as indicated above using two replications. At each sampling time, bacterial isolation was also carried out from one of the seven leaves collected in each orchard, by dilution-plating on KBCA followed by isolate purification and characterization as described above (Figure 1).

Canes showing symptoms of dieback were selected during winter pruning (26 March 2018) in orchard No. 3 (Supplementary Figure S3) and sectioned in 20 cm fragments. Twenty sections were immediately taken to the laboratory while 60 sections were placed in four gauze-enclosed frames (15 sections/frame) and buried 5 cm deep into the ground along four different plant rows as described by Tyson *et al.*, (2012). Forty-two (May), 77 (June) and 117 (July) days after pruning (dap), 20 sections (five sections/frame) were retrieved from the orchard floor. Bacterial isolation on KBCA, total nucleic acids extractions, BIO-PCRs and qPCRs were performed as described above (Figure 1). Two replications per each DNA sample were used.

Chi-square contingency table (2X2) analysis was performed to examine differences in the distribution of Psa3 and Pv7 across the different types of leaf symptoms (ts, gs, al), or for each of the two bacteria, according to the indexing methodology (qPCR vs. dilution plating or qPCR vs. BIO-PCR). Yates correction for continuity was applied (Zar, 1999).

Artificially inoculated leaves

In May 2020 (bud break occurred on 30 March), presence of Psa3 and Pv in the leaves of six, 4-year-old female potted plants of AcdH maintained at the University of Florence was assessed using the two qPCR protocols, and by isolation onto KBCA and NSA amended with 60 mg L^{-1} cycloheximide. In June, strains Pv7aKL317 and Psa3KL318, which had been isolated from the same leaf in 2018 at Lutirano (Supplementary Table S1), were grown for 72 h on NA plates at 27°C, suspended in sterile distilled water to a concentration of 10^8 CFU mL^{-1}, and individually inoculated (on 8 June 2020) onto 40 leaves of each of two plants per bacterial strain. Each leaf was inoculated by rubbing two 10 µL droplets of bacterial suspension on the two opposite sides of the adaxial midrib of a fully expanded leaves using gloved fingers (Renzi *et al.*, 2012). The rubbed area was labeled with a marker, and two control plants were inoculated in a similar manner with sterile distilled water. Each leaf was then enclosed in a plastic bag for 24 h, and all plants were then maintained outdoor and watered daily. The plants were sprayed once a month with boscalid (Cantus®, BASF, Italy) from June to September as a prophylactic treatment for gray mold disease (*Botrytis cinerea*). Four, 11, 18 (June), 42 (July) and 85 (September) days after inoculation (dai), 12 leaves from each treatment were inspected for symptoms (ts, gs) and were then collected. Approx. 1 g of leaf blade tissue including the labeled areas of inoculation, was excised with a sterile scalpel inside a laminar flow hood, and isolation on NSA, total nucleic acids extraction from growth plates and BIO-PCR indexing, were performed as described above. Nucleic acids extraction from the leaves and qPCR indexing were carried out as previous-

ly described (Figure 1). Two replications per each total nucleic acid sample were used. To verify that sequence amplicons of qPCR for Psa3 and Pv7 corresponded to their target, the respective amplification products were purified from a selection of qPCR reactions showing different Cqs, and these were Sanger sequenced in forward and reverse (Figure 1). Sequence identity (%) was evaluated by comparison with the homologous sequences of strains Pv7aKL317 and Psa3KL318.

RESULTS

Phylogeny of the Pseudomonas *sp. community inhabiting AcdH in Tuscany (Italy)*

cts sequence comparison of the 240 isolates recovered from kiwifruit on KBCA between 2014 and 2020 showed the existence of 73 alleles (Supplementary Table 1). Neighbor joining analysis indicated that 45 alleles (169 strains) could be ascribed to the Pssc, and 30 of these could be classified both to PG and subclade level according to Berge *et al*., (2014). These were: PG01 (three alleles in clade b), PG02 (14 alleles of which one was in clade a, nine in clade b, one in clade c and three in clade d), PG07 (four in clade a), PG12 (two alleles in clade b) and PG13 (seven alleles in clade a). For alleles-18 and -19, the subclade of classification within PG02 could not be determined. Neighbor joining analysis indicated that 13 alleles, although ascribable to the Pssc following the procedure of Berge *et al*., (2014), could not be allocated into canonical PGs (Supplementary Table 1). Overall, among the *cts* alleles found within the Pssc associated with kiwifruit in Tuscany, 23 were novel (based on GenBank database searches, https://www.ncbi.nlm.nih.gov/genbank), 18 were found in only one of the Pssc strains that were typed. Allele-1 was the most common, being shared by 24 strains isolated from five of six orchards at Lutirano in different years, seasons, and from different plant tissues. Based on the *Pseudomonas* sp. used as reference in the *cts* identification scheme, 20 of the remaining 28 alleles could be ascribed to this genus (59 strains). The remaining eight alleles (12 strains) were distantly related to the references included in the analyses, although results of identity searches carried out in GenBank indicated closest similarities to *Pseudomonas* (data not shown). Altogether, 24 alleles that we ascribed to *Pseudomonas* sp. were new, 18 were found only in one kiwifruit strain, and one (allele-50), within 14 strains isolated from kiwifruit leaves in different years and orchards, was the most prevalent. Evidence was found showing that members of six of the 13 phylogroups forming the Pssc, were associated to AcdH in Tuscany.

qPCRs specificity testing

Of the 78 reference strains used in this study (Table 1), 57 of which were Pssc, only Psa3 strains (ICMP 18708, ICMP 18884, ICMP 19076, CFBP 7286, CFBP 7906 and CFBP 8302) were successfully amplified with the qPCRPsa3 assay, indicating 100% specificity. When qPCRPv7 was used, only the DNA of the Pv and Ps strains that belong to PG07/a and 07/b according to *cts*, or to PG07 according to *rpoD* analysis results (Table 1), could be amplified. Indeed, no qPCRPv7 signal was obtained from strains ICMP 13105 (PG02), ICMP 13110 (PG02), ICMP 13302 (PG03), ICMP 13303 (PG03), CFBP 4476 (PG02), TOMA3-02 (PG01), TOME9-02 (PG01), TOMU4-02 (PG01) and OrSU-MM (PG03), although all of these are all classified as Pv in their respective collections of origin. Since the DNA of Pv CFBP 8511 (PG08) was also discriminated, the qPCRPv7 assay is considered to be 100% specific for Pv members of the PG07 clade.

To accurately assess specificity against the background *Pseudomonas* sp. community associated with kiwifruit in Tuscany, qPCR assays were used on the DNA extracted from 240 strains isolated from AcdH leaves (143 isolates), sap (23) and canes (74), on KBCA between 2014 and 2020 and on NSA in 2020 (Supplementary Table S1). When tested with the qPCRPsa3 assay, only the DNA from 24 strains isolated from leaves (22) or canes (two) in different years and orchards at Lutirano, could be amplified. For all of these isolates, *cts* and *pfk* gene sequence analyses showed 100% identity with the homologous sequences of the Psa3 pathotype strain (ICMP 9617). Moreover, they all tested positive to the Psa-specific PCR protocol developed by Gallelli *et al.* (2011), their LOPAT profile was Ia, they all weakly hydrolyzed aesculin, produced acid from sucrose, did not produce fluorescence on KB, and grew with mannitol and glucose but not with D(-) tartrate as sole carbon sources (data not shown). Based on these results, analytical specificity of the qPCRPsa3 assay was 100% also when tested against representative isolates of the cultivable fraction of the *Pseudomonas* sp. community associated to kiwifruit in Tuscany.

For the qPCRPv7 assay, only the DNA of nine strains that were isolated in different years and orchards at Lutirano, was amplified. Seven strains were isolated from leaf tissues and two from canes after 77 days of permanence in orchard No. 3 floor. These strains were the only ones that, according to *cts* (four alleles in clade a) and *rpoD* (four alleles) NJ analyses, belong to PG07/a and PG07, respectively (Supplementary Table 1 and Figure S4). With the exception of strains UCR-1 (PG07/a)

and TOMP2-02 (PG07/b), a Pv T-PAI fragment (Bartoli *et al.*, 2014), was successfully amplified from the PG07 strains used in the present study. Sequencing of these products confirmed the existence of high variability, not only between Pv reference strains, which were collected from different hosts and from different continents, but also within the Pv7a population resident in Lutirano. These strains were placed in seven clades by NJ analysis, with the five strains that shared the *cts* allele-24 partitioned in four of them (Supplementary Figure S5). In accordance with *P. viridiflava*, all PG07 strains isolated at Lutirano hydrolyzed aesculin, did not produce acid from sucrose, and grew with mannitol, glucose and D(-) tartrate as sole carbon source (data not shown). At last, according to the LOPAT scheme, strains KL24, KL48, KL317, KL396 and KL397 (*cts*-allele 24) can be ascribed to group II (*P. viridiflava*), while strains KL5, KL6, KL332 and KL345 (*cts* allele-23, -21, -22) could not, since they did not induce HR on tobacco (Lelliot and Stead, 1987).

Based on strains and isolates characterization carried out herein, qPCRPv7 assays has a theoretical 100% analytical specificity for Pv7.

In vitro *sensitivity of the qPCR assays*

When adding Psa3KL103 or Pv7aKL5 DNA to either water, a background (4 ng) of non-target bacterial DNA (Psa3KL103 or Pv7aKL5), or non-target nucleic acids extracted from plant (AcdH or *N. tabacum*), the LOD was 60 fg for both qPCRPsa3 and qPCRPv7 (Table 3). In general, addition of non-target nucleic acids (non-target bacteria or plants) increased the sensitivity of qPCRs, with the exception of when either type of plant DNA was added to qPCRPsa3. For both qPCR assays, R^2 values were greater then 0.997 in all reaction conditions, while E varied in a range from 99.3% (qPCRPsa3 with presence of AcdH nucleic acids) to 113.1% (qPCRPv7 with presence of Psa3KL103 DNA).

When leaf or cane tissues were spiked with Pv7aKL5 or Psa3KL103 cells and total nucleic acids co-extracted using CTAB (leaf) or boiling lysis (canes) methods (Table 4), the derived R^2 values indicated that both assays were suitable for quantifying the bacterial targets (R^2> 0.980; Bustin and Huggett, 2017), showing linearity over the nominal range of 10^8 to $10^4/10^3$ CFU g^{-1}. However, while both assays had good amplification efficiency (within the 95–105% range; Bustin and Huggett, 2017) for nucleic acids extracted from leaf tissues using CTAB, the efficiency of both assays decreased when applied to nucleic acids extracted from cane tissues using boiling lysis (Table 4).

In vivo *sensitivity and specificity of the qPCR assays*

Naturally infected leaves

A total of 210 leaves were collected from the six kiwifruit orchards that were monitored during the 2018 growing season. Of these, 39 were recorded as ts, 85 as gs, and 86 were al. At the first sampling time, approx. 21 d from bud break, no ts symptoms could be found in any orchard and the incidence of gs was low (four leaves out of 42) (Table 5). In subsequent samplings, the presence of ts or gs was always observed, albeit their relative abundance varied between orchards (data not shown).

Table 3. Limit of detection of the qPCR assays developed in this study on Psa3KL103 and Pv7aKL5 nucleic acids in different substrate backgrounds.

Substrate[a]	Range[b] (fg/reaction)		qPCRPsa3						qPCRPv7					
			LOD[c]		Linear regression[e]				LOD		Linear regression			
	From	To	Cq[d]	SD	k	q	E	R^2	Cq	SD	k	q	E	R^2
water	6*10^6	60	35.88	±1.4	-3.323	41.6	99.93	0.999	35.36	±0.6	-3.282	41.09	101.6	0.999
bacterium	6*10^6	60	34.78	±1.1	-3.077	40.6	111.3	0.997	35.06	±0.7	-3.043	40.31	113.1	0.998
AcdH	6*10^6	60	38.35	±0.3	-3.338	44.1	99.32	0.999	34.57	±0.5	-3.308	40.49	100.5	0.999
Nt	6*10^6	60	38.30	±0.4	-3.209	43.9	104.9	0.999	34.21	±0.4	-3.160	39.94	107.2	0.999

[a] Background substrate of water, 4 ng of non-target bacterial DNA (Pv7aKL5, qPCRPsa3; Psa3KL103, qPCRPv7), 4 ng of non-infected kiwi (AcdH) or tobacco (Nt) leaf nucleic acids.
[b] Psa3KL103 or Pv7aKL5 DNA per 15 µL reaction mixture.
[c] Limit of Detection.
[d] Average Cq values were calculated for four PCRs from the lowest bacterial concentration detected.
[e] Linear regression of all positive samples: k and q, slope and intercept of the standard regression line; E, average efficiency of amplification; R^2, coefficient of determination.

Table 4. Limit of detection of qPCR analyses of kiwifruit leaf and cane extracts, spiked with bacterial cell suspensions of Psa3KL103 or Pv7aKL5 prior to nucleic acids extraction using the CTAB (leaves) or boiling lysis (canes) methods .

PCR	Spiked leaves								Spiked canes							
	Range[a] (CFU g^{-1})		LOD[b]		Linear Regression[d]				Range (CFU g^{-1})		LOD		Linear Regression			
			Cq[c]	SD	k	q	E	R^2			Cq	SD	k	q	E	R^2
qPCRPsa3	10^8	10^3	37.99	±0.5	-3.419	40.20	96.08	0.999	10^8	10^4	37.17	±0.8	-3.957	44.40	78.94	0.989
qPCRPv7	10^8	10^3	35.72	±0.3	-3.308	37.44	100.5	0.999	10^8	10^3	38.05	±0.2	-3.078	39.39	111.2	0.992

[a] Concentration of Psa3KL103 or Pv7aKL5 cells per 1 g of healthy leaf or cane tissue, added prior to DNA extraction.
[b] Limit Of Detection.
[c] Average Cq values were calculated for four PCRs from the lowest bacterial concentration detected.
[d] Linear regressions of all positive samples: k and q, slopes and intercepts of the standard regression lines; E, average efficiencies of amplification; R^2, coefficients of determination.

Table 5. Analyses of symptomatic and asymptomatic kiwifruit leaves collected from six orchards during the 2018 vegetative season 21, 57, 92, 162 or 190 days after bud break (DABB). At each time point from each of seven plants in a row/orchard, one leaf was collected, scored as asymptomatic (al) or symptomatic (ts, typical symptoms, gs, generic symptoms; see Figure S3 and text for description). Presence of Psa3 and Pv7 was verified using qPCR, and by dilution plating of plant tissue extracts on KBCA followed by purification and characterization of single bacterial colonies (one leaf/orchard/time point).

DABB	Numbers of positive leaves /total numbers of leaves analyzed					
	qPCR			Isolates characterization		
	ts	gs	al	ts	gs	al
Psa3						
21	0/0	1/4	1/38	0/0	0/0	0/6
57	13/14	4/12	0/16	3/4	0/1	0/1
92	12/15	0/9	0/18	2/2	0/3	0/1
162	5/8	2/26	0/8	1/1	1/5	0/0
190	2/2	0/34	0/6	0/0	0/6	0/0
TOTAL	32/39	7/85	1/86	6/7	1/15	0/8
Pv7						
21	0/0	1/4	11/38	0/0	0/0	0/6
57	7/14	3/12	7/16	1/4	0/1	0/1
92	5/15	1/9	1/18	0/2	1/3	0/1
162	0/8	1/26	1/8	0/1	0/5	0/0
190	0/2	2/34	1/6	0/0	0/6	0/0
TOTAL	12/39	8/85	21/86	1/7	1/15	0/8

Ts symptoms were most commonly observed at 57 dabb (June) and 92 dabb (July), while gs symptoms were greatest toward the end of the season, at 162 and 190 dabb.

Based on qPCRPsa3 results, 40 leaves (19%) were positive for Psa3 throughout the season. Of these, 32 and seven were recorded as ts or gs, respectively. Only one leaf was scored as asymptomatic. According to qPCRPv7, 41 leaves were positive for the presence of Pv7 DNA. Of these, 12 and eight were showing ts or gs, respectively, while 21 were asymptomatic. The overall frequency of detection differed ($P < 0.001$) significantly between Psa3 and Pv7 in ts leaves, as well as in asymptomatic leaves, but not in leaves showing gs ($P > 0.50$).

Results obtained from dilution plating on KBCA followed by strain typing were in agreement ($P > 0.90$) with those obtained by qPCRPsa3. Twenty-two out of 30 leaves were negative and six were positive in both procedures, one leaf was positive to qPCRPsa3 only, and one leaf was positive only in Psa3 standard isolation. The results of the two approaches were also in agreement for Pv7 ($P > 0.05$). Twenty-three leaves tested positive and two leaves were negative, from both procedures, five leaves tested positive to qPCRPv7 only, and no leaves were positive for isolation only (Supplementary Table S2).

Naturally infected canes

qPCRPsa3 results confirmed the widespread presence of Psa3 in symptomatic canes (bc) collected in Lutirano at the time of winter pruning, with 19 out of 20 segments testing positive (Table 6). Thereafter, a sharp decrease in the frequency of Psa3 positive samples was recorded during the permanence of canes in the orchard floor, until the DNA of the bacterium became undetectable 117 dap. Selection and characterization results of the most common bacterial colony morphotypes, indicated that pruning was the only time at which Psa3 could be separated from the rest of the microorganisms that grew on KBCA plates. Nevertheless, when the same plates were washed and the growing microbial mass was analyzed with BIO-PCRPsa3 (culturable cells), evidence of Psa3 presence in the isolation plates was found until

Table 6. Analyses of dieback cane sections collected in orchard No. 3 at pruning time (0) or 42, 77 or 117 days after pruning (DAP), during which time cane sections were maintained buried at 5 cm depth in the orchard soil. Presence of Psa3 and Pv7 was verified using qPCR, and by dilution plating of the extracts on KBCA followed by BIO-PCR or by purification and characterization of single bacterial colonies.

DAP	Numbers positive sections/total numbers of sections analyzed					
	Psa3			Pv7		
	qPCR	BIO-PCR	Isolates characterization	qPCR	BIO-PCR	Isolates characterization
0	19/20	13/20	1/20	15/20	1/20	0/20
42	4/20	2/20	0/20	10/20	4/20	0/20
77	5/20	5/20	0/20	5/20	5/20	2/20
117	0/20	0/20	0/20	5/20	3/20	0/20
TOTAL	28/80	20/80	1/80	35/80	13/80	2/80

77 dap (Table 6). Overall frequencies of Psa3 detection were not statistically different ($P > 0.1$) between qPCRPsa3 (viable, culturable, and dead cells) and BIO-PCRPsa3 (culturable cells).

According to qPCRPv7, Pv7 was also a common inhabitant of bc canes at the time of pruning, as 15 out of 20 assessed cane sections were found positive. As for Psa3, the concentration of Pv7 DNA decreased, although less steeply, during the permanence of the canes in the orchard floor, but unlike Psa3, Pv7 remained detectable until 117 dap, when five out of 20 assessed cane sections tested positive. Only at the 77 dap time point, Pv7 colonies were successfully separated from the bacterial mass growing on KBCA plates, although BIO-PCRPv7 indicated that culturable Pv7 was occasionally present in the plates from pruning time (one section out of 20) to the end of the trial. Overall frequency of Pv7 detection by qPCRPv7 was significantly different ($P < 0.001$) from that obtained with BIO-PCRPv7.

Artificially inoculated leaves

Nucleic acids extracted from leaves of six AcdH potted plants in May 2020, just prior to inoculation with Psa3KL318 and Pv7aKL317, tested negative in the qPCR and BIO-PCR assays. However, standard isolation on both NSA and KBCA followed by isolate characterization at that time indicated the presence of bacteria belonging to PG02/b and PG02/d of Pssc (Supplementary Table S1). None of the inoculated or non-inoculated leaves showed bacterial disease symptoms throughout the trial. Taking into account their respective LOD values estimated *in vitro* (Cq=37.99, qPCRPsa3; Cq=35.72, qPCRPv7a), when qPCRPsa3 and qPCRPv7 were applied to the nucleic acids extracted from leaves, the frequency of detection of the two bacteria (Table 7) was greatest at 4 dai (six leaves tested positive to qPCRPsa3 and ten leaves were positive for qPCRPv7). Thereafter, detection decreased until the end of the trial (85 dai), when none (qPCRPsa3) or one (qPCRPv7) of the sampled leaves tested positive. According to qPCRPsa3 and qPCRPv7, the amounts of the two bacteria per g of leaf, when detected, were stable and similar until 42 dai, varying from 3.96 to 3.83 log CFU g^{-1} for Psa3 and 4.3 to 3.5 log CFU g^{-1} for Pv7.

Results obtained by selection and identification of colony morphotypes growing on NSA plates showed only three leaves as infected with Psa3 (two sampled at

Table 7. Analyses of artificially infected leaves from kiwi potted plants, 4, 11, 18, 42 or 85 days after inoculation (DAI) with strains Psa3KL318 or Pv7aKL317. Presence of Psa3 and Pv7 was verified using qPCR, and by dilution plating of the extracts on NSA followed by BIO-PCR or by purification and characterization of single bacterial colonies.

DAI	Psa3					Pv7a				
	No. positive leaves/total No. of leaves analyzed			Cq[a] (SD)	Log CFU g^{-1} [b] (SD)	No. positive leaves/total No. of leaves analyzed			Cq (SD)	Log CFU g^{-1} (SD)
	Isolates characterization	BIO-PCR	qPCR			Isolates characterization	BIO-PCR	qPCR		
4	0/12	1/12	6/12	35.45 (±1.68)	3.96 (±0.46)	0/12	1/12	10/12	31.71 (±2.01)	4.30 (±0.59)
11	0/12	3/12	2/12	36.36 (±2.19)	3.65 (±0.59)	2/12	11/12	8/12	33.53 (±1.46)	3.77 (±0.47)
18	2/12	9/12	2/12	35.68 (±1.34)	3.93 (±0.27)	3/12	12/12	5/12	34.52 (±0.46)	3.48 (±0.10)
42	0/12	7/12	1/12	36.1 (-)	3.83 (-)	0/12	6/12	5/12	34.54 (±1.22)	3.49 (±0.40)
85	1/12	3/12	0/12	-	-	0/12	6/12	1/12	31.96 (-)	4.18 (-)
TOTAL	3/60	23/60	11/60			5/12	36/60	29/60		

18 dai; one at 85 dai) and five leaves infected with Pv7 (two sampled at 11 dai; three at 18 dai). Nevertheless, according to BIO-PCRPsa3 and BIO-PCRPv7, the two viable bacteria were often present on/in the sampled leaves, being culturable on NSA isolation plates. The frequency of leaves from which Psa3 and Pv7 could be cultured was minimum at four dai, increased until 18 dai, and then decreased until the end of the trial (85 dai). Based on overall detection frequencies, sensitivities of qPCRPsa3 ($P < 0.025$) and qPCRPv7 ($P > 0.05$) were, respectively, less than and equal to that of their BIO-PCR variant, but greater than isolate characterization following dilution plating on NSA ($P < 0.001$). When qPCR amplicons were sequenced to test the specificity of qPCRPsa3 (eight amplicons; Cq range from 33.4 to 36.6) and qPCRPv7 (eight amplicons; Cq range from 29.8 to 36.5), 100% identity was found with the respective homologous sequences of Psa3KL318 and Pv7aKL317 (Supplementary Table S3).

DISCUSSION

In this work we present two new TaqMan qPCR-based approaches for sensitive and accurate detection of Psa3 (qPCRPsa3) and Pv PG07 (qPCRPv7) in kiwifruit, and the procedure followed for their validation against the *Pseudomonas* sp. background microflora of kiwifruit.

The lineage of Psa3 responsible for the bacterial canker pandemic, is pathogenic to *Actinidia* sp. and possibly few other species (Liu *et al*., 2016), and has just recently started to diversify (McCann *et al*., 2017; Firrao *et al*., 2018). In pursuing the development of a new specific test for the detection of the Psa3 pandemic lineage, the recently characterized *lscγ* gene was targeted. The gene codes for a functional levansucrase with a peculiar signature of 15 amino acids at the N-terminal region, which, based on whole genome comparisons, has only been found in pandemic isolates of the bacterium (Luti *et al*., 2021). More challenging was development of a qPCR assay for Pv. This bacterium is a heterogenic saprophyte, is prone to recombination, and has a complex evolutionary history, being currently allocated in PGs 7 and 8, of Pssc (Billing, 1970; Goss *et al*., 2005; Bartoli *et al*., 2014; Bull and Koike, 2015). To account for intrataxon variability, the present study focused on developing an assay for PG07 (Pv7), which represents most of the strains classified as *P. viridiflava* in previous studies (Berge *et al*., 2014; Lipps and Samac, 2022), and exploited the polymorphisms of the HK *rpoD* gene.

In vitro analyses showed that both assays were sensitive and specific. However, when qPCRPv7 was tested against a collection of 78 reference bacterial strains from kiwifruit and other hosts, the DNAs of 10 strains catalogued as "*P. viridiflava*" in their collections of origin, failed to give the expected signal. Further *cts* and *rpoD* sequence analyses indicated that none of them belonged to PG07, confirming the robustness of the protocol for discriminating within the Pss complex according to the *rpoD* phylogenetic signal (Parkinson *et al*., 2011). For a more *ad hoc* verification, the two approaches were assessed on the DNA of 240 *Pseudomonas* sp. strains that we isolated in different years, seasons and from different kiwifruit tissues, 169 of which could be classified within the Pss complex (PGs 01, 02, 03, 07, 12 and 13) according to their *cts* derived phylogeny. Both assays were highly specific as they distinguished Psa3 (24 isolates) and Pv7 (nine isolates).

To properly evaluate these results, it is important to recall that the range of Pssc PGs and subclades that has been found associated to kiwifruit worldwide, is much vaster than what detected in the present study. For example, the presence of PG5 and PG10 has been detected in orchards in France, but never in Tuscany (Italy). Similarly, PG03, which was very rarely isolated from kiwifruit in Tuscany in the present study, is widespread in New Zealand where it causes bacterial blight (Young *et al*., 1997).

Phylogeographic structuring possibly exists in the Pssc metapopulation associated to *Actinidia* sp. (Bastardo *et al*., 2017), but there is also circumstantial evidence that the decision to use a semi-selective medium (KBCA) to set-up the reference collection may have led to underestimate the variability of the *Pseudomonas* sp. community in Tuscany. KBCA was originally developed to isolate Ps pvs. syringae, tomato and pisi (Mohan and Schaad, 1987). Since bacterial isolation may be difficult without the use of a semi-selective media, use of KBCA has become a common practice to investigate the composition of Pssc in different environmental samples, as well as a standard when indexing for Psa3 (Riffaud and Morris, 2002; Morris *et al*., 2008; Loreti *et al*., 2018; Parisi *et al*., 2019). Keeping in mind that also "all-purpose" media are selective to some extent, NSA and KBA amended only with cycloheximide (King *et al*., 1954) were used in the preliminary phases of the present study to isolate Pssc bacteria from naturally infected leaves and canes. However the mass of microorganisms growing in the first 72 h of culturing was often so abundant that isolation of Pv, and especially of Psa3, was strongly hindered (data not shown). Although the use of KBCA reduced this problem, the trade-off was possibly a data bias towards a simplified *Pseudomonas* sp. population structure (i.e. underestimation of variability), as has

been previously shown for Pssc and specifically for Pv (Gitaitis *et al.*, 1997; Morris *et al.*, 2008). The present study then evaluated the effective degree of specificity of the two assays in three validation experiments, using plant tissues that contained natural or artificial populations of the target organisms as well as of their background microflora. These tissues included naturally infected leaves or canes collected in different orchards during the vegetative growth season, or artificially infected leaves of nursery plants.

Results from orchard leaves obtained by means of qPCRs or dilution plating on KBCA followed by isolate typing (*cts*, *rpoD* and *pfk* genes) were in good agreement and indicated that during the 2018 vegetative season in Tuscany there was an association of Psa3, but not of Pv7a, with leaves showing ts symptoms. Previous research has indicated that these symptoms can be caused by both target bacteria (Young *et al.*, 1988, 1997; Serizawa *et al.*, 1989; Balestra *et al.*, 2009; Vanneste *et al.*, 2011). As previously reported in New Zealand and Japan, the frequency of ts leaves in Lutirano (Tuscany) greatly increased from late spring (3 weeks after bud break), when leaves were nearly all asymptomatic, to mid-summer, and then progressively decreased until the end of the kiwifruit growing season (late Autumn in Tuscany). In contrast, frequency of gs increased and of asymptomatic leaves decreased with leaf aging (Vanneste *et al.*, 2011). Accordingly, qPCRPsa3 results indicated that the bacterium was significantly associated to ts leaves, rarely to gs leaves, and never with asymptomatic leaves, highlighting the specificity of the assay also when tested against the kiwifruit leaves background microflora present in each orchard.

Pv is a common member of the Pssc associated with kiwifruit phylloplane in Central Italy, where its population reaches a major peak in spring and a secondary one in autumn (Balestra and Varvaro, 1998). However, the capability of Pv to induce ts symptoms on kiwifruit leaves is controversial. In California and in Central Italy, Pv (LOPAT II), together with *P. syringae* (LOPAT Ia), is reported as the agent of kiwifruit bacterial blight, a disease characterized by the development of ts leaf spots and flower bud rot. In New Zealand, after further characterization of the pathogenic isolates, it was concluded that the agent of bacterial blight, a bacterium with a LOPAT II profile, was misidentified as Pv and should be classified in the PG03, *P. savastanoi*, of the Pssc (Conn *et al.*, 1993; Young *et al.,* 1997; Hu *et al.*, 1999; Visnovsky *et al.*, 2019). Based on qPCRPv7 results the present study confirmed that Pv7 is a common inhabitant of kiwifruit leaves in Tuscany. However, the present study data also show that in the Tuscan environment, in contrast to Psa3, Pv7 is a leaf saprophyte rather than a pathogen, since: i) its frequency in/on the sampled leaves was high 21 days after bud break when nearly all leaves were asymptomatic; ii) its frequency in/on ts leaves was approximately 1/3 of that of Psa3; and iii) as for Psa3, its presence in/on leaves showing generic spot symptoms (gs) was only sporadic.

Following a procedure that was previously used in New Zealand orchards (Tyson *et al.*, 2012), in the second validation experiment we monitored the fate of Psa3 and, for the first time of Pv7, in pruned canes with symptoms of dieback after burying them to 5 cm depth in the orchard soil.

Since attempts to purify and characterize colony morphotypes from KBCA was hampered by the abundance of the growing microbial mass, we verified the presence of Psa3 and Pv7 in the plates by BIO-PCR, an approach that enhances likelihood of detecting the bacterial target, if in a culturable state (Schaad *et al.*, 1995, 1999). Although, with the exception of pruning time, Psa3 could not be recovered according to isolate typing results, we found that this bacterium was often effectively growing in the isolation plates, and that qPCRPsa3 and BIO-PCRPsa3 results were overall in strict agreement. While Psa3 was widely colonizing the woody tissues at pruning, thereafter Psa3 population rapidly diminished and became undetectable by both detection methods after approximately 4 months in the orchard floor. These results are concordant with previous findings by Tyson *et al.* (2012) using BIO-PCR in New Zealand , which showed that in early spring nearly all symptomatic canes were systematically infected, and that viable Psa could be detected only until 11 weeks of permanence of canes in the orchard floor. The present study gave similar results using BIO-PCRPsa3 and qPCRPsa3 at 77 dap (June). In the case of Pv7, which was recently found associated with canker tissues of plum and apricot (Parisi *et al.*, 2019; Bophela *et al.*, 2020), the qPCRPv7 and BIO-PCR-Pv7 approaches gave conflicting results at pruning. With qPCRPv7, 75% of the cane sections were infected by the bacterium, while according to BIO-PCRPv7, culturable Pv7 was nearly absent (5%), in accordance with isolate typing results. Over successive sampling dates, qPCRPv7 and BIO-PCRPv7 results were in closer agreement, with both methods indicating that 15% of cane sections were infected at 77 dap and 25% were infected at 117 dap. These results indicate that at pruning, nearly all Pv7 cells in the sampled canes were dead or were non-culturable on KBCA (low frequencies of positive according to BIO-PCRPv7), although their DNA recovered from woody tissues was amplifiable (high frequencies of infected canes according to qPCRPv7). Subsequently, however, either

the original Pv7 population found suitable conditions to proliferate in the decaying tissues, or the canes became re-infected. Pv is a water cycle-related bacterium, so spring rains, which are frequent and abundant in Lutirano, could have easily dispersed Pv inoculum from the phylloplane, where the bacterium was present in 2018 at least since May, to the soil surface. Then, water movements on/in the soil, where there is circumstantial evidence that this bacterium can survive, may have facilitated its contact with the buried canes and their subsequent infection (Gitaitis *et al.*, 1997; Bartoli *et al.*, 2015; Borshinger *et al.*, 2016). Of course, Psa3 could have used the same route to reach the canes but, even so, our data indicate that the dead tissues were not a substrate where its proliferation was possible.

In the final *in vivo* experiment, fully expanded kiwifruit leaves were inoculated at the end of spring, with the aim of evaluating usefulness of the two qPCR assays for detecting asymptomatic infections. Susceptibility of kiwifruit leaves to Psa1 in Japan and to Psa3 in New Zealand, was strongly correlated with leaf age, being maximum when leaves reached 2 cm in length or are 1-3 weeks old, and then decreased progressively during aging. Spray-inoculated 7-week-old cv. Hayward leaves never showed spots or fleck symptoms (Serizawa and Ichikawa, 1993; Tyson *et al.,* 2015). Petriccione *et al.* (2014) showed that after inoculation of Psa3 CRA-FRU 8.43 on fully expanded AcdH leaves, the bacterium entered a biotrophic phase (asymptomatic) that may last several weeks, during which it was unable, at least temporarily, to overcome plant host defenses. This impedes infection progress, as indicated by stable Psa3 population in leaf apoplast, as well as absence of disease symptoms (necrotrophic phase). More recently, the infection threshold value for the transition from epiphytic phase to apoplastic invasion on AcdH leaves by Psa3 (CFBP7286), was estimated to be 4.4×10^4 CFU g^{-1} of leaf blade tissues (Donati *et al.*, 2020). The present results confirm these previous observations for Psa3KL318, but also show that Pv7aKL317 behaved in a similar fashion. Although both strains did not cause leaf symptoms throughout the trial, both survived in/on aging leaves during summer, as indicated by BIO-PCR and, for Psa3, by re-isolating the bacterium 85 dai.

The inability of Psa3KL318 to progress from biotrophic to necrotrophic phases in order to grow and multiply at higher densities in host leaves, was also evident by interpolating qPCR Cq values against the corresponding standard curves developed in this study. The average population of Psa3KL318 per leaf was nearly constant and never exceeded 10^4 CFU g^{-1}, which is in the order of magnitude of population densities reached by Psa3 CRA-FRU 8.43 in asymptomatic leaves (Petriccione *et al.*, 2014). Keeping in mind that Pv7aKL317 and Psa3KL318 were co-infecting the same leaf in early spring of 2018, the finding that Pv7aKL317 can also sustain itself on the phylloplane at a density of 10^4 to 10^3 CFU g^{-1}, confirms that the kiwifruit phyllosphere is a niche that *Pseudomonas* spp. bacteria cohabit under a wide range of environmental conditions. Given the apparently common presence of this heterogenic community worldwide, its existence should be fully accounted for when developing specific culture-independent diagnostic assays for *Pseudomonas* sp. on kiwifruit.

In conclusion, this research has demonstrated the usefulness of TaqMan-based qPCR for sensitive and specific detection of Pv7 and Psa3 from symptomatic and asymptomatic kiwifruit tissues. Several molecular diagnostic assays for Psa3 have been previously developed, including two SYBR green qPCR protocols. The qPCRPsa3 described here will be a valuable alternative for confirmatory analyses as it is based on different genomic targets (Gallelli *et al.,* 2014; Andersen *et al.,* 2018; EPPO PM7\120[2], 2021). Due to their reliability as monitoring tools to estimate target populations in the phyllosphere, qPCRPsa3 and qPCRPv7, which represent the first molecular assays specifically developed for PG07, should facilitate future studies on colonization and survival of these pathogens in orchard conditions.

ACKNOWLEDGMENTS

The authors declare no conflict of interest. We thank Dr. Sally Miller (OSU), Dr. Caroline Roper (UCR), and Dr. Melodie Putnam (OrSU) who generously provided strains for this study, and two anonymous reviewers for their valuable input and constructive criticism.

FUNDING

This study was supported by the Phytosanitary Service of the Tuscany Region (Italy).

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Sara Campigli: Conceptualization, Data curation, Investigation, Writing-original draft; **Simone Luti**: Investigation, Methodology; **Tommaso Martellini**: Investigation; **Domenico Rizzo**: Formal analysis, Review; **Linda Bartolini**: Investigation; **Claudio Carrai**: Formal analysis, Funding acquisition; **Jeyaseelan**

Baskarathevan: Resources, Review; **Luisa Ghelardini**: Conceptualization, Review & editing; **Francesca Peduto Hand**: Supervision, Writing, Review & editing; **Guido Marchi**: Conceptualization, Data curation, Supervision, Writing, Review & editing.

LITERATURE CITED

Andersen M.T., Templeton M.D., Rees-George J., Vanneste J.L., Cornish D.A., … Rikkerink E.H.A., 2018. Highly specific assays to detect isolates of *Pseudomonas syringae* pv. *actinidiae* biovar 3 and *Pseudomonas syringae* pv. *actinidifoliorum* directly from plant material. *Plant Pathology* 67: 1220–1230. https://doi.org/10.1111/ppa.12817

Angelini E., Clair D., Borgo M., Bertaccini A., Boudon-Padieu E., 2001. Flavescence dorée in France and Italy-Occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. *Vitis* 40: 79–86. https://doi.org/10.5073/vitis.2001.40.79-86

Araki H., Tian D., Goss E.M., Jakob K., Halldorsdottir S.S.,… Bergelson J., 2006. Presence/absence polymorphism for alternative pathogenicity islands in *Pseudomonas viridiflava*, a pathogen of *Arabidopsis*. *Proceedings of the National Academy of Sciences* 103: 5887–5892. https://doi.org/10.1073/pnas.0601431103

Balestra G.M., Mazzaglia A., Quattrucci A., Renzi M., Rossetti A., 2009. Occurrence of *Pseudomonas syringae* pv. *actinidiae* in Jin Tao kiwi plants in Italy. *Phytopathologia Mediterranea* 48: 299–301. DOI: 10.14601/Phytopathol_Mediterr-2821

Balestra G.M., Varvaro L., 1998. Seasonal fluctuations in kiwifruit phyllosphere and ice nucleation activity of *Pseudomonas viridiflava*. *Journal of Plant Pathology* 80: 151–156. http://dx.doi.org/10.4454/jpp.v80i2.812

Baltrus D.A., McCann H.C., Guttman D.S., 2017. Evolution, genomics and epidemiology of *Pseudomonas syringae*: challenges in bacterial molecular plant pathology. *Molecular Plant Pathology* 18: 152–168. https://doi.org/10.1111/mpp.12506

Bartoli C., Berge O., Monteil C.L., Guilbaud C., Balestra G.M., … Morris C.E., 2014. *The Pseudomonas viridiflava* phylogroups in the *P. syringae* species complex are characterized by genetic variability and phenotypic plasticity of pathogenicity-related traits. *Environmental Microbiology* 16: 2301–2315. https://doi.org/10.1111/1462-2920.12433

Bartoli C., Lamichhane J.R., Berge O., Varvaro L., Morris C.E., 2015. Mutability in *Pseudomonas viridiflava* as a programmed balance between antibiotic resistance and pathogenicity. *Molecular Plant Pathology* 16: 860–869. https://doi.org/10.1111/mpp.12243

Bastardo A., Balboa S., Romalde J.L., 2017. From the Gene Sequence to the Phylogeography through the Population Structure: The Cases of *Yersinia ruckeri* and *Vibrio tapetis*. In: *Genetic Diversity* (L. Bitz ed.), InTech, Rijeka, Croatia, Chapter 4, 57–87 http://dx.doi.org/10.5772/67182

Berge O., Monteil C. L., Bartoli C., Chandeysson C., Guilbaud C., … Morris C.E., 2014. A User's Guide to a Data Base of the Diversity of *Pseudomonas syringae* and Its Application to Classifying Strains in This Phylogenetic Complex. *PLoS ONE* 9: e105547. doi:10.1371/journal.pone.0105547

Billing E., 1970. *Pseudomonas viridiflava* (Burkholder, 1930; Clara 1934). *Journal of Applied Microbiology* 33: 492–500. https://doi.org/10.1111/j.1365-2672.1970.tb02225.x

Biondi E., Galeone A., Kuzmanović N., Ardizzi S., Lucchese C., Bertaccini A., 2013. *Pseudomonas syringae* pv. *actinidiae* detection in kiwifruit plant tissue and bleeding sap. *Annals of Applied Biology* 162: 60–70. https://doi.org/10.1111/aab.12001

Bophela K.N., Petersen Y., Bull C.T., Coutinho T.A., 2020. Identification of *Pseudomonas* isolates associated with bacterial canker of stone fruit trees in the Western Cape, South Africa. *Plant Disease* 104: 882–892. https://doi.org/10.1094/PDIS-05-19-1102-RE

Borschinger B., Bartoli C., Chandeysson C., Guilbaud C., Parisi L., … Morris C.E., 2016. A set of PCRs for rapid identification and characterization of *Pseudomonas syringae* phylogroups. *Journal of Applied Microbiology* 120: 714–723. https://doi.org/10.1111/jam.13219

Bull C.T., Koike S.T., 2015. Practical benefits of knowing the enemy: modern molecular tools for diagnosing the etiology of bacterial diseases and understanding the taxonomy and diversity of plant-pathogenic bacteria. *Annual Review of Phytopathology* 53: 157–180. https://doi.org/10.1146/annurev-phyto-080614-120122

Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., … Wittwer C.T., 2009. The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments. *Clinical Chemistry* 55: 611–622. https://doi.org/10.1373/clinchem.2008.112797

Bustin S., Huggett J., 2017. qPCR primer design revisited. *Biomolecular Detection and Quantification* 14: 19–28. https://doi.org/10.1016/j.bdq.2017.11.001

Chapman J.R., Taylor R.K., Weir B.S., Romberg M.K., Vanneste J.L., … Alexander B.J.R., 2012. Phylogenetic relationships among global populations of *Pseu-*

domonas syringae pv. *actinidiae*. *Phytopathology* 102: 1034–1044. https://doi.org/10.1094/PHYTO-03-12-0064-R

Conn K.E., Gubler W.D., Hasey J.K., 1993. Bacterial blight of kiwifruit in California. *Plant Disease* 77: 228–230. DOI: 10.1094/PD-77-0228.

Dillon M.M., Thakur S., Almeida R N., Wang P.W., Weir B.S., Guttman D.S., 2019. Recombination of ecologically and evolutionarily significant loci maintains genetic cohesion in the *Pseudomonas syringae* species complex. *Genome Biology* 20: 1–28. https://doi.org/10.1186/s13059-018-1606-y

Donati I., Cellini A., Sangiorgio D., Vanneste J.L., Scortichini M., … Spinelli F., 2020. *Pseudomonas syringae* pv. *actinidiae*: Ecology, infection dynamics and disease epidemiology. *Microbial Ecology* 80: 81–102. https://doi.org/10.1007/s00248-019-01459-8

Dye D.W., Bradbury J., Goto M., Hayward A.C., Lelliott R.A., Schroth M.N., 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153–168.

EPPO Bulletin 2021. Volume 51, Issue 3, PM 7/120 (2) *Pseudomonas syringae* pv. *actinidiae*. EPPO STANDARD – DIAGNOSTICS. DOI: 10.1111/epp.12782

FAOSTAT, 2020. Food and Agriculture Organization of the United Nations. Available at: http://www.fao.org/faostat/en/#home. Accessed March 16, 2021.

Firrao G., Torelli E., Polano C., Ferrante P., Ferrini F., … Ermacora P., 2018. Genomic structural variations affecting virulence during clonal expansion of *Pseudomonas syringae* pv. *actinidiae* biovar 3 in Europe. *Frontiers in Microbiology* 9: 656. https://doi.org/10.3389/fmicb.2018.00656

Gallelli A., Talocci S., L'Aurora A., Loreti S., 2011. Detection of *Pseudomonas syringae* pv. actinidiae, causal agent of bacterial canker of kiwifruit, from symptomless fruits and twigs, and from pollen. *Phytopathologia Mediterranea* 50: 462–472.

Gallelli A., Talocci S., Pilotti M., Loreti S., 2014. Real-time and qualitative PCR for detecting *P seudomonas syringae* pv. *actinidiae* isolates causing recent outbreaks of kiwifruit bacterial canker. *Plant Pathology* 63: 264–276. https://doi.org/10.1111/ppa.12082

Gardan L., Shafik H., Belouin S., Broch R., Grimont F., Grimont P.A.D., 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov.(ex Sutic and Dowson 1959). *International Journal of Systematic and Evolutionary Microbiology* 49: 469–478. https://doi.org/10.1099/00207713-49-2-469

Gitaitis R., Sumner D., Gay D., Smittle D., McDonald G., … Hung Y., 1997. Bacterial streak and bulb rot of onion: I. A diagnostic medium for the semiselective isolation and enumeration of *Pseudomonas viridiflava*. *Plant Disease* 81: 897–900. https://doi.org/10.1094/PDIS.1997.81.8.897

Goss E.M., Kreitman M., Bergelson J., 2005. Genetic diversity, recombination and cryptic clades in *Pseudomonas viridiflava* infecting natural populations of *Arabidopsis thaliana*. *Genetics* 169: 21–35. https://doi.org/10.1534/genetics.104.031351

Hirano S.S., Upper C.D., 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews* 64: 624–653. DOI: 10.1128/MMBR.64.3.624-653.2000

Hu F.P., Young J.M., Jones D.S., 1999. Evidence that bacterial blight of kiwifruit, caused by a *Pseudomonas sp*., was introduced into New Zealand from China. *Journal of Phytopathology* 147: 89–97.

King E.O., Ward M.K., Raney D.E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* 44: 301–307.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547–1549. https://doi.org/10.1093/molbev/msy096

Lelliott R.A., Stead D.E., 1987. *Methods for the Diagnosis of Bacterial Diseases of Plants*. Blackwell Scientific Publications, Oxford, United Kingdom, 162 pp.

Lipps S.M., Samac D.A., 2022. *Pseudomonas viridiflava*: An internal outsider of the *Pseudomonas syringae* species complex. *Molecular Plant Pathology* 23: 3–15. https://doi.org/10.1111/mpp.13133

Liu P., Xue S., He R., Hu J., Wang X., … Zhu L., 2016. *Pseudomonas syringae* pv. *Actinidiae* isolated from non-kiwifruit plant species in China. *European Journal of Plant Pathology* 145: 743–754. DOI 10.1007/s10658-016-0863-4

Loreti S., Cunty A., Pucci N., Chabirand A., Stefani E., … Poliakoff F., 2018. Performance of diagnostic tests for the detection and identification of *Pseudomonas syringae* pv. *actinidiae* (Psa) from woody samples. *European Journal of Plant Pathology* 152: 657–676. https://doi.org/10.1007/s10658-018-1509-5

Luti S., Campigli S., Ranaldi F., Paoli P., Pazzagli L., Marchi G., 2021. Lsc**β** and lsc**γ**, two novel levansucrases of *Pseudomonas syringae* pv. actinidiae biovar 3, the causal agent of bacterial canker of kiwifruit, show different enzymatic properties. *International*

Journal of Biological Macromolecules 179: 279–291. https://doi.org/10.1016/j.ijbiomac.2021.02.189

McCann H.C., Li L., Liu Y., Li D., Pan H., … Huang H., 2017. Origin and evolution of the kiwifruit canker pandemic. *Genome Biology and Evolution* 9: 932–944. https://doi.org/10.1093/gbe/evx055

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

Mohr T.J., Liu H., Yan S., Morris C.E., Castillo J.A., … Vinatzer B.A., 2008. Naturally occurring nonpathogenic isolates of the plant pathogen *Pseudomonas syringae* lack a type III secretion system and effector gene orthologues. *Journal of Bacteriology* 190: 2858–2870. https://doi.org/10.1128/JB.01757-07

Moore E., Arnscheidt A., Krüger A., Strömpl C., Mau M., 2004. Simplified protocols for the preparation of genomic DNA from bacterial cultures. *Molecular Microbial Ecology Manual* 1: 3–18.

Morris C.E., Kinkel L.L., Xiao K., Prior P., Sands D.C., 2007. Surprising niche for the plant pathogen *Pseudomonas syringae*. *Infection, Genetics and Evolution* 7: 84–92. https://doi.org/10.1016/j.meegid.2006.05.002

Morris C.E., Sands D.C., Vinatzer B.A., Glaux C., Guilbaud C., … Thompson B.M., 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME journal* 2: 321–334. DOI: 10.1038/ismej.2007.113

Morris C.E., Sands D.C., Vanneste J.L., Montarry J., Oakley B., … Glaux C., 2010. Inferring the evolutionary history of the plant pathogen *Pseudomonas syringae* from its biogeography in headwaters of rivers in North America, Europe, and New Zealand. *MBio* 1: 00107–00110. https://doi.org/10.1128/mBio.00107-10

Morris C.E., Lamichhane J.R., Nikolić I., Stanković S., Moury B., 2019. The overlapping continuum of host range among strains in the *Pseudomonas syringae* complex. *Phytopathology Research* 1: 1–16. https://doi.org/10.1186/s42483-018-0010-6

Parisi L., Morgaint B., Blanco-Garcia J., Guilbaud C., Chandeysson C., … Morris C.E., 2019. Bacteria from four phylogroups of the *Pseudomonas syringae* complex can cause bacterial canker of apricot. *Plant Pathology* 68: 1249–1258. https://doi.org/10.1111/ppa.13051

Parkinson N., Bryant R., Bew, J., Elphinstone J., 2011. Rapid phylogenetic identification of members of the *Pseudomonas syringae* species complex using the *rpoD* locus. *Plant Pathology* 60: 338–344. https://doi.org/10.1111/j.1365-3059.2010.02366.x

Petriccione M., Salzano A. M., Di Cecco I., Scaloni A., Scortichini M., 2014. Proteomic analysis of the *Actinidia deliciosa* leaf apoplast during biotrophic colonization by *Pseudomonas syringae* pv. *actinidiae*. *Journal of Proteomics* 101: 43–62. https://doi.org/10.1016/j.jprot.2014.01.030

Renzi M., Copini P., Taddei A. R., Rossetti A., Gallipoli L., … Balestra G.M., 2012. Bacterial canker on kiwifruit in Italy: anatomical changes in the wood and in the primary infection sites. *Phytopathology* 102: 827–840. https://doi.org/10.1094/PHYTO-02-12-0019-R

Riffaud C.H., Morris C.E., 2002. Detection of *Pseudomonas syringae* pv. *aptata* in irrigation water retention basins by immunofluorescence colony-staining. *European Journal of Plant Pathology* 108: 539–545. https://doi.org/10.1023/A:1019919627886

Sarkar S.F., Guttman D.S., 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology* 70: 1999–2012. DOI:10.1128/AEM.02553-07

Sawada H., Fujikawa T., 2019. Genetic diversity of *Pseudomonas syringae* pv. actinidiae, pathogen of kiwifruit bacterial canker. *Plant Pathology* 68: 1235–1248. https://doi.org/10.1111/ppa.13040

Schaad N.W., Cheong S.S., Tamaki S., Hatziloukas E., Panopoulas N.J., 1995. A combined biological amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* 85: 243–248. DOI: 10.1094/Phyto-85-243

Schaad N.W., Berthier-Schaad Y., Sechler A., Knorr D., 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. *Plant Disease* 83: 1095–1100. DOI: 10.1094/PDIS.1999.83.12.1095

Schaad N.W., Jones J.B., Chun W., 2001. *Laboratory Guide for the Identification of Plant Pathogenic Bacteria*. 3th ed. American Phytopathological Society (APS Press), USA, 373 pp.

Serizawa S., Ichikawa T., Takikawa Y., Tsuyumu S., Goto M., 1989. Occurrence of bacterial canker of kiwifruit in Japan. *Japanese Journal of Phytopathology* 55: 427–436 (in Japanese). https://doi.org/10.3186/jjphytopath.55.427

Serizawa S., Ichikawa T., 1993. Epidemiology of bacterial canker of kiwifruit. 2. The most suitable times and environments for infection on new canes. *Annals of the Phytopathological Society of Japan* 59: 460–468 (in Japanese). DOI 10.3186/jjphytopath.59.460

Straub C., Colombi E., Li L., Huang H., Templeton M.D., … Rainey P.B., 2018. The ecological genetics of *Pseudomonas syringae* from kiwifruit leaves. *Environmental Microbiology* 20: 2066–2084. https://doi.org/10.1111/1462-2920.14092

Tyson J.L., Rees-George J., Curtis C.L., Manning M.A., Fullerton R.A., 2012. Survival of *Pseudomonas syringae* pv *actinidiae* on the orchard floor over winter. *New Zealand Plant Protection* 65: 25–28. https://doi.org/10.30843/nzpp.2012.65.5420

Tyson J.L., Horner I.J., Curtis C.L., Blackmore A., Manning M.A., 2015. Influence of leaf age on infection of *Actinidia* species by *Pseudomonas syringae* pv *actinidiae*. *New Zealand Plant Protection* 68: 328–331. https://doi.org/10.30843/nzpp.2015.68.5829

Vanneste J.L., Yu J., Cornish D.A., Max S., Clark G., 2011. Presence of *Pseudomonas syringae* pv *actinidiae* the causal agent of bacterial canker of kiwifruit on symptomatic and asymptomatic tissues of kiwifruit. *New Zealand Plant Protection* 64: 241–245. https://doi.org/10.30843/nzpp.2011.64.5948

Vanneste J.L., 2017. The scientific, economic, and social impacts of the New Zealand outbreak of bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*). *Annual Review of Phytopathology* 55: 377–399. https://doi.org/10.1146/annurev-phyto-080516-035530

Visnovsky S.B., Marroni M.V., Pushparajah S., Everett K.R., Taylor R.K., … Pitman A.R., 2019. Using multilocus sequence analysis to distinguish pathogenic from saprotrophic strains of *Pseudomonas* from stone fruit and kiwifruit. *European Journal of Plant Pathology* 155: 643–658. https://doi.org/10.1007/s10658-019-01799-8

Wilkie J.P., Dye D.W., Watson D.R.W., 1973. Further hosts of *Pseudomonas viridiflava*. *New Zealand Journal of Agricultural Research* 16: 315–323. https://doi.org/10.1080/00288233.1973.10421110

Young J.M., Cheesmur G.J., Welham F.V., Henshall W.R., 1988. Bacterial blight of kiwifruit. *Annals of Applied Biology* 112: 91–105. https://doi.org/10.1111/j.1744-7348.1988.tb02044.x

Young J.M., Gardan L., Ren X.Z., Hu F.P., 1997. Genomic and phenotypic characterization of the bacterium causing blight of kiwifruit in New Zealand. *Plant Pathology* 46: 857-864. https://doi.org/10.1046/j.1365-3059.1997.d01-72.x

Zar J.H., 1999. *Biostatistical Analysis*, 4th ed. Prentice-Hall International, London, UK, 663 pp.