Detection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen samples

J.L. Vanneste[1], D. Giovanardi[2], J. Yu[1], D.A. Cornish[1], C. Kay[3], F. Spinelli[4] and E. Stefani[2]

[1]*The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand*
[2]*Dipartimento di Scienze Agrarie e degli Alimenti, Universita degli Studi di Modena e Reggio Emilia, via J.F. Kennedy, 17 - 42122 Reggio Emilia, Italy*
[3]*ZESPRI International Limited, via dei Rangers, 04012 Cisterna di Latina (LT), Italy*
[4]*Dipartimento di Colture Arboree, University of Bologna, viale Fanin 46 - 40127, Bologna, Italy*
Corresponding author: Joel.Vanneste@plantandfood.co.nz

Abstract Presence of *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, in pollen samples collected from infected and non infected orchards in Italy and in New Zealand was determined by polymerase chain reaction (PCR) and by direct bacterial isolation. Psa was isolated only from pollen samples collected in Italy, including pollen collected from two uninfected orchards, which the following year showed signs of infection. Psa was also detected in pollen collected from male and female vines in an Italian infected orchard. Pollen samples from Italy, but not from New Zealand, were collected with a vacuum device. Psa could not be isolated from any of the 25 New Zealand pollen samples analysed. This is the first report of Psa being associated with pollen. There is currently no evidence that artificial pollination leads to increased infection or that pollen has been responsible for the introduction of Psa in a previously Psa-free area.

Keywords bacterial canker of kiwifruit, pollen, artificial pollination, epidemiology, green fluorescent protein, flower infection.

INTRODUCTION

Most species of *Actinidia*, including *A. deliciosa* and *A. chinensis,* the two commercially most important species of kiwifruit in the world, are dioecious. This means a kiwifruit vine produces either male or female flowers. Fruit is produced when pollen from a male vine fertilises a flower from a female vine. Export quality kiwifruit contains between 1000 and 1400 seeds. Each seed is the result of a successful fertilisation by a single pollen grain. Therefore production of an export quality kiwifruit requires that a large number of viable pollen grains are deposited on the stigmas of a female flower (Howpage et al. 2001). Although kiwifruit flowers are fragrant, they do not produce nectar and therefore they are not very attractive to pollinating insects such as honey bees, yet pollination needs to occur in the relatively short period of time (ca 6 days) when the stigmas are receptive to pollen (Gonzalez et al. 1995). Since pollination dictates the size and

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the quality of the crop, in addition to honey bees, some kiwifruit growers have been using artificial pollination. This consists of spraying or dusting female flowers with pollen harvested either in male-only orchards or from the male vines located in commercial orchards.

Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) has had a major impact on the Italian production of kiwifruit, especially on *A. chinensis*, since an outbreak of this disease in 2009 (Vanneste et al. 2011). Although pollen of *Actinidia* spp. has never been shown to carry and/or vector plant bacterial pathogens, because artificial pollination is relatively widespread in the kiwifruit industry, it is important to determine whether pollen could be responsible for the dispersal of the inoculum and consequently of the disease. Pollen has been reported to be one of the sources of bacterial inoculum for *Xanthomonas arboricola* pv. *juglandis*, the causal agent of the bacterial canker of walnut (Ark 1944; Garcin et al. 2001). In this study samples of New Zealand pollen commercially available in New Zealand, pollen samples commercially available in Italy (including pollen originating from New Zealand), and pollen harvested from infected vines in Italy were analysed for presence of Psa.

MATERIALS AND METHODS

Pollen samples

Some pollen samples were collected in Italy. Those collected by Italian phytosanitary officers or by inspectors of the Italian extension services were analysed in Italy. The other samples were collected by the authors and were sent to the PC2 laboratory of Plant & Food Research in Hamilton, New Zealand. The samples analysed in Italy were representative of commercial pollen lots and were collected in male kiwifruit orchards in Emilia Romagna during the spring of 2010 (4 samples) or the spring of 2011 (8 samples). No symptoms of bacterial canker had been observed in any of the orchards at the time of collection. Two samples of New Zealand pollen commercially available on the Italian market in spring 2009 were also included in the study.

Samples sent to New Zealand for analysis were from commercial orchards of *A. deliciosa* 'Hayward' (three samples from male vines and one sample from female vine) and *A. chinensis* 'Jin Tao' (two samples) in which symptoms of the disease had been observed at the time of collection. These orchards were located in the Latina province, which is the major kiwifruit growing area in Italy and is the area where the Psa outbreak was first detected. Samples were collected using a vacuum device in the same way as pollen is collected commercially in Italy.

Twenty-five commercial samples of pollen harvested in 2010 from New Zealand were also analysed.

Isolation of Psa from pollen samples

Isolation of Psa from pollen samples was carried out in Italy and in New Zealand using two slightly different protocols. The small differences between these protocols reflect that they were developed independently. Both protocols aim to wash the bacteria off the pollen grains and use this washing for plating and/or PCR, but the protocol carried out in Italy contains an additional concentration step. The advantage or disadvantage of adding that step has not been examined.

For samples analysed in Italy, 1.5 g of dry pollen was washed with 20 ml of sterile saline solution (0.85% NaCl) in a 50-ml Falcon tube. The tubes were shaken for 60 min on a rotary shaker at 120 rpm. The pollen was then allowed to precipitate before the supernatant was filtered through a sterile gauze and centrifuged at 10,000 g for 20 min at 6°C. The supernatant was discarded and the pellet was rehydrated with 1 ml of sterile water. This solution is referred to as the final concentrate (FC). Ten to 20 µl of the FC and of a 10-fold dilution made in sterile distilled water were spread onto two plates of nutrient sucrose agar (NSA medium). Plates were incubated at 24°C for up to 72 h. From each plate five Psa-like colonies were chosen for further characterisation. Each pollen sample was analysed twice. When Psa was found in a sample, Psa-like colonies were counted after dilutions in order to assess the possible degree of contamination.

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For samples analysed in New Zealand, 500 mg of dry pollen was washed in a 30-ml bottle with 10 ml of sterile water and vortexed regularly for 30 min until the pollen was fully rehydrated. The rehydrated pollen was then dispensed in Eppendorf tubes and centrifuged for 90 s at 13,000 rpm. The supernatant and a 10-fold dilution made in sterile distilled water were used for PCR assays or for bacterial isolation. For bacterial isolation, 100 µl of the suspension to analyse was spread on plates of King's B medium (King et al. 1954) supplemented with 5 ml/litre of a 1% solution of cycloheximide. After 48 h of incubation at 28°C, five to six Psa-like bacteria, when present, were selected for further characterisation. When presence of Psa was confirmed, dilutions of the pollen washings were used to determine the degree of contamination by counting colonies showing a Psa-like morphology.

Biochemical characteristics of strains of *Pseudomonas* isolated from kiwifruit pollen

Psa-like bacterial colonies were characterised using biochemical assays that allow the differentiation of Psa strains from strains of other *Pseudomonas* species and strains of other *P. syringae* pathovars. Psa strains produce levan, induce a hypersensitive reaction (HR) when infiltrated in tobacco plants, do not have a cytochrome c oxidase or an arginine dehydrolase, and do not rot potato. These assays were conducted as previously described (Vanneste et al. 2010).

Detection and identification of Psa by polymerase chain reaction (PCR) in Italy

In Italy, presence of Psa in pollen samples was carried out using DNA extracted from the FC solution and DNA extracted as part of a Bio-PCR protocol. DNA from the FC solution was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's guide. In the Bio-PCR protocol, NSA plates inoculated with FC or a dilution of FC were grown for 48 h at 24°C before being washed with 1-2 ml sterile saline solution. DNA was extracted by adding 100 µl of a 0.5 M NaOH solution to 900 µl of this washing, and boiling the mixture at 95°C for 5 min. The suspension was then immediately cooled on ice for 30 min. Two µl of the boiled suspension was used directly for PCR.

Presence of Psa was detected by PCR using the primers KNF (5'-CACGATACAT GGGCTTATGC-3') and KNR (5'-CTTTTCAT CCACACACTCCG-3') developed by Koh & Nou (2002). Each reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM Promega dNTPs, 0.8 µM of each primer, 1.25 µl of DMSO and 0.2 µl of Promega Flexi Taq polymerase. After a pre-denaturation step of 5 min at 94°C, 30 cycles of 60 s denaturation at 94°C, 60 s annealing at 48°C and 60 s elongation at 72°C were carried out. The amplicons were purified using the i-PCR Purification Kit (Metabion, Martinsried, Germany) and separated by electrophoresis on a 2% agarose gel. The Psa strains NCPPB 3739 (also known as ICMP9617 and which is the pathotype of Psa) and DAFS 470 (a strain isolated in Italy in 2009) were used as positive controls. PCR was also performed with the primers PsaF3 (5'-ACCTTGGTGAAGTTGGTCAGAGC-3') and PsaR4 (5'-CGCACCCTTCAATCAGGATG -3'), which yield a 170 bp band with strains of Psa (Rees George et al. 2010), using the PSA Detection Kit (Ipadlab, International Plant Analysis & Diagnostics, Lodi, Italy).

BOX-PCR was used to confirm that strains isolated on plates were Psa. The BOX primer BOXA1R(5'-CTACGGCAAGGCGACCTGACG -3') was used with the BOX-PCR programme described by Louws et al. (1994).

Detection and identification of Psa by polymerase chain reaction (PCR) in New Zealand

Total DNA was isolated from pollen, pollen washes or from bacterial cultures with a cell disruptor FastPrep®-24 from MP™ using the ZR fungal/bacterial DNA kit™ from Zymo Research, USA. PCRs were performed on an Eppendorf Mastercycler® Gradient. The PCRs were carried out in 30 µl of 1× PCR buffer supplied with the DNA polymerase, containing 50 to 100 ng of

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DNA of the strain of interest, 0.8 μM of each primer, 2.5 mM of each dNTP and 1 unit of i-Taq™ from INtRON Biotechnology, Inc. A negative control, in which the DNA solution was replaced by the same volume of water, and a positive control, in which the DNA was that of Psa strain NCPPB 3739, were both used for each experiment.

The PCR products were separated by horizontal gel electrophoresis using a Biokey Super Screener 120 Electrophoresis system (Innovation Sciences Limited, Dunedin, New Zealand) on 1% or 2% agarose containing 10 ng/ml of ethidium bromide. From each reaction, 15–30 μl were loaded on the agarose gel and the DNA bands were visualised under UV light. On each gel, a DNA ladder (ZR 1 kb DNA ladder from Zymo Research, USA) was used for size comparison.

To identify strains of Psa, the primers PsaF1 (5'-TTTTGCTTTGCACACCCGATTTT-3') and PsaR2 (5'-CACGCACCCTTCAATCAGG-ATG-3') were used, as they yield an amplicon of 280 bp when tested on DNA from strains of Psa. The programme published earlier (Rees-George et al. 2010) was employed as modified by Vanneste et al. (2010). When BOX-PCR fingerprinting was used to confirm that strains isolated on plates were Psa, it was carried out as described above.

RESULTS AND DISCUSSION

Detection and identification of Psa in New Zealand pollen commercially available in Italy and in New Zealand

Only a few Psa-like colonies were isolated from the two commercial samples of New Zealand pollen available in Italy. However, further analysis showed that none of these colonies was Psa. In addition, no amplicon of the expected size was found after PCR using DNA extracted from the FC and using the Koh & Nou (2002) primers, or after Bio-PCR (Figure 1). Those pollen samples did not harbour Psa. Similar results were obtained in New Zealand with other New Zealand samples of pollen. Psa could not be detected by PCR on pollen washing from any of the 25 commercial samples of New Zealand pollen analysed in this study. No Psa-like colonies were isolated from any of the 17 of those 25 samples analysed by plating on King's B medium. When pollen was directly used for DNA extraction and PCR, in 10 out of 12 samples analysed a faint band of the expected size was observed. The fact that no Psa colonies were isolated from those same samples could simply reflect that in those samples Psa was dead or was below the threshold of detection. However, one cannot rule out that the positive PCR was a false positive, i.e. a non-Psa bacterium giving an amplicon of the expected size with the primers PsaF1/R2. Several strains of *P. syringae* belonging to pathovars other than *actinidiae* have been found to give a 280 bp fragment by PCR when using those primers (Rees-George et al. 2010; J.L. Vanneste, unpublished data). Previous experience in trying to detect and identify other plant pathogenic bacteria, such as *Erwinia amylovora* the fire blight pathogen, shows that PCR should not be used by itself for determining the presence of a specific bacterium in a sample (Vanneste 2008).

Figure 1 Agarose gel electrophoresis after polymerase chain reaction (PCR) using the primers specific for *Pseudomonas syringae* pv. *actinidiae*. Lane 1 and Lane 12: DNA ladder, Lane 2: *P. s.* pv. *actinidiae* NCPPB 3739 Lane 3: *P. s.* pv. *actinidiae DAFS 470*, Lane 4-7: New Zealand pollen samples commercially available in Italy, Lane 8-11: direct PCR on Italian pollen samples (2010), Lane 13-16: Bio-PCR on same samples as in Lanes 8-11, Lane 17-19: three Italian pollen samples collected in 2011, Lane 20: water control.

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Detection and identification of Psa in Italian pollen samples

The presence of Psa was detected by PCR in the two pollen samples collected in 2009 in a commercial 'Jin Tao' orchard in Latina, and in the two samples collected in 2010 from male opened flowers in 'Jin Tao' and 'Hayward' orchards. Psa was also detected by PCR in non viable pollen collected from female flowers. From those samples no colonies of Psa were found after plating. As mentioned previously this could reflect that in those samples Psa was dead or was below the threshold of detection but it cannot be ruled out that those results are false positives.

Other samples collected in Latina gave a positive reaction by PCR and gave bacterial colonies that were confirmed to be Psa by biochemical (produced levan, induced HR when infiltrated in tobacco plants, did not have a cytochrome c oxidase or an arginine dehydrolase and did not rot potato) and molecular characteristics (BOX-PCR). From one pollen sample collected with a vacuum device from a 'CK2' vine in an infected orchard, the population of Psa was estimated to be ca 10^6 colony forming units (cfu)/g pollen.

In two of the four samples collected in Emilia Romagna in 2010 and in one of the eight samples collected in 2011, Psa was detected after PCR and Bio-PCR. However, Psa was isolated from only the two samples collected in 2010. In the sample collected in 2011, as stated earlier, Psa was dead or below the threshold of detection or this was a false positive. In the samples from 2010, the concentration of Psa was estimated to be ca 7×10^3 cfu/g of pollen. The cultures obtained from those samples were confirmed to be Psa by biochemical (produced levan, induced HR when infiltrated in tobacco plants, did not have a cytochrome c oxidase or an arginine dehydrolase and did not rot potato) and molecular characteristics (BOX-PCR).

Two different BOX-PCR electrophoretic patterns have been described for Psa (Ferrante & Scortichini 2010; Vanneste et al. 2010). The strains of Psa isolated from Italy after 2008 have a pattern that is slightly different from the pattern of strains isolated from Asia or from Italy before 2008. All the strains isolated from pollen and characterised by BOX-PCR showed the same pattern as the strains isolated from Italy after 2008.

Potential role of pollen in the epidemiology of bacterial canker of kiwifruit

In two cases, Psa was found associated with pollen from Italian orchards where no symptom of bacterial canker had been detected at the time the pollen was harvested. A survey of the four orchards from which the pollen samples were harvested in 2010 was conducted in 2011. Symptoms of bacterial canker were present in the two orchards from which pollen was found contaminated with Psa, but not in the two orchards from which the pollen was free of Psa. This suggests that one of the first signs that an orchard is infected by Psa could be the presence of the pathogen on pollen. Alternatively, symptoms might have been present but not detected in those two orchards.

All the pollen samples from which live cells of Psa were found are samples originating from Italy, where pollen is collected by vacuum. One cannot rule out that the presence of Psa in some of those samples was the result of collecting extraneous material itself contaminated with Psa. However, on one occasion live cells of Psa were also isolated from pollen harvested from one unopened male flower (data not shown); if this can be confirmed it would suggest that Psa is able to colonise flower buds before they open. Whether Psa is directly associated with the pollen or collected at the same time as the pollen, pollen collected from an infected orchard could contain Psa and therefore presents the risk of distributing the pathogen to orchards not yet infected. So far, there is no proof that this ever happened.

The role of contaminated pollen in the spread of the disease has yet to be demonstrated, as is the role of the flower in the epidemiology of the disease. If Psa can infect the flower, pollen contaminated with Psa would be of a greater risk of propagating the disease. Using a derivative of Psa that expresses a green fluorescent protein

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(gfp) visible under UV light, it is possible to follow how Psa enters the plant and which tissues are colonised by the pathogen (F. Spinelli, unpublished data). Using such a derivative, it seems that Psa is able to colonise different parts of the flower and in particular the anthers, stigmas and calyx. It could be that Psa present on pollen somehow follows the germinating pollen and enters the flower through the stigma to the ovary. The use of this gfp-expressing Psa derivative should provide answers to those significant questions on the epidemiology of this economically important pathogen.

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REFERENCES

Ark PA 1944. Pollen as a source of walnut bacterial blight infections. Phytopathology 34: 330-334.

Ferrante P, Scortichini M 2010. Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. Plant Pathology 59: 954-962.

Garcin A, El-Maataoui M, Tichadou S, Prunet J.P, Ginibre T, Penet C 2001. Walnut blight, new knowledge for an old disease: summary of research (1995-2000). Infos-Ctifl 171: 27-30.

Gonzalez MV, Coque M, Herrero M 1995. Papillar integrity as an indicator of stigmatic receptivity in kiwifruit (*Actinidia deliciosa*). Journal of Experimental Botany 46: 263-269.

Howpage D, Spooner-Hart S, Vithanage V 2001. Influence of honey bee (*Apis mellifera*) on kiwifruit pollination and fruit quality under Australian conditions. New Zealand Journal of Crop and Horticultural Science 29: 51-59.

King EO, Ward MK, Raney DE 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory Clinical Medicine 44: 301–307.

Koh YJ, Nou IS 2002. DNA markers for identification of *Pseudomonas syringae* pv. *actinidiae*. Molecules and Cells 13: 309-314.

Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Applied and Environmental Microbiology 60: 2286–2295.

Rees-George J, Vanneste JL, Cornish DA, Pushparajah IPS, Yu J, Templeton MD, Everett KR 2010. Detection of *Pseudomonas syringae* pv *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S-23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. Plant Pathology 59: 453–464.

Vanneste JL 2008. Challenges in tracking the fire blight pathogen (*Erwinia amylovora*): a case study. In: Froud KJ, Popay AI, Zydenbos SM ed. Surveillance for biosecurity: Pre-border to pest management. New Zealand Plant Protection Soc. Inc., Christchurch, New Zealand. Pp. 29-36.

Vanneste JL, Kay C, Onorato R, Yu J, Cornish DA, Spinelli F, Max S 2011. Recent advances in the characterisation and control of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker on kiwifruit. Acta Horticulturae In Press.

Vanneste JL, Yu J, Cornish DA 2010. Molecular characterisations of *Pseudomonas syringae* pv. *actinidiae* strains isolated from the recent outbreak of bacterial canker on kiwifruit in Italy. New Zealand Plant Protection 63: 7-14.

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