Presence of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit, on symptomatic and asymptomatic tissues of kiwifruit

J.L. Vanneste¹, J. Yu¹, D.A. Cornish¹, S. Max² and G. Clark²

¹The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand

²ZESPRI International Limited, PO Box 4034, Mt Maunganui, New Zealand

Corresponding author: Joel.Vanneste@plantandfood.co.nz

Abstract *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, has recently been found in New Zealand. This pathogen has been the object of few studies and little is known about its epidemiology. Yet the development of an effective strategy of control requires an understanding of the life cycle of the pathogen, in particular determining the location of the bacteria in tissues. In this study, the presence of Psa on flowers, symptomatic and asymptomatic leaves and different parts of canes showing symptoms was determined by polymerase chain reaction (PCR) or by direct bacterial isolation. Psa was found associated with angular necrotic leaf spots, with white exudate and in canes. In canes, Psa seemed to be either in the parenchyma leading to production of exudate or in the vascular system leading to wilting. Psa was also found on asymptomatic tissues, where it was probably surviving as an epiphyte.

Keywords epidemiology, life cycle, control.

INTRODUCTION

A recent outbreak of bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), in Latina (Italy) has caused considerable damage to the Italian kiwifruit industry (Vanneste et al. 2011). The economic importance of this outbreak reflects that little is known about the epidemiology of the disease and emphasises that only a limited number of measures are available to control bacterial plant diseases. These control measures mostly focus on reducing the inoculum by cutting and removing the infected material from the orchard, and on preventing new infections by spraying antibacterial compounds before infection periods. Unfortunately, the number of

antibacterial compounds available for control of plant pathogenic bacteria is relatively limited and infection periods for Psa are not fully known. A better understanding of the epidemiology of the disease would help to limit its incidence by providing more information on the correct timing for the application of protective sprays and improved targeting of the appropriate plant material for removal to minimise inoculum.

Psa has only recently been detected in New Zealand (EPPO 2010) and this study was aimed specifically at determining, under New Zealand conditions, from which plant tissues, including asymptomatic tissues, the pathogen could be isolated. This will constitute the basis for future

epidemiology studies, which will allow better control of this devastating kiwifruit disease.

MATERIALS AND METHODS

Plant material analysed for presence of *Psa*

Plant material was sampled from commercial kiwifruit orchards located in the Bay of Plenty area of New Zealand. The samples were double bagged immediately after collection and sent by courier in a polystyrene chilly bin to the PC2 Plant & Food Research Laboratory located in Hamilton, New Zealand. Flowers with no obvious signs of infection were collected from *Actinidia deliciosa* 'CK2' male vines on 18 November 2010 from an infected orchard. Several hundred leaf samples were collected from early November 2010 until April 2011 from orchards in which *Psa* had been isolated or in which its presence was suspected (i.e. orchards located close to orchards where *Psa* had been isolated). Results for about 150 leaves from 64 orchards are presented. Most leaves were symptomatic, i.e. they had leaf spots. Leaf spots were characterised based on their morphology as elliptical, angular or irregular; large or pinpoint; with or without halo (Figure 1). However, some leaves without leaf spots were also analysed. Most of the asymptomatic leaves were from orchards where the disease or the pathogen had not been detected at the time of the sampling. However, asymptomatic leaves from two different orchards infected by *Psa* were also analysed. Ten canes were also sampled between November 2010 and April 2011. They were from infected commercial orchards from vines that showed symptoms of *Psa* either as leaf spots and cracking of the bark and necrosis (4 samples) or as shoots wilting (6 samples). White milky exudate found on infected canes was also analysed.

Isolation of *Psa* from plant tissues

Bacterial isolations from leaves were made by washing the leaves individually in a sterile bag containing 10 ml of sterile distilled water. After vigorous rubbing for 2 min, the bags were kept on an orbital shaker and then 100 µl of the washing was plated on King's B medium (King et al. 1954) containing 5 ml/litre of a 1% solution of

cycloheximide. After 28 h of incubation at 28°C, *Psa*-like colonies were purified twice on King's B medium before being characterised.

Isolations from flowers were made by washing together flowers from the same cluster. The petioles were removed but the sepals and petals were kept. A total of four lots of flowers were analysed. In lot 1, 24 flowers were washed in 30 ml of sterile distilled water. In lots 2, 3 and 4, 14, 8 and 20 flowers respectively were washed together in 20 ml of sterile distilled water. From each of the washings, 250 µl was plated onto plates of King's B medium supplemented with cycloheximide. Bacterial growth was swiped after 24 h of incubation and the total DNA was extracted for analysis by polymerase chain reaction (PCR) as described below.

Isolations from cane tissues were made by teasing out tissues to analyse in sterile water and streaking the resulting suspension on plates of King's B medium supplemented with cycloheximide. Tissues were taken at different distances (20 to 50 cm) from the symptoms. After 48 h of incubation at 28°C, *Psa*-like colonies were purified twice at 28°C on King's B medium before being identified and characterised by PCR as described below. All the strains were maintained on King's B medium at 28°C.

Identification of *Psa* by polymerase chain reaction (PCR) experiments

Identification of *Psa* by PCR was carried out as described earlier (Vanneste et al. 2010). Total DNA was isolated with a cell disruptor FastPrep®-24 from MP™ using the ZR fungal/bacterial DNA kit™ from Zymo Research, USA. Polymerase chain reactions (PCRs) were performed on an Eppendorf Mastercycler® Gradient. The PCRs were carried out in 30 µl of buffer supplied with the DNA polymerase containing 50 to 100 ng of DNA of the strain of interest, 10 pmol of each primer, 200 µM of each dNTP, and 1 unit of i-Taq™ from INtRON Biotechnology, Inc. For identification of *Psa*, the primers *Psa*F1 (5'-TTTTGCTTTGCACACCCGATTTT-3') and *Psa*R2 (5'-CACGCACCCCTCAATCAGGATG-3'), which yield a 280 bp amplicon with DNA from

strains of *Psa*, were used following the protocol described by Rees George et al. (2010) and modified by Vanneste et al. (2010). 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 a strain already identified as *Psa*, were both used for each experiment. For characterisation of the strains of *Psa*, a BOX-PCR was carried out using the BOX primer BOXA1R (5'-CTACGGCAAGGCGACCTGACG-3') with the BOX-PCR programme described by Louws et al. (1994).

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 to 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.

RESULTS AND DISCUSSION

Isolation of *Psa* in flowers

Presence of *Psa* was detected by PCR using the primers *Psa*F1/R2 in three of the four samples of flowers analysed (lots 1, 3 and 4). No attempt to isolate *Psa* was made. Presence of *Psa* on flowers is consistent with reports of symptoms on flowers caused by *Psa* (Serizawa et al. 1989). However, the flowers analysed in this study did not have obvious symptoms, suggesting that *Psa* might have been present as an epiphyte. The role of the flower in the epidemiology of the disease is not yet clearly understood.

Isolation of *Psa* in leaves

Psa was isolated from 83 of the 127 leaves showing angular necrotic spots delineated by the leaf veins (Figures 1a & 1b). On some occasions, the spots were surrounded by a yellow halo (Figure 1c). This halo is thought to be caused by bacterial toxins (Vanneste et al. 2011). At least two different toxins produced by *Psa* have been identified. Strains isolated from Japan have been found to produce phaseolotoxin but not coronatine while the

strains isolated from Korea produce coronatine but not phaseolotoxin (Han et al. 2003). Strains isolated from New Zealand have not been found to produce any of those two toxins (data not shown). Consequently, the cause of those haloes is not known. The size of the necrotic lesions from which *Psa* was regularly isolated varied from very large (Figures 1d & 1e) to very small (almost pin point) (Figure 1f). Sometimes the majority of the leaf surface was necrosed. These large necrotic lesions occurred as many small necrotic areas merged into each other. These symptoms can be called typical *Psa* symptoms. However, other pathogens, such as *Pseudomonas* sp. the causal agent of blossom blight on kiwifruit, can cause similar symptoms. Therefore, leaf spots should not be used as the only guide for the diagnosis of bacterial diseases on kiwifruit. The occasional inability to isolate *Psa* from such typical symptoms could be due to the symptoms being caused by another pathogen or to *Psa* being dead or below the threshold of detection because of some of the treatments carried out in the orchard before sampling (such as copper treatments).

Psa was isolated only in four of the 23 leaves showing a blackening of the veins (Figure 1g) or from leaves showing elliptical necrosed areas (Figures 1h & 1i). Often the elliptical necrosed areas crossed over leaf veins. These symptoms are not believed to be caused by *Psa*. The occasional isolation of *Psa* from such symptoms could be caused by the presence of this pathogen as an epiphyte (i.e. being present on the surface of the leaf without causing disease). Pathovars of *P. syringae* have been shown to be able to colonise host plant leaf tissues (Hirano & Upper 2000). It is therefore logical to assume that *Psa* could be present as an epiphyte on kiwifruit leaves. In support of this hypothesis, *Psa* was isolated from two symptomless leaves (leaves without any leaf spot) collected from orchards where the disease was present. *Psa* was never isolated from symptomless leaves sampled from orchards where the disease was not found.

The ability to multiply on the surface of the host plant without causing any symptoms has been demonstrated for many pathovars of

P. syringae and is central to the life cycle of those pathovars (Rudolph 2000). In the case of *Psa* it would allow the pathogen to multiply and colonise new parts of a vine or new vines, and to spread within the orchard inconspicuously. Those epiphytic populations can also constitute a source of inoculum that can be transferred by the movement of symptomless plants.

Isolation of *Psa* in canes

Psa was easily isolated from canes showing blackening, cracking of the bark and necrosis, indicating that those symptoms were probably caused by *Psa*. In two of the four canes analysed that showed those symptoms, *Psa* could also be isolated up to 50 cm away from the symptoms. The only bacteria recovered on King's B plates after streaking the white milky exudate produced by some canes were *Psa*. Therefore this exudate was a pure culture of *Psa*. To be present as a drop

of pure culture on the outside of the plant, *Psa* must have been in the cortical parenchyma below the bark. Other plant pathogenic bacteria, such as *Erwinia amylovora* the fire blight pathogen, produce a similar exudate and the presence of this exudate is explained by a rapid multiplication of the pathogen in the intercellular spaces of the parenchyma, leading to the bacteria being squeezed out of the intercellular space (Billing 2011). The bacteria move according to the path of least resistance, which in some cases will bring the bacteria further up or further down the cane, and in other cases will lead to the bacteria being pushed out of the plant as a drop of white exudate. These drops of pure bacterial culture therefore indicate that *Psa* was present and multiplying in the cortical parenchyma.

When analysing the six wilting shoots, *Psa* could not be isolated from the part that was wilted but was found in the samples taken

Figure 1 Kiwifruit leaves showing symptoms usually associated with presence of *Pseudomonas syringae* pv. *actinidiae* (*Psa*) (a to f), and symptoms usually not associated with presence of *Psa* (g to i).

further away from the shoot tip of the cane where the wood joined older wood. This suggests that the wilting was not caused directly by *Psa*, but was a consequence of the vascular system being blocked. In these instances, *Psa* must have been in the vascular system.

CONCLUSIONS

This study suggests that *Psa* can live or survive as an epiphytic bacterium on kiwifruit flowers or leaves. The inconsistent isolation of *Psa* from leaf spots with similar morphology indicates that presence of leaf spots is not a reliable diagnostic tool if used on its own. When present in the canes, *Psa* can either multiply in the parenchyma, leading to canker and production of a white milky exudate, or invade and block the vascular system, leading to wilted canes. These results support the hypothetic life cycle presented earlier (Vanneste et al. 2011), and might help in the development of management strategies for *Psa*.

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