Survival of *Pseudomonas syringae* pv. *actinidiae* on *Cryptomeria japonica*, a non-host plant used as shelter belts in kiwifruit orchards

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Abstract *Pseudomonas syringae* is a group of plant pathogenic bacteria that can survive in the environment outside their host plants. The environment can therefore act as a reservoir for the pathogen. The goal of this study was to determine whether *Cryptomeria japonica*, one of the most common plant species used in the Bay of Plenty as shelter belts between blocks of kiwifruit, could harbour *Pseudomonas syringae* pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit. To determine whether *C. japonica* could constitute a source of inoculum, potted plants maintained in a PC2 laboratory were inoculated with different concentrations of a streptomycin-resistant derivative of Psa. Populations of Psa were determined at regular intervals up to 18 days after inoculation. In all experiments, populations of Psa declined over time. Therefore, under laboratory conditions, *C. japonica* did not support the multiplication of the pathogen.

Keywords bacterial canker of kiwifruit, source of inoculum, epiphyte, epidemiology.

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), the causal agent of bacterial canker of kiwifruit, is currently the most economically important pathogen affecting kiwifruit production in the world. Originally identified in Japan (Takikawa et al. 1989), until 2010 it had been isolated only in Korea (Koh et al. 1994), Italy (Scortichini 1994) and China (Wang et al. 1992). Since 2010 the pathogen has been isolated from France (Vanneste et al. 2011c), Portugal (Balestra et al. 2010), Spain (Abelleira et al. 2011), Turkey (Bastas & Karakaya 2011), Chile (EPPO 2011a), Switzerland (EPPO 2011b), Australia (EPPO 2011c) and New Zealand (Everett et al. 2011; Vanneste et al. 2011a). In New

Zealand two populations of this pathogen have been identified and characterised (J.L. Vanneste, unpublished data). The most virulent strains present in New Zealand, called Psa-V, produce necrotic leaf spots, shoot die-backs and cankers. Vines of the most susceptible cultivars, such as 'Hort16A', can be killed within a season (Vanneste et al. 2011b). The economic importance of this disease can be illustrated by the fact that a year and half after its discovery Psa-V has infected 56% of the area grown with 'Hort16A' in New Zealand (Kiwifruit Vine Health 2012).

In New Zealand the pathogen was isolated from vines showing leaf spots or shoot die-back but also

from asymptomatic kiwifruit leaves, suggesting it was surviving as an epiphyte (Vanneste et al. 2011a). This ability of Psa to survive as an epiphyte on its host plant is a characteristic shared by many other pathovars of *P. syringae* (Hirano & Upper 1990; Rudolph 1995; Hirano & Upper 2000). Some strains of *P. syringae* can also survive epiphytically on non-host plants (Hirano & Upper 2000). This creates reservoirs of plant pathogenic bacteria that can act as a source of inoculum. Understanding whether such reservoirs exist in the case of Psa, and where they are, is very important to understanding the lifecycle of this pathogen and combating it more effectively.

This study aimed to determine whether Psa could multiply on *Cryptomeria japonica*, a non-host plant used extensively as a shelter belt in the Bay of Plenty. If Psa can multiply on this plant, then shelter belts of *C. japonica* or shelter belts containing this plant could constitute a reservoir of the pathogen, contributing to its spread and increasing the incidence of the disease. On the other hand, if Psa cannot multiply on this plant, then such shelter belts might protect the orchard from outside sources of inoculum and slow the rate of spread between orchards and orchard blocks.

MATERIALS AND METHODS

Strains and media

The strain 10627 of Psa from our laboratory collection was isolated in New Zealand in November 2010 from a 'Hort16A'-infected orchard. A spontaneous streptomycin-resistant derivative of that strain, called Psa 10627 Sm^R, was selected and used for all the experiments in this study. In King's B medium (King et al. 1954), Psa 10627 Sm^R was found to grow as fast as the wild type strain. Strains were grown and maintained at 28°C on King's B medium supplemented with streptomycin (100 ppm) when necessary.

Inoculation and determination of the bacterial population on *C. japonica* plants

Potted *C. japonica* were purchased from local nurseries and inoculated in the Physical Containment level 2 (PC2) laboratory of The

New Zealand Institute for Plant & Food Research Ltd at the Ruakura Research Centre. Inoculations were made by applying a suspension of Psa in sterile distilled water using a handheld sprayer. In the first two experiments, the suspensions were sprayed until run off. In the third experiment, the suspensions were sprayed only until leaf wetness. In each experiment, a plant similar in size and age to the plant or plants inoculated with Psa was treated with water only, as a negative control. After inoculation, the plants were kept in the PC2 laboratory at ambient temperature for the duration of the experiments. Kiwifruit inoculated with Psa and kept under similar conditions developed symptoms of bacterial canker within a few weeks. The population of Psa present on the plants at the different sampling times was determined by washing 1-g samples of plant tissues in 5 ml of water and by plating 1/10th dilutions of the washings made in 10 mM MgSO₄ onto King's B agar plates supplemented with streptomycin (100 ppm). The bacterial colonies were recorded after 48 h of incubation at 28°C.

The first experiment was a preliminary experiment to establish the methodology and how long Psa could be detected on *C. japonica*. A 90-cm plant was inoculated with Psa 10627 Sm^R at 6.6×10^8 colony forming units (cfu)/ml. Psa populations were determined daily on a single sample of plant material until 18 days after inoculation. In the second experiment, a 40 cm tall *C. japonica* was inoculated with Psa 10627 Sm^R at 9.6×10^9 cfu/ml. For each time point, three samples of 1 g each were taken and washed in 5 ml of sterile water as described above. In the third experiment, three different concentrations of Psa 10627 Sm^R containing 9.6×10^9 , 1.2×10^8 and 1.7×10^7 cfu/ml respectively, were sprayed onto two *C. japonica* plants. Populations were estimated daily from three replicates.

Identification of Psa by polymerase chain reaction (PCR)

Confirmation that the bacteria isolated from the *C. japonica* were Psa was carried out by polymerase chain reaction (PCR) using the primers PsaF1 (5'-TTTTGCTTTGCACACCCGATTTT-3') and

PsaR2 (5'-CACGCACCCTTCAATCAGGATG-3'), which yield a 280 bp amplicon with DNA from strains of Psa (Rees-George 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. PCRs were performed on an Eppendorf Mastercycler® Gradient, in 30 µl of the buffer supplied with the DNA polymerase containing 50 to 100 ng of DNA, 10 pmol of each primer, 200 µM of each dNTP, and 1 unit of i-Taq™ from INTRON Biotechnology, Inc. The protocol used was that 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 from a strain already identified as Psa, were included in 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 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

Psa or Psa-like colonies were not recovered from any of the plants treated with water only. Nonetheless, the identity of the bacteria recovered from inoculated plants was confirmed as Psa by PCR. In the first experiment, the bacterial population recovered from *C. japonica* inoculated with Psa 10627 Sm^R decreased rapidly after inoculation (Figure 1), falling from 5×10^6 cfu/g at 1 day after inoculation to 7×10^3 cfu/g at day 3. Between day 3 and day 14, the populations recovered from the sample plant varied widely, being several times below the level of detection (1.6×10^2 cfu/g), but at four sampling times, including at day 14, reaching populations above 2×10^3 cfu/g. This could indicate either that Psa was multiplying on this non-host plant or that it was unevenly distributed on the plant and the population being detected varied according to the sample being analysed.

To determine whether Psa was unevenly distributed on the inoculated *C. japonica* or was multiplying epiphytically on this non-host plant, in the second experiment three independent

Figure 1 Population of *Pseudomonas syringae* pv. *actinidiae* 10627 Sm^R (cfu/g leaf tissue) recovered from *Cryptomeria japonica* at different times after being sprayed until run off with a bacterial suspension containing 6.6×10^8 cfu/ml. The limit of detection is indicated by the horizontal dotted line.

samples were taken from the same plant at each time point. The population of Psa recovered at each sampling time point varied greatly between the replicate samples (Figure 2). In the most extreme cases, the population recovered from one sample was 100 times higher than that of another sample taken at the same time (e.g. days 1 and 4, Figure 2). This demonstrated that the distribution of Psa on the leaves of *C. japonica* was not uniform. This result suggests that the variability detected between sampling times between day 3 and day 14 in the first experiment was more likely to be the result of uneven distribution of the organism on the plant rather than its multiplication on the plant. The drooping habit of young *C. japonica* plants and the relatively tight overlapping spiral arrangement of the leaves could both contribute to the uneven distribution of inoculum on those plants following spray application.

In both experiments, the Psa populations decreased with time. In the first experiment, Psa population did not fall below the limit of detection until 11 days after inoculation (Figure 1), while in the second experiment, Psa could still be detected 9 days after inoculation (Figure

2). To determine whether the length of time Psa was detectable on *C. japonica* was related to the inoculum concentration being applied, three different concentrations of Psa 10627 Sm^R were sprayed until leaf wetness on to *C. japonica* plants. As expected, the higher the concentration of the inoculum, the higher the population of Psa recovered from the plant 2 h after inoculation (Figure 3). When lower inoculum concentrations were used, the Psa population was below the limit of detection (1.6×10^2 cfu/g) in the majority of the samples analysed 3 days after inoculation. However, independently of the concentration of the inoculum used to treat the plants, relatively large populations of Psa were recovered from random samples several days after bacterial populations in the majority of the samples were below the level of detection. This could indicate that some parts of the plant are more favourable than others for the survival of Psa. Nevertheless, results from the third experiment suggest that in the two previous experiments, the length of time Psa could be detected after application was in part the result of the concentration and volume of inoculum being applied to those plants.

Figure 2 Population of *Pseudomonas syringae* pv. *actinidiae* 10627 Sm^R (cfu/g leaf tissue) recovered from *Cryptomeria japonica* after being sprayed until run off with a bacterial suspension containing 9.6×10^9 colony forming units/ml. Three random 1-g samples were analysed at each time point; each open circle represents the population recovered from one sample. The limit of detection is indicated by the horizontal dotted line.

Figure 3 Population of *Pseudomonas syringae* pv. *actinidiae* (Psa) 10627 Sm^R (cfu/g leaf tissue) recovered from *Cryptomeria japonica* inoculated with different concentrations of bacteria. Each panel corresponds to the bacterial population recovered over time from a single plant. Panels (a) and (b) correspond to plants inoculated with a low concentration of Psa (1.7×10^7 cfu/ml), panels (c) and (d) to plants inoculated with a medium concentration of Psa (1.2×10^8 cfu/ml) and panels (e) and (f) plants inoculated with a high concentration of Psa (9.6×10^9 cfu/ml). Each open circle represents the population recovered from a 1-g sample of plant tissue. The limit of detection is indicated by the horizontal dotted line.

In all three experiments, the population of Psa recovered from *C. japonica* decreased over time, often falling below the level of detection before the end of the experiment. These experiments were carried out under environmental conditions that result in infection of kiwifruit by Psa and therefore in conditions conducive for Psa multiplication. The length of time Psa could be recovered from the plants was related to the initial amount of inoculum applied. It is concluded that the apparent increase in populations of Psa over time, as seen in the first experiment, was the result of variability between samples. There was no evidence of multiplication of Psa on *C. japonica*. In agreement with these results, Psa has never been isolated from shelter belts surrounding infected orchards during a limited survey conducted in the Bay of Plenty (J.L. Vanneste, unpublished data).

If the results of this laboratory study can be validated in the field, shelter belts of *C. japonica* could be beneficial by slowing the movement of Psa between orchards and orchard blocks. Shelter belts of *C. japonica* might capture Psa when airborne, preventing it from landing on kiwifruit plants. Once on the shelter belt, the Psa population might decrease and disappear as it has done under laboratory conditions. This would mean that shelter belts could provide some protection against Psa. However, a shelter belt is unfortunately not enough to prevent infection of a kiwifruit orchard by Psa, even if it might reduce the inoculum landing in the orchard. The creation of a microclimate by the shelter belt, which could result in either a decrease or an increase of infection, has not been looked at in this study. In conclusion, *C. japonica* might act as a temporary refuge for the pathogen but this laboratory study has shown that it may not constitute a long-term reservoir of Psa.

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