Preliminary investigations of the risk of plant debris as a *Pseudomonas syringae* pv. *actinidiae* inoculum source

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Abstract In New Zealand, the causal agent of kiwifruit canker, *Pseudomonas syringae* pv. *actinidiae* (Psa), can survive in overwintered kiwifruit plant debris on the orchard floor at least until spring. This study used trap plates and trap plants to determine the importance of fallen leaves and winter prunings on the orchard floor as inoculum sources. Trap plates were positioned 15, 50 and 100 cm above the debris. Psa was detected only when the plates were exposed during rain events and was found to be splashed upwards in small amounts from the debris during rain. Leaf lesions developed only on the trap plants exposed during the first week of the 20-week trial. In this trial, there is some evidence that plant debris is not a significant inoculum source and would not have posed a major risk for re-infection of any surrounding kiwifruit vines at the time of budburst in spring.

Keywords *Pseudomonas syringae* pv. *actinidiae*, Psa, bacterial canker, inoculum survival

INTRODUCTION

Kiwifruit canker, caused by the bacterial plant pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa), is a devastating disease of *Actinidia* spp. in a number of countries around the world. Psa biovar 3 was first found in New Zealand in 2010, in Te Puke in the Bay of Plenty region (Everett et al. 2011). In subsequent years, further infected orchards and regions were identified across the country. Psa is an ongoing threat to the New Zealand kiwifruit industry and a significant amount of research is under way to reduce the impact of Psa on kiwifruit production including identification of potential sources of inoculum within kiwifruit orchards.

Many pathovars of *Pseudomonas syringae*, particularly those associated with vegetable crops,

have been found to survive in plant debris. For example, *Pseudomonas syringae* pv. *lachrymans*, the cause of angular leaf spot of cucumber, can survive in plant debris for at least eight months after harvest (Bhat 2009), while Hollaway & Bretag (1997) confirmed that *Pseudomonas syringae* pv. *pisi* can survive on field pea trash for two seasons (78 weeks) and that infected pea trash is a potential source of inoculum for a following pea crop.

Bacterial canker of cherry trees can be caused by two different pathogens: *Pseudomonas syringae* pv. *syringae* (Pss) and *Pseudomonas syringae* pv. *morsprunorum* (Psm). Latorre & Jones (1979) found that Pss was able to be isolated from plant debris collected from under cherry trees, whereas Psm was not. They concluded that plant refuse

was a possible source for primary inoculum of bacterial canker caused by Pss, but not by Psm.

Previous work in New Zealand has shown that Psa survived in kiwifruit plant debris on the orchard floor for at least 15 weeks until spring (Tyson et al. 2012), while Aguín et al. (2015) found that in Galicia (Spain), Psa survived in the leaf litter for more than two months. Importantly, it is not known if this material poses a risk for re-infection of kiwifruit vines. This study used trap plates and trap plants to determine the importance of fallen leaves and winter prunings on the orchard floor as potential inoculum sources for reinfection of vines in the spring.

MATERIALS AND METHODS

Four wooden frames containing fallen kiwifruit leaves and pruning debris with symptoms of Psa infection were set up at Te Puke Research Orchard (TPRO) on Friday 27 July 2012. The trial area was at least 100 m away from the nearest kiwifruit canopy. This allowed the risk of debris as an inoculum source to be assessed in relative isolation from the risk of inoculum from infected vines. Buildings, roadways and shelterbelts adjacent to the trial area provided a further barrier to potential inoculum exposure from kiwifruit canopies in the vicinity.

Each frame was 1.2 m x 1.2 m and filled to a depth of approximately 20 cm with kiwifruit leaves with Psa leaf spot symptoms and cane/leader wood with dieback or orange ooze/cankers. Plant debris was contained in the frames with wire netting (mesh size = 5 mm).

Sets of trap plates and trap plants were exposed in the field from 30 July 2012 until 14 December 2012 (20 weeks).

Trap plates

Methods have been previously developed to capture and identify Psa from the air using passive aerosol traps (Curtis et al. 2013; Tyson et al. 2013). These methods rely on Petri plates containing an agar medium semi-selective for *Pseudomonas syringae*, a slight modification of KBC (Mohan & Schaad 1987), using sucrose

nutrient medium as the base instead of King's B medium. Two types of trap were used:

- 1) A metal stake with fixed positions for inverted plates of modified KBC medium at three heights (15 cm, 50 cm, 100 cm above the debris) to detect upwards 'splash-dispersal' of the bacteria, and,
- 2) A swinging vane aerosol trap with a Petri plate of modified KBC medium attached to a weather vane on the top of a 1 m metal stake to detect windborne inoculum.

One set of fixed downward facing traps was placed in the centre of each debris frame. Weather vane-style traps were set up around the outside of the frames (3 m distance) in four positions (N, S, E and W).

Plates were put out four days in each week (Monday–Thursday) and removed after 24 h (0900 h - 0900 h), for the duration of the 20-week trial. After removal from the traps, the plates were incubated at 20°C for three days.

DNA extractions were made on the mixed-colony plates. These were then tested for the presence of Psa by quantitative Polymerase Chain Reaction (qPCR), as detailed below.

Trap plants

Tissue-cultured plantlets of *Actinidia chinensis* var. *chinensis* 'Hort16A' were potted into 8-cm diameter planting bags and grown in a glasshouse in a Psa-free area (average temperature of $25 \pm 5^\circ\text{C}$). At approximately 3 months post-tissue culture, plants were 30-40 cm high and with 10-15 leaves. To obtain plants as uniform as possible, plants were tipped above 40 cm in height. One plant was placed in each frame each week. The plants were placed in the frames each Monday morning and were collected on Friday morning. After exposure in the field, the plants were incubated in isolation from further infection for 21 days and then assessed for symptom expression (presence/absence of leaf spots). Bacterial isolations were made from any leaves that developed leaf spots typical of Psa to confirm the presence of the pathogen.

Bacterial isolation and enrichment were carried out as follows: pieces of leaf tissue were aseptically

Table 1 Upwards dispersal of viable *Pseudomonas syringae* pv. *actinidiae* (Psa) to inverted Petri plates from kiwifruit plant debris held in frames over the period 30 July–14 December 2012.

Location	Height above plant debris (cm)	Petri plates testing positive for Psa*
Position 1	15	4
Position 2	50	2
Position 3	100	0

*Live Psa was detected on plates in weeks 2, 3, 4 and 10

Table 2 Capture of viable *Pseudomonas syringae* pv. *actinidiae* (Psa) splashed from kiwifruit plant debris held in frames in the orchard using trap plants and inverted petri plates over the period 30 July–14 December 2012.

Beginning of each 4-day exposure period	Trap plants testing positive for Psa	Petri plates testing positive for Psa	Total rain over each 4-day exposure period (mm)
30 Jul 2012	2	0	106.2
06 Aug 2012	0	1	14.0
13 Aug 2012	0	2	11.1
20 Aug 2012	0	2	16.0
27 Aug 2012	0	0	1.4
03 Sep 2012	0	0	25.2
10 Sep 2012	0	0	1.1
17 Sep 2012	0	0	0.1
24 Sep 2012	0	0	2.2
01 Oct 2012	0	0	5.5
08 Oct 2012	0	1	12.8
15 Oct 2012	0	0	16.4
23 Oct 2012	0	0	0.4
29 Oct 2012	0	0	0.0
05 Nov 2012	0	0	0.0
12 Nov 2012	0	0	8.4
19 Nov 2012	0	0	0.0
26 Nov 2012	0	0	0.0
03 Dec 2012	0	0	21.5
10 Dec 2012	0	0	15.7

excised and macerated in 2 ml bacteriological saline (BS) (0.85% NaCl in sterile distilled water), and left for at least 5 min. A 200 µl aliquot of the resulting suspension was streaked onto modified KBC medium. Plates were incubated at ca 20°C for 48 h.

The isolation plates were incubated at 20°C for 72 hours and then assessed for bacterial growth. DNA extractions were made on the mixed-colony plates and qPCR identification carried out.

Debris samples

Isolations and qPCR identification were carried out weekly on the plant debris below the traps (the inoculum source). Single leaves were taken from each frame at each sampling date and processed as for the trap plant leaf spots.

DNA extraction and qPCR analysis

DNA extraction, and qPCR conditions and analysis, were as described by Tyson et al. (2012), using the primers PsaF3 and PsaR4 developed by Rees-George et al. (2010). In addition, bacterial 23S primers (Anthony et al. 2000) were used in qPCR as an internal control to check that the DNA was PCR-competent.

In this study, a Cp value (crossing point or

threshold value) below 30 was interpreted as a Psa-positive result, 30–35 as a weak positive, and a Cp value above 35 as a negative result.

Weather data

Weather data, including hourly temperatures, rainfall and wind direction, were collected over the period of trapping from a NIWA weather station immediately adjacent to the trial site, and compared with trap data. The weather data were downloaded from the National Institute of Water and Atmospheric Research Ltd, the National Climate Database (CliFlo) for the period of the trial (<http://cliflo.niwa.co.nz/>).

RESULTS

Trap plates

Few trap plates tested positive for Psa over the 20-week trial (Table 1). Psa was identified from trap plates exposed during the first 4 weeks of the trial (30 July–20 August 2012), but only once thereafter. Psa was detected on the fixed inverted trap plates only during rain events. However, they were not found during every rain period. Psa was not detected

Figure 1 Frequency of isolation of viable *Pseudomonas syringae* pv. *actinidiae* (Psa) from four separate samples of kiwifruit leaf litter held in frames in the orchard over the periods 25 May–1 September 2011 and 30 July–14 December 2012. The 2011 data are from Tyson et al. (2012).

on the fixed plates 100 cm above the debris and only rarely on plates 15 and 50 cm from the debris.

Trap plants

Psa leaf spotting developed only on plants exposed during the first week of the trial (Table 2). During the four days that these plants were exposed, 106 mm of rain was recorded, over four times as much as the next wettest week.

Debris samples

The level of Psa in the leaves appeared to be at or below the level of detection over the 20-week period and consequently there were very few detections of live Psa from the leaf debris isolations. A comparison of these data and the 2011 leaf debris isolation results is shown in Figure 1.

DISCUSSION

Psa is known to be able to survive in kiwifruit plant debris on the orchard floor for several months (Tyson et al. 2012; Aguin et al. 2015), but it is not known if this material poses a risk for re-infection of kiwifruit vines. This trial aimed to determine the importance of overwintered kiwifruit plant debris as a Psa inoculum source in spring.

Psa was splashed upwards from the debris during rain, albeit rarely, and it was never splashed higher than the second trap plate (50 cm above the debris). A typical pergola-grown kiwifruit canopy is approximately 180 cm above the orchard floor, well above the highest detected splashed Psa. Furthermore, Psa was detected on the trap plants only during the first week of the trial.

In a similar study on the *Pseudomonas syringae* pathovar causing tip dieback and leaf spotting of maple, Malvick & Moore (1988) used a marked strain to demonstrate upwards dispersal of the bacterium from inoculated grass in a maple nursery. Nearly all the airborne bacteria detected were captured 12 cm above grass during wind-driven rain or sprinkler irrigation.

The survival of Psa in fallen leaves and cane prunings was previously investigated during winter 2011 (Tyson et al. 2012). In this trial, for practical reasons only the leaves were assayed for live Psa

over time. The current trial was started 10 weeks later in the season than the 2011 trial in order to coincide with budburst, and the first assays corresponded with the time at which there had been a significant decline in recovery of Psa in the 2011 trial. In both trials, Psa appeared to be at or below the level of detection by that time.

The plant debris in this trial appears to have constituted a low risk in terms of an inoculum source and would not have posed a major risk for re-infection of any surrounding kiwifruit vines at the time of budburst (September). Further research is required to determine if there are specific weather conditions that allow commercial kiwifruit vines to be infected by Psa-infected plant debris. Based on the personal observations of the authors, sources of inoculum that are likely to pose a greater risk are the bacterial exudates from cankers and subsequently the exudates that can form on the underside of Psa leaf spots from early spring and through the summer.

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