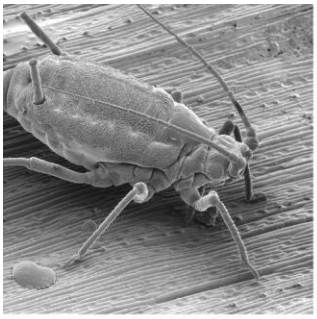
PFR SPTS No. 10209

Psa leaf spots as inoculum sources

Casonato S, Bent S, Horner I

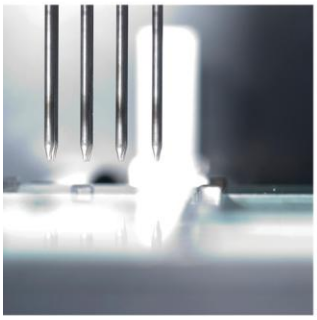
June 2014





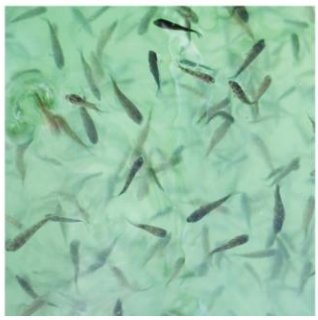
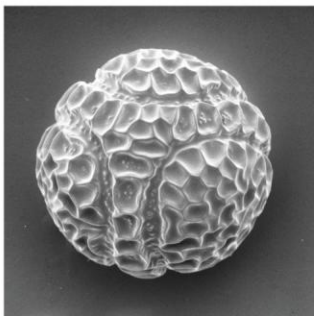










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Contents

Executive summary

Psa leaf spots as inoculum sources

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Many kiwifruit vines in *Pseudomonas syringae* pv. *actinidiae* (Psa) infected orchards have leaves with Psa spots. In some 'Hayward' orchard more than 50% of leaves have spots caused by Psa. If these lesions are producing Psa bacteria, these are potentially massive inoculum sources.

This project has shown that leaf spots continually produced inoculum between November 2013 and March 2014. Under humid conditions, even 'old' leaf spots were capable of producing ooze at the edge of the lesion, containing large numbers of viable bacteria. Thus, leaf spots are a continual source of Psa inoculum during the spring and summer seasons.

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1 Introduction

Controlling inoculum is a critical aspect of disease management. Inoculum can be produced from a variety of sources for any one disease. On many kiwifruit (*Actinidia* spp.), *Pseudomonas syringae* pv. *actinidiae* (Psa) produces cankers that produce bacterial ooze as well as multiple leaf spots. Emphasis has been placed on removing kiwifruit vines with oozing cankers as a means of reducing inoculum, with Psa leaf spots gaining much less attention.

Very high numbers of leaf spots may be present within Psa-infected kiwifruit orchards. The aim of this project was to determine the role of leaf spots found on 'Hayward' vines as sources of inoculum during the spring and summer periods and for how long individual spots were able to produce inoculum.

2 Materials and Methods

2.1 Field sampling of leaf spots

The production of Psa inoculum from leaf spots on *Actinidia deliciosa* 'Hayward' vines grown in the Te Puke Research Orchard was assessed from November 2013 to March 2014. Individual spots were marked as they appeared on two flushes of leaves in late October/early November (cohort one) and late November/early December (cohort two). Each leaf was tagged and photographed (Figure 1). Spots ranged in size from c. 0.5. to 1 mm^2 when they were first marked. Each marked leaf spot (68 in total) was swabbed once on the upper surface of the leaf and once on the lower surface of the leaf, using a sterile swab (cultiplast® SWAB, LP Italiana Spa). Swabs were returned to a tube containing 2-mL sterile bacterial saline and stored at -20°C for subsequent validation by polymerase chain reaction (PCR). Repeated swabs of each marked leaf spot were made every two to four weeks until February 2014. In total, 68 leaves were swabbed: 39 leaf spots from cohort one were tracked from 11 November 2013, and 29 spots from cohort two were tracked from 12 December 2013.

Figure 1. An *Actinidia deliciosa* 'Hayward' leaf with the spot (circled with red) swabbed each sampling time.

2.2 Bacterial viability

Bacterial viability was tested on cohort one leaves from three sampling dates: 11 November 2013, 23 December 2013, and 5 February 2014. Samples were plated onto Kings B-C medium and colonies that had similar features to a known Psa control plate were counted. For each sampling date, 10% of the samples were randomly chosen to confirm the colonies being counted were Psa using qPCR.

2.3 DNA extraction

2.3.1 Swab extractions

For DNA analysis of leaf spot swabs, each swab was thawed and the end of the swab (15 mm, diameter 4 mm) was cut off and placed directly into a 1.5 mL Eppendorf tube containing 200 µL of 10% Chelex (BioRad Chelex 100) and vortexed vigorously. The Chelex extraction process was as follows: the sample was vortexed vigorously for 30-60s. The tube was heated to 100°C for 10 minutes, removed and vortexed vigorously and then heated for a further 10 minutes at 100°C. The sample was centrifuged at 13,500 rpm for 10 minutes and the supernatant was removed and placed in a clean, new tube for further processing.

2.3.2 Leaf washing

At the same time the initial swab samples were taken in November 2013, ten leaves without any visible leaf spots were selected and washed with 10 mL sterile bacterial saline, with the runoff captured in sterile tubes. For each of the samples 100 µL was added to 200 µL of 10% Chelex. The samples underwent the Chelex extraction procedure outlined above. The supernatant was removed and placed into a new tube which was stored at -20°C for subsequent PCR validation.

2.3.3 Excised leaf spots

On 15 April 2014 all marked leaves which were still present on the vines were detached and photographed (Figure 2). Each marked leaf spot was excised, ground in liquid nitrogen in two freeze/thaw cycles and deposited into a 1.5 mL Eppendorf tube that was placed in -20°C. Samples had DNA extracted on the 29 and 30 April 2014. The homogenised material was removed from the freezer and thawed. Plant material was placed into 200 µL of 10% Chelex and underwent the Chelex extraction procedure outlined above. The supernatant was removed and placed into a new tube for processing with qPCR. (Figure 2 below over two pages.)

A

B

C

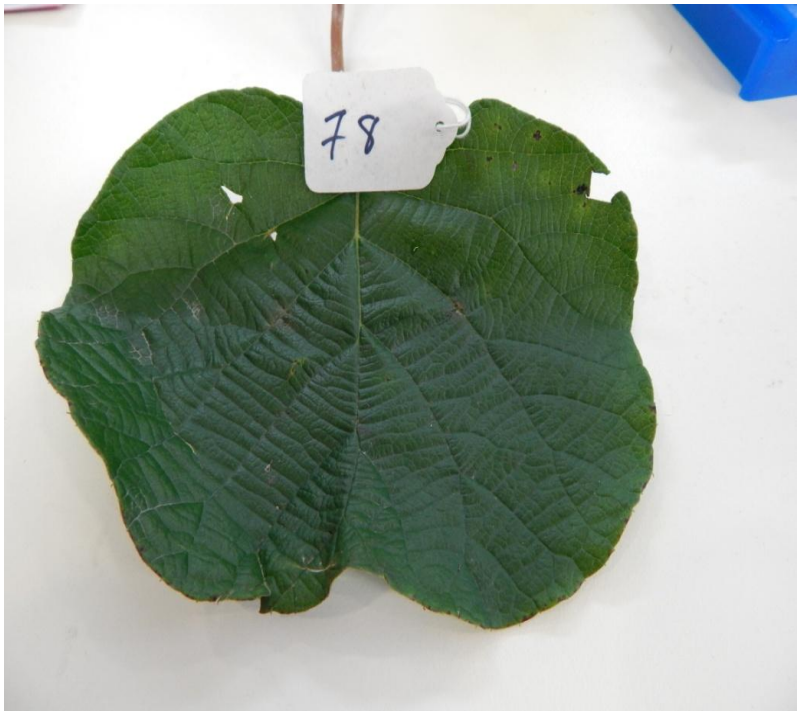

Figure 2. *Actinidia deliciosa* 'Hayward' Leaf 78 from cohort two when leaf spots began to emerge in late November/early December 2013. A. Leaf 78 in the field in December 2013. B. Leaf 78 at the conclusion of the trial with the leaf removed in April 2014 before excision of leaf spot. C. Leaf 78 post leaf spot excision in April 2014.

2.4 Quantitative polymerase chain reaction (qPCR)

qPCR and data analysis were performed on a Rotor Gene 2-plex. The qPCR was performed using the Rotor-Gene® SYBR Green® PCR kit (Qiagen). The primers used were hopA1F2: 5'-GCCTCGATGTCGGCGC-3' and hopA1R1: 5'-ATTCGATAGAAGAACTTCTTTGCGTTT-3' (Rikkerink et al. 2011). Each 10 µL reaction contained 2.5 µL DNA template, 5 µL 1x Rotor-Gene SYBR Green, 0.5 µL 10 µM hopA1F2, 0.5 µL 10 µM hopA1R1, and 1.5 µL sterile water. qPCR conditions were as follows: 10 min at 95°C to activate the hot-start *Taq* polymerase, 35 cycles of 95°C for 5 s, 64°C for 10 s and 72°C for 15 s, with fluorescence acquisition following each 72°C step. Following this a melt was performed during which the temperature was increased at 1°C per 5 seconds from 72°C to 95°C, with continual fluorescence acquisition. Each run consisted of 36 samples, which included a six-point standard curve and a non-template reagent control. Data were analysed using QIAGEN Rotor-Gene software version 2.1.0. For quantification analysis a threshold of 0.05 was applied to each run.

Approximate colony forming unit (cfu) concentrations per mL were calculated by comparing the crossing threshold (Ct) value obtained from the known bacterial standards of Psa that were made to $10^{8,}$ $10^{7,}$ 10^{6}, 10^{5}, 10^{4}, 10^{3} cfu/mL. A calculation was made using the Rotor-Gene software that compared the known concentration of the standards against the Ct value derived from the qPCR run. The unknown sample Ct values were obtained from the run and the estimated cfu per mL of each sample was calculated.

2.5 Copper treatment

On 19 December 2013 a Nordox® 75WG + DuWett® mix was applied to the orchard by the Te Puke Research Orchard staff. Half the trial leaves were covered with plastic bags one hour before the spray application to prevent exposure to the chemical. Bags were removed 24 hours post spray application. There were no other copper applications to the trial block during the course of the trial.

2.6 Leaf incubation

In November, December, January and March, leaves (total of 24) with spots were detached from 'Hayward' vines in the trial area. The leaves came from the same shoots as those marked for swabbing. They were incubated in a humid chamber for 48 hours then removed and observed microscopically for bacterial ooze. Areas of ooze were sampled and plated onto Kings B-C medium to confirm the presence of Psa. Colonies resembling Psa that grew on the Kings B-C medium were removed and placed into sterilised water and put into -20ºC for subsequent processing. The Chelex extraction method was used to extract DNA before amplification using qPCR.

2.7 Statistical analysis

Before statistical analysis, the concentration value obtained for each of the samples by qPCR was transformed by $\log_{10}$(concentration +1). The logged data were analysed using restricted maximum likelihood (REML). The models that were examined included fixed effects for leaf cohort (cohort one in November 2013, or cohort two in December 2013), copper treatment (no copper or copper applied), and collection date (date of leaf spot swabbing). Leaves sampled came from two distinct cohorts: cohort one initially sampled in November 2013 when the first leaf spots appeared, and cohort two on a later flush of leaves, initially sampled in December 2013 when the first leaf spots appeared. The cohorts also differed in their potential exposure to the copper spray (see above). The leaf from which the swab was obtained was treated as a random effect in the analysis.

3 Results and discussion

Leaf spots produced inoculum throughout the spring/summer season and the bacteria within the leaf spots were viable.

Checks of bacterial viability in November, December and February indicated that on each occasion viable bacteria could be detected in leaf spot swabs. This demonstrated the presence of viable inoculum on these leaf spots throughout the season.

Detached leaves placed in a humid chamber showed that Psa was present on the leaves throughout the spring and summer seasons and the bacteria were viable. Ooze was produced even on spots that were 2 or 3 months old. The concentration of the bacteria in the leaf spot ooze was estimated at 2×10^8 cfu/mL. This result indicates that under suitable conditions, bacterial ooze can be produced on or around leaf spots. The usual pattern is for ooze to be produced from the green tissue at the margin of the spot, which is the only place the pathogen can be active. The centre of the spot, where there is necrotic tissue, is not amenable to bacterial multiplication. Thus, leaf spots can be inoculum sources for further infections.

Background populations of Psa on 10 symptomless leaves on 11 November 2013 indicated that Psa was present even on unspotted leaves.

There were significant ($P<0.001$) differences in the number of Psa colonies found at each sampling time, regardless of whether they were from cohort one or two (Figure 3). For each of the cohorts separately, there were no significant ($P>0.05$) differences in Psa colony numbers that were calculated against the known Psa standards used in the qPCR. The trends were similar for each of the cohorts, with a high initial population of Psa that remained for at least a month after the appearance of the spots, then a gradual decline in the numbers of Psa bacteria being produced.

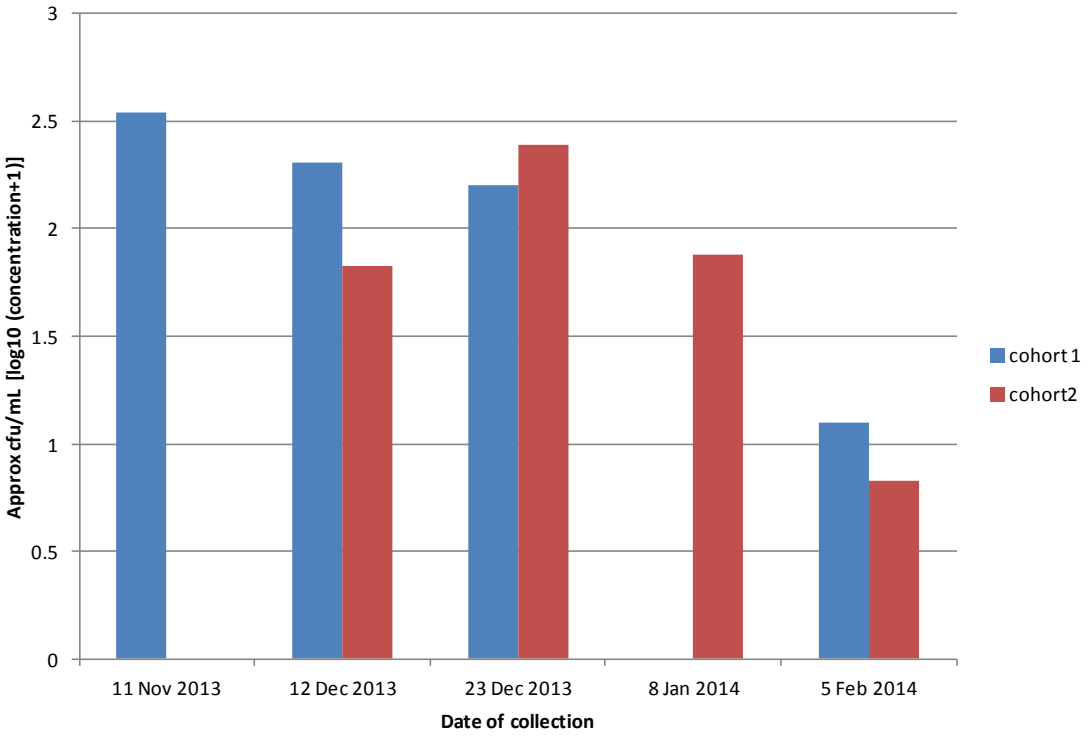

Figure 3. The approximate number of colony forming units of *Pseudomonas syringae* pv. *actinidiae* (Psa) per m, presented as log-transformed data. Leaf spots on 'Hayward' kiwifruit vines were swabbed on the lower and upper surface (in total two swabs) of the leaf at five collection dates. Leaves sampled came from two cohorts: cohort one initially sampled in November 2013 when the first leaf spots appeared, and cohort two on a later flush of leaves, initially sampled in December 2013 when the first leaf spots appeared.

The trial leaves that remained attached to the vine at the conclusion of the trial had the marked leaf spot excised in April 2014 and processed using qPCR. Despite there being very few Psa bacteria detected on leaf spots at the conclusion of the swabbing trial (February 2014), analysis of excised leaf spots showed that Psa were present inside the leaf (Figure 2). This indicates that the Psa had penetrated into the leaf and survived for long periods.

The copper treatment applied on 19 December 2013 had no effect in reducing the number of Psa bacteria detected by the leaf spot swabs. Analysis of each cohort separately indicated there was no statistical difference (P>0.05) in the bacterial counts between the copper and non-copper-treated leaves (Table 1).

Table 1. Log-transformed concentrations (cfu per mL) of *Pseudomonas syringae* pv. *actinidiae* (Psa) bacteria swabbed from leaf spots from two cohorts of *Actinidia deliciosa* 'Hayward' leaves, representing differing Psa leaf spot emergence times, over five periods from November 2013 until February 2014. Leaf treatments were with and without the application of copper. Nordox® 75WP was applied to the treated leaves on 19 December 2013.

		cohort1	cohort2
11 November 2013	No copper	2.668	*
	Copper	2.407	*
12 December 2013	No copper	2.407	2.687
	Copper	2.21	1.024
23 December 2013	No copper	2.211	2.419
	Copper	2.188	2.358
8 January 2014	No copper	*	1.585
	Copper	*	2.178
5 February 2014	No copper	1.161	0.278
	Copper	1.036	1.297

4 Concluding remarks

This project has shown that during spring and summer, leaf spots are an ongoing source of viable Psa inoculum in 'Hayward' kiwifruit. The numbers of bacteria produced by the spots remained high for a month or two after spot appearance, then generally decreased over the rest of the season. Under suitable environmental conditions, spots are capable of producing large numbers of bacteria. Thus, Psa spots on leaves constitute significant sources of inoculum over the growing season in kiwifruit orchards, and this needs to be factored into any management regime in a kiwifruit orchard.

5 Reference

Rikkerink E, Andersen M, Rees-George J, Cui W, Vanneste J, Templeton M. 2011. Development of a rapid tool for the molecular characterisation of Psa haplotypes. A report prepared for Zespri Group Limited. Ref VI1256. SPT No. 6361.

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