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VI1505: Determine the source of leaf inoculum in Hayward: from bud break to senescence

Casonato S, Kabir S, Parry B, Bent S, Fullerton RA

December 2015















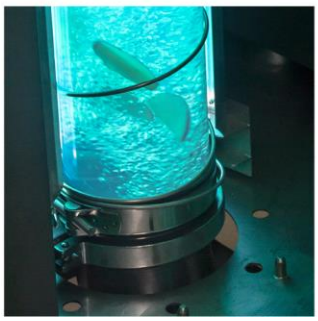


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CONTENTS

EXECUTIVE SUMMARY

VI1505: Determine the source of leaf inoculum in Hayward: from bud break to senescence

Casonato S[1], Kabir S[1], Parry B[1], Bent S[1], Fullerton RA[2]
Plant & Food Research: [1]Te Puke, [2]Auckland

December 2015

This project is aimed at understanding the sources of inoculum *Pseudomonas syringae* pv. *actinidiae* biovar 3 Psa (Psa) in *Actinidia chinensis* var. *deliciosa* found on 'Hayward' kiwifruit during the time from bud break to leaf senescence.

The work was carried out at the Plant & Food Te Puke Research Orchard (Block 50) under field conditions during 2014–2015.

The aims of the study were:

1. to determine whether the first leaf spotting seen in the spring is caused by bacteria coming from bark and cankers on the vines or from bacteria surviving inside the buds --
2. to examine how to manage inoculum from external sources
3. to determine the viability of Psa in leaf lesions from bud break to leaf senescence.

Psa associated with buds. The results show that bacteria is present on both the outside of the buds and within the buds themselves as they were expanding in the spring. The overall amount of Psa detected was low however Psa was found more frequently from juvenile tissues from inside the buds (10.3%) than from the outside (5.1%). Consequently it appears that leaf spotting on the first expanding young leaves in the spring could be caused be either Psa from within the bud or Psa arriving on the expanding leaves. Critically from this work as Psa was found in the protected environment under the bud scales meaning that protectants applied prior to bud burst may not provide adequate control and growers need to consider protecting vines from both internal and external Psa sources.

Psa associated with leaf spots. Psa was found associated with leaf spots throughout the whole period of the study. The bacteria were typically found in the healthy tissues surrounding the spot and are exuded at the margin of the lesion. Bacteria were detected more often and in greater numbers in the early spring. By mid-summer very few viable bacteria were detected on the surface of lesions. Leaf spots appear to be a source of Psa, particularly in spring, controlling Psa from leaf spots is likely to be important in minimising reinfection or movement onto other vines.

Effect of covering shoots to exclude inoculum. Covering of young shoots, though incomplete, did result in reduced the amount of bacteria on the leaf surface compared when compared to uncovered shoots.

Future considerations

1. More work is needed to confirm an endophytic origin should focus on assays of excised cane tissue below buds and on internal tissue of petioles of juvenile leaves.

2. The method used to assess viability in this study was subject to error related to the limits of detection of low populations of bacteria using swabs. Future work should focus on the development and application of viability PCR technology, a method to discriminate between DNA from living and dead organisms. That technology is now available at Te Puke Research Station and able to be used in future studies of this kind.

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1 INTRODUCTION

Kiwifruit leaf spotting caused by *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa) first appears on young leaves from early spring. Some early spring infections are being caused by Psa inoculum coming from the surrounding orchard environment most likely from canes and cankers on stems (Tyson et al. 2015). However field observations on the distribution of leaf spotting in orchards in early spring on *Actinidia chinensis* var*. chinensis* 'Hort 16A' have shown that in some cases there is extensive leaf spotting on new leaf clusters soon after bud break on canes in one vine but none on immediately adjacent canes from a different vine (Figure 1. Horner unpublished data).

Figure 1. Adjacent vines of Hort 16A, vine on left with leaf spots and wilting of young leaves and vine on right healthy. Photo Ian Horner.

That pattern indicates that the symptoms in the vines on the left were caused by bacteria growing inside the cane and moving out into the leaves as they formed and not from airborne bacteria directly infecting leaves.

In a study over the 2013–14 season, Casonato et al. (2015) showed that large amounts of bacteria were produced on leaf lesions. It is formed as bacterial ooze at the margins of leaf spots. The amount of ooze and the viability of bacterial cells was highest during the first two months of the season and declined as the lesions aged. However, viable inoculum could be produced on lesions throughout the summer. While few viable bacteria could be detected on the surface of lesions by February 2014, viable bacteria could be detected readily from excised spots. That study showed that bacteria can survive the full season in the tissues immediately surrounding the leaf spots.

In a study of Psa in canes and buds of *A. chinensis* var. *deliciosa* 'Hayward' over the winter period May to October 2013, Tyson et al. (2015) showed that Psa could be detected intermittently on the outside of dormant buds and occasionally (but very rarely) on the inside. The extent to which these bacteria located on and within buds contributes to leaf spot symptoms is unknown. The current study aimed to investigate the question of whether the first leaf spots in the spring are the result of infections from external inoculum or whether they are the result of internal colonisation of buds prior to expansion.

The aims of the research were:

1. to determine whether initial leaf spotting in spring is caused by bacteria spread from the outside of canes in the orchard or from bacteria surviving inside the buds.
2. to examine how to manage inoculum from external sources
3. to determine the viability of Psa in leaf lesions from bud break to leaf senescence.

The study was for the period from bud break in 2014 to mid-summer (February) 2015.

2 MATERIALS AND METHODS

2.1 Treatments and field sampling

The research was conducted on vines of a 'Hayward' in block 50 of the Plant & Food Research Te Puke Research Orchard (TPRO) over the 2014–2015 growing season.

Studies were carried out on inoculum associated with buds before bud burst, and inoculum production by individual lesions over the period from spring to mid-summer.

Inoculum associated with buds. Buds were assayed for the presence of Psa during the early stages of bud swelling. Over the period from 26 to 30 September 2014, five buds were collected from each of 35 vines providing 175 individual buds. Swabs were taken on the outside of each bud using a sterile medical swab (Cultiplast® SWAB, LP Italiana Spa) that had previously been dipped in sterile bacteriological saline (BS) and replaced in the tube to await use. The end of the swab was rubbed over the surface of the expanding bud and returned to the tube for later assay. After swabbing, the outer bud scales were peeled from the bud, leaving the growing point surrounded by a cluster of minute leaf primordia. The exposed juvenile tissue was washed with ethanol then pinched out with fine forceps and placed in 2mL Phosphate Buffered Saline (PBS) for DNA extraction and bacterial quantification. The swabs and the internal tissue samples were frozen and held at -20°C until processed.

Populations and viability of Psa associated with individual lesions.

Thirty-five vines were selected in the orchard block. On each vine a single leader cane was selected and three treatments were applied to young expanding shoots on that cane (Figure 1).

The treatments were:

1. Canes from which inoculum was excluded and where artificial cover was used over the shoot to protect it from external inoculum sources
2. Non-excluded canes – normal orchard management (including one application of copper)
3. Non-excluded, no copper treatment.

To exclude inoculum, pheromone trap covers (Delta trap; E-Tech Ltd.) were placed over the selected canes on the 8 October 2014. At that stage the leaves were still expanding, 2–3 cm across and no lesions were present on any of the covered shoots. Swabs were taken from the leaf surfaces of a selection of three leaves from each of 24 vines for quantification of Psa to provide an indication background populations of Psa at the beginning of the trial period.

Conditions of temperature and rainfall prevailing over the trial period are shown in Figure 1a in Appendix

After the first lesions appeared in early November 2014, a single lesion was marked with a felt pen on each of three leaves on each treated shoot on each cane. These were used for successive sampling and assay of Psa surface populations and viability over the season.

Sampling of the marked lesions commenced on 13 November 2014 and continued at approximately fortnightly intervals until 18 February 2015. Samples were taken on 13 November, 25 November, 15 December, 06 January, 19 January, 03 February and 18 February. On each occasion, each marked lesion spot was swabbed using the method outlined for external bud swabs above.

2.2 Psa detection, viability and quantification

After collection, each swab was wiped onto the surface of King's B culture medium to test for bacterial viability then the swabs held at -20°C to await DNA extraction and Psa quantification. Viability was recorded as presence of absence of Psa colonies on the culture medium (recognised by comparison of colony characteristics of a known Psa colony).

For Psa detection and quantification each frozen swab (kept in -20°C) 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 100 µL of 10% Chelex (BioRad Chelex 100). 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, again vortexed vigorously, 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 (Casonato et. al. 2014).

Quantitative PCR (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 (Psa biovar 3 specific) used were hopZ2bF2-L: 5'- ACAACTTCAGGCTACAATACTTACGC-3' and hopZ2bR2: 5'- CTCAGGATGCGTTTCGGTTAC' (Rikkerink et al. 2011). Each 10 µL reaction contained 2.5 µL DNA templates, 5 µL 2x Rotor-Gene SYBR Green, 1 µL 5 µM hopZ2bF2-L, 1 µL 5 µM hopZ2bR2, and 0.5 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 72 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 concentrations per mL (cfu/mL) were calculated by comparing the crossing threshold (Ct) value obtained from the known bacterial standards of Psa biovar 3 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 /mL of each sample was calculated.

This procedure provided a measure of the numbers of bacteria on each swab. This in turn was accepted as an indicator of the relative population of bacteria on the surface of the lesion from which the swab was taken on each occasion. This value, termed here the population value, allowed a comparison of populations on the surface of the lesions over the course of the season.

2.2.1 Statistical analysis

The statistical analysis was run in SAS 9.4 using the mixed procedure. There was some non-detectable Psa cell forming unit (cfu/ml) values for each time point. These non-detectable values were replaced by half the observed minimum value of a time point. Prior to analysis, the amount of Psa load (cell forming unit/ml) was log10 transformed to stabilise the residual variance. Paired t-test was conducted for each treatment with control canes.

Percentage of buds in Figure 2 was calculated based on total population of 175 (35 biological replicates× 5 buds from each biological replicate) and that of leaves in Figure 4 was calculated based on total population of 105 (35 biological replicates × 3 leaves from each biological replicate).

3 RESULTS AND DISCUSSION

3.1 Association of Psa with buds prior to bud break

Psa was detected on both the outside and the inside of buds prior to bud break. The results are shown in Figure 2. Although the overall detection rate for Psa was relatively low, the organism was detected more frequently from the juvenile tissues from inside the buds (10.3%) than from the outside (5.1%). In only one sample was Psa found both outside and inside the bud.

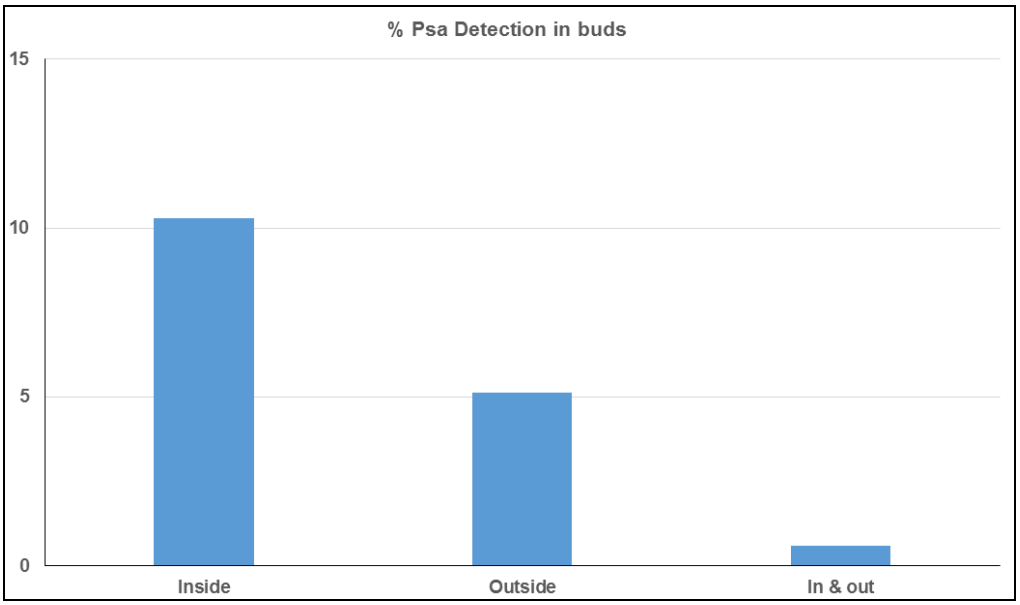

Figure 2. Proportions of buds from which *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa) was detected from the inside of the bud, the outside of the bud and both inside and outside from a sample of 175 buds. Note that the outside assays was made by extracting DNA from swabs and the inside assays by DNA extraction from the whole bud after treatment with ethanol, both followed by qPCR analysis. 'In and out' represents when it was present in both inside and outside of bud.

Although the primary focus of the study was to establish the presence or absence of Psa on and within buds, for interest the populations of bacteria on swabs and in internal tissues were quantified. The overall mean of population values for swabs taken from the outside of the bud was 10,143 cfu/ml. Quantification of bacteria inside the buds, based on extracted DNA, was quite variable but indicated a mean population of 11,263,326 cfu/ml. The different methods used to sample the outside and inside of buds do not allow a direct comparison of the numbers from the two positions but do provide some indication of numbers of bacteria present, particularly inside the contaminated buds.

These results are consistent with the results of Tyson et al (2015) who showed that Psa could be present within developing buds during the winter months. The method used here to detect the organism in buds clearly shows that Psa was present in the protected environment under the bud scales and could infect the emerging leaves. Importantly Psa under the bud scales would be protected from protectant sprays applied prior to bud burst. However the study does not provide evidence as to if the bacteria under the bud scales was the result of a systemic infection of the plant; that is, it had a truly endophytic origin. In a current study in the same block Tyson et al. (2015) have found that airborne inoculum can be detected at any time of the year

and is always associated with rain events. Thus all developing buds will be exposed to airborne inoculum during rain events. As buds are developing or breaking out and swelling, there is the opportunity for the ingress of bacteria between the bud scale leaves and for their multiplication in the protected environment of the primordial bud.

The differences between numbers of detection on the outside and insides of buds may be related to the differences in the detection sensitivity between swabbing and PCR detection applied to the different parts of the bud. To confirm that there is true endophytic colonisation of buds, it would be necessary to aseptically excise vascular traces and cortical tissue from the cane immediately below the bud, and from the internal tissues of the petioles of symptomatic leaves, tissues which could not have been colonised by airborne inoculum.

3.2 Populations of Psa on leaf lesions

The baseline population value of Psa on leaves, measured on 10 October 2014 prior to lesion development, was $2.8 \times 10^3 \pm 2.5 \times 10^3$ cfu/ml. Subsequently one set of canes was covered by pheromone trap holders while those of the other two treatments (non-excluded with copper; non-excluded no copper) remained uncovered. When population comparisons were made on 30 November from swabs taken from lesions on the leaves, the population values for excluded and the non-excluded no copper treatments were 8.5×10^6 and 10.1×10^6 cfu/ml respectively, each more than 1000 times greater than the baseline. This dramatic increase in surface populations most likely reflects the production of bacterial ooze at the margins of leaf lesions (Figure 3).

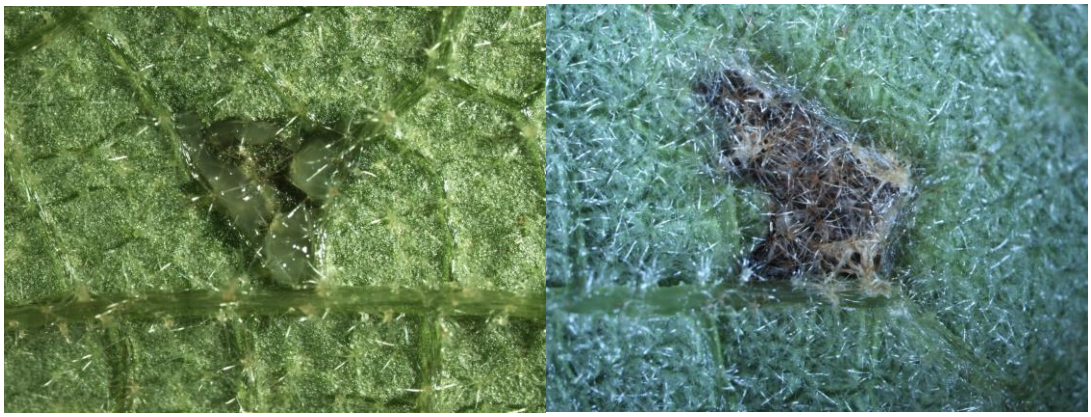

Figure 3. Examples of *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa) bacterial ooze at the margins of leaf lesions. Left: Psa oozing from the margin of a young leaf lesion on *Actinidia chinensis* var. *chinensis* 'Hort16A' after artificial inoculation. Right: Dried bacterial ooze on underside of leaf of *A. chinensis* var. *deliciosa* in field (photos Bob Fullerton).

3.3 Psa incidence and viability on leaf lesions

The incidence and viability of Psa on lesions was assessed over the period from late spring (13 November 2014) to late summer (18 February 2015). Swabs were taken from leaf surfaces, streaked onto King's B-C medium to check for viability then DNA extracted to confirm the presence of Psa by PCR. The proportion of swabs from which Psa was detected by PCR, and the proportion from which Psa was cultured at each sampling date are shown in Figure 4.

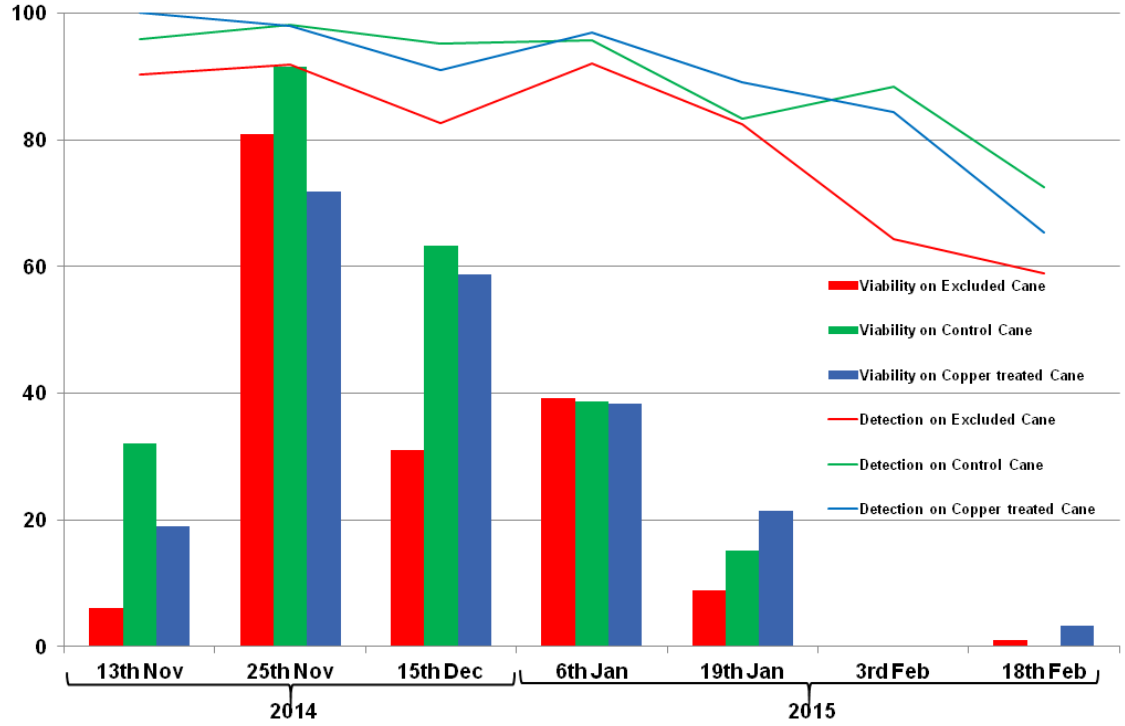

Figure 4. Average percentage of samples from lesions from which *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa) was detected (line graph) and percentage of samples with viable Psa (bar graph) at each sampling date during the 2014–15 season. Percentages based on 105 observations.

Psa was detected by PCR on swabs from lesions of all treatments throughout the whole period of the study. The percentages of positive samples were highest during the early part of the season with a gradual decline over the summer months. In general there was a lower percentage detection in the covered leaves than in the uncovered leaves. The highest proportion of samples which yielded cultures of Psa (viability test) was found on 25 November followed by a gradual decline in viability as the season progressed. Very few samples yielded viable bacteria after 18 February. The percentage of samples that yielded bacterial cultures (the measure of 'viability') recorded on the first sampling period on 13 November was very low compared with that 12 days later. This apparently anomalous result may be related to the stage of lesion development at that time. Lesions were just becoming visible in the days prior to 13 November, and there were no lesions expressed on the covered shoots at the time of covering. Potentially, very low numbers on the leaf surface, and hence on swabs, associated with those very young lesions were able to be detected by PCR, but the numbers on the swabs may have been too low to be consistently transferred to the culture medium and detected as colonies. Thus the result is most likely related to bacterial cell numbers and detection thresholds than to the viability of the organism at that stage, with the limit of detection on a swab by PCR much lower than that of streaking onto culture medium. Differences in detection frequency between the two methods at the end of the season are, however, more likely to be the result of loss of viability of surface bacteria. The lesions had been shown to produce significant amounts of inoculum earlier in the season. The decline at the end of the season may be related to both the decline in numbers of viable bacteria on the surface and a drop in multiplication rate of bacteria in the leaf tissue surrounding the lesion. As noted above, for the late-season assays, PCR would detect DNA from dead bacteria on the surface of the lesion but the plate test would fail to produce cultures. In order to discriminate between populations of living and dead bacteria on

the plant surface the application of viability PCR technology would be necessary. This technology distinguishes between living and dead microorganisms based on loss of membrane integrity in dead cells. It uses the compound propidium monazide which, after photoactivation with a defined wavelength intercalates and binds with the DNA of dead cells. The modified DNA will not amplify in subsequent PCR thus reducing the amplification signal compared with total DNA extraction.

The reduction of viable inoculum on leaves during the course of the summer found in this trial is consistent with the results of Casonato & Bent (2014) confirming a pattern of low inoculum production during the hot, drier conditions of mid-summer period. Nevertheless that previous study confirmed that the bacterium was surviving within the leaf tissues and may have the capacity to resume multiplication during the cooler autumn period. A study by Tyson et al. (2012) found copious exudates of Psa on leaves of *A. chinensis* in May 2011, and viable bacteria could be retrieved from those leaves both while on the plant prior to leaf drop and subsequently through to September 2011 as the leaves decomposed on the orchard floor. Collectively these studies show that, apart from a period of low leaf inoculum production during mid-summer, leaf lesions have the capacity to contribute high amounts of inoculum to the orchard environment throughout the growing season.

In general the percentage of positive samples determined both by PCR and culturing (viability) was consistently lower for lesions on the covered leaves than on the exposed leaves. The use of pheromone trap covers did not provide absolute protection of the covered leaves. While they would prevent contamination of leaves from direct rain splash, they would be exposed to inoculum in the form of aerosols or fine wind-blown mist carrying bacterial cells. Nevertheless there was a consistent difference between both detection percentage and population value between leaves under covers and those that were fully exposed. This difference may be indicative of the degree of protection from direct rain afforded by the covers with populations on exposed leaves representing a combination of external and locally produced inoculum while populations on covered leaves were primarily of local origin. This result is consistent with earlier studies (Casonato & Bent 2014) that demonstrated that exclusion of rain by protective covers was effective at reducing infection of plants under orchard conditions.

4 KEY RESULTS

1. Psa is present both on the outside and inside of expanding buds in the spring but at a relatively low incidence. There was a greater incidence of bacteria inside the buds than on the outside. Critically Psa was found in the protected environment under the bud scales meaning that protectants applied prior to bud burst may not provide adequate control and growers need to consider protecting vines from both internal and external Psa sources.

2. Psa can be produced on leaf lesions throughout the period from spring to mid-summer. Leaf spots appear to be a source of Psa, particularly in spring, controlling Psa from leaf spots is likely to be important in minimising reinfection or movement onto other vines.

3. The incidence and apparent viability of surface bacteria associated with leaf lesions is highest early in the season and declines to very low levels in mid-summer.

4. Covering of shoots in the early spring does provide a measure of protection from external inoculum and helps us to understand why covered structures work (or similar)

5 ACKNOWLEDGEMENTS

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APPENDIX 1: WEATHER PARAMETERS AT THE TE PUKE RESEARCH ORCHARD

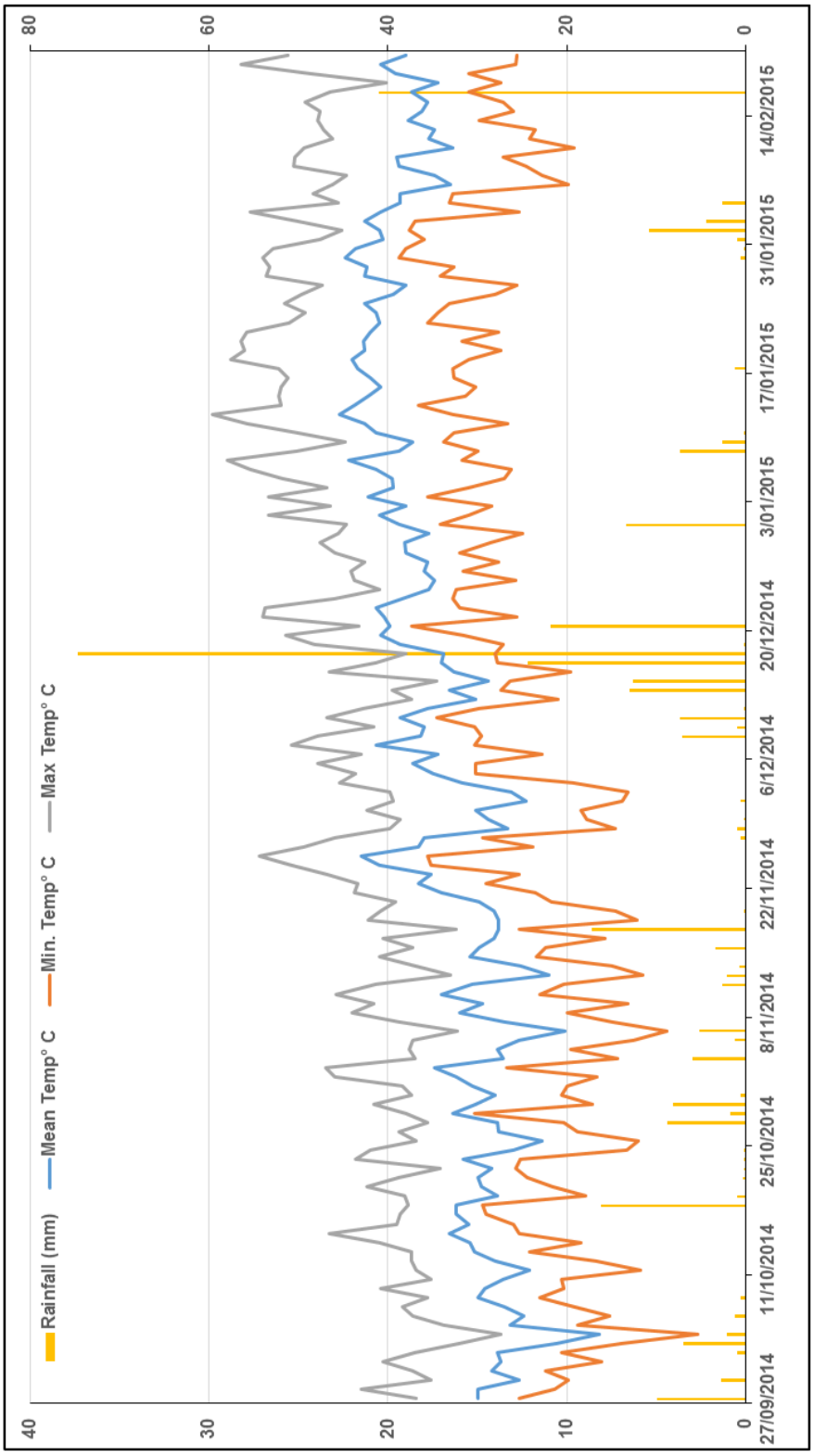

Figure A1. Weather parameters at the Te Puke Research Orchard from 27 September 2014 to 14 February 2015.

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