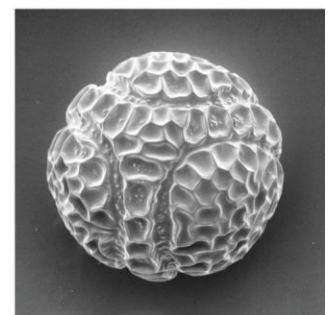
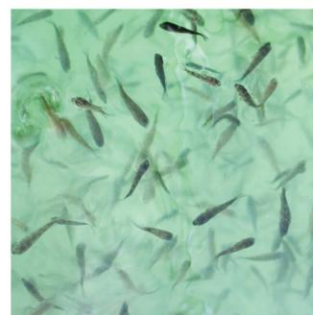
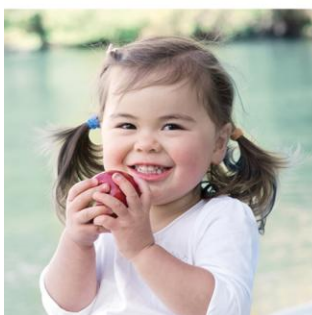
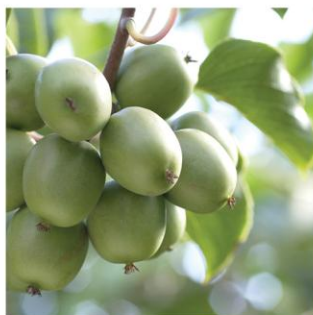
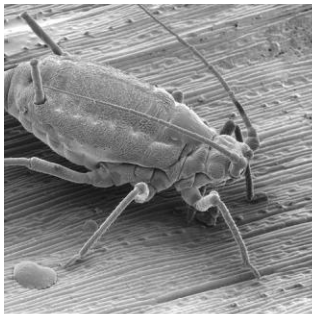


Testing limit of detection for Psa-V in kiwifruit canes

Everett KR, Pushparajah IPS, Casonato SG, Bent S.

July 2014



Confidential report for:
Kiwifruit Vine Health, Matt Dyck

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Executive summary

Testing limit of detection for Psa-V in kiwifruit canes

Everett KR¹, Pushparajah IPS¹, Casonato SG², Bent, S²

Plant & Food Research: ¹Auckland, ²Te Puke

July 2014

- Five canes from each of five kiwifruit vines systemically infected with Psa-V were sampled
- The canes were cut up, and 10 'disc' equivalents were weighed and washed. DNA was extracted from the washings.
- Healthy cane discs were 'spiked' with a 10-fold dilution series of $10^9 - 10^6$ cfu/mL Psa-V
- After Psa-V was dried onto healthy cane tissue, DNA was extracted from the washings
- Psa-V was detected from extracted DNA using the F3/R4 and the HopZ2b primers in quantitative PCR (qPCR).
- The concentration of Psa-V was also determined using a dilution series on King's medium B and counting colonies
- The most sensitive detection method was the F3/R4 primers in a qPCR reaction. However, the amount of Psa-V detected in infected 'Hort16A' kiwifruit vines sampled in June was very low. Five canes from one of the five sampled vines yielded an average of only 45 cfu/mL Psa.
- The amount of Psa-V from two of the infected vines was below the limit of detection of the HopZ2b primers. It was also below the limit of detection of the F3/R4 primers for nine of the 50 canes that were sampled. This was despite pooling 10 cane discs per replicate.
- The amount of Psa-V was unevenly distributed within the canes and within the vines which made detection unreliable.
- The results of artificially contaminating cane discs with Psa-V showed that 36.2% of the applied bacterial cells were able to be recovered using the washing method and the most sensitive detection method. Some improvement of detection would therefore be possible if canes were macerated instead of washed. If the numbers of Psa detected were increased by 36.2%, the Ct value remains in the 'negative' range (>35). Therefore, because of the low amounts of Psa detected in infected vines, a 36.2% increase in sensitivity would not increase the Ct values into a 'positive' (<30) or weak positive (30-35) range.
- Psa-V was not able to be reliably detected in symptomatic 'Hort16A' kiwifruit vines planted in an orchard when sampled in late June using the methodology described here. This was due to low numbers of Psa and uneven distribution between individual canes from the same vine, and between vines.
- Because of the low numbers of Psa-V present in symptomatic kiwifruit vines, sampling canes during winter for a high-health validation scheme cannot be recommended.

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1 Introduction

KVH is developing a 'Kiwifruit Plant Certification Scheme' to achieve the following goal:

To supply kiwifruit plants free of high risk pests and diseases, supporting long term success and future growth of the New Zealand kiwifruit industry.

KVH is striving to develop a robust scheme underpinned by sound science, and one that can evolve over time as science delivers new knowledge and improvements.

Phase 1 of the project has been completed. That is, a customised sampling/monitoring plan has been developed for *Pseudomonas syringae* pv. *actinidiae* (Psa). Existing information on limit of detection (LOD) was used to design the appropriate sampling strategy. This information, in conjunction with an understanding of the distribution of Psa in a nursery and the most useful experimental unit, was used to assign confidence levels to each test. A brief best practice guideline was written for the monitoring of Psa in the nursery.

The sampling strategy was based on information already obtained about sampling from leaves (Everett & Pushparajah 2013; Everett & Pushparajah 2014; Pushparajah et al. 2014). KVH want to accelerate the process by allowing sampling of cane material during winter. However, there is no existing information on the limit of detection in canes infected with Psa-V. This project was conducted to obtain that information.

2 Methods

2.1 Sampling kiwifruit vines

2.1.1 Artificially contaminated vines

Five canes from five symptomless 'Hort16A' kiwifruit plants obtained from a Psa-free zone (Kerikeri Research Orchard in 2012) and maintained in a growth room at MARC were sampled on 7 July 2014. Ten cane discs approximately 1-1.5mm thickness were cut, weighed and placed in five sterile Petri plates (Figure 1). A 10-fold dilution series of Psa-V from 10^9 to 10^6 cfu/mL (determined spectrophotometrically) (Pushparajah et al. 2014) was prepared and aliquots of 10 μ L of each dilution placed on each of the 10 cane discs in the five Petri plates. The concentration of the inoculum was confirmed by counting colonies on King's medium B (Barbosa et al. 1995). The inoculum on discs was air dried inside a laminar flow hood. Each replicate of 10 discs was placed in a 30 mL PS V base vial containing 2 mL of 0.85% NaCl and vortexed for at least 20 seconds, twice. Aliquots of 1 mL of the solution were placed into an Eppendorf tube for DNA extraction, and 20 μ L into another Eppendorf tube for counting colonies.

2.1.2 Infected vines

Five canes from five Psa-V infected vines were sent to Mt Albert Research Centre (MARC) of Plant & Food Research (PFR) from the PFR Te Puke Research Orchard (TPRO) on 27 June 2014 by overnight courier. Upon arrival, the canes were placed in a 2°C coolstore until processing. On 7 July 2014 the canes were sprayed with 70% ethanol to minimise cross-contamination. After the ethanol had evaporated, 10 cane discs approximately 1-1.5mm thickness were cut and placed in a Petri plate. After weighing, discs were transferred to a 30 mL PS V base vial (Thermo Fisher Scientific) containing 2mL of 0.85% NaCl. Every vial was vortexed for at least 20 seconds twice. Aliquots of 1 mL of the solution were pipetted into Eppendorf tubes for DNA extraction.

2.2 Quantification

A 1 mL aliquot was removed and centrifuged (13K, 10 min). The pellet was resuspended in 180 μ L Buffer ATL of a Qiagen[®] DNeasy kit, and, after extraction and washing, was eluted in 100 μ L. Psa-V was quantified using the HopZ2b primers of Rikkerink et al. (2011) and the PsaF3/R4 primers of Rees-George et al. (2010) according to the methodology developed in Everett et al. (2013).

2.3 Real-time polymerase chain reaction (RT PCR)

The 10 μ L/well reaction consisted of 2.5 μ L of DNA, 5 μ L SYBR Green I Master, 1.5 μ L GIBCO[™] water and 5 μ M of each forward and reverse primers, and was conducted in the LightCycler[®] 480 Real-Time PCR System under the following conditions: 95°C for 10 min, 45 cycles of 95°C for 5 s, 60°C for 7 s, 72°C for 7 s, followed by melting-curve analysis with a temperature profile slope from 65°C to 97°C with continuous fluorescence measurement.

2.4 Conversion to bacterial numbers

Crossing threshold (Ct) values from real-time PCR results were converted to colony-forming unit (cfu) equivalents using a 10-fold dilution series of Psa-V (from 10^9 to 10^4 cfu/mL). The concentration was determined spectrophotometrically, and confirmed by counting colonies on

King's medium B. DNA was extracted using the Qiagen DNeasy kit as above and qPCR reactions conducted as described using the F3/R4 and HopZ2b primers.

2.5 Data analysis

The generalised linear model of Minitab[®] was used for data analysis. Graphs were generated using Microcal[®] Origin.

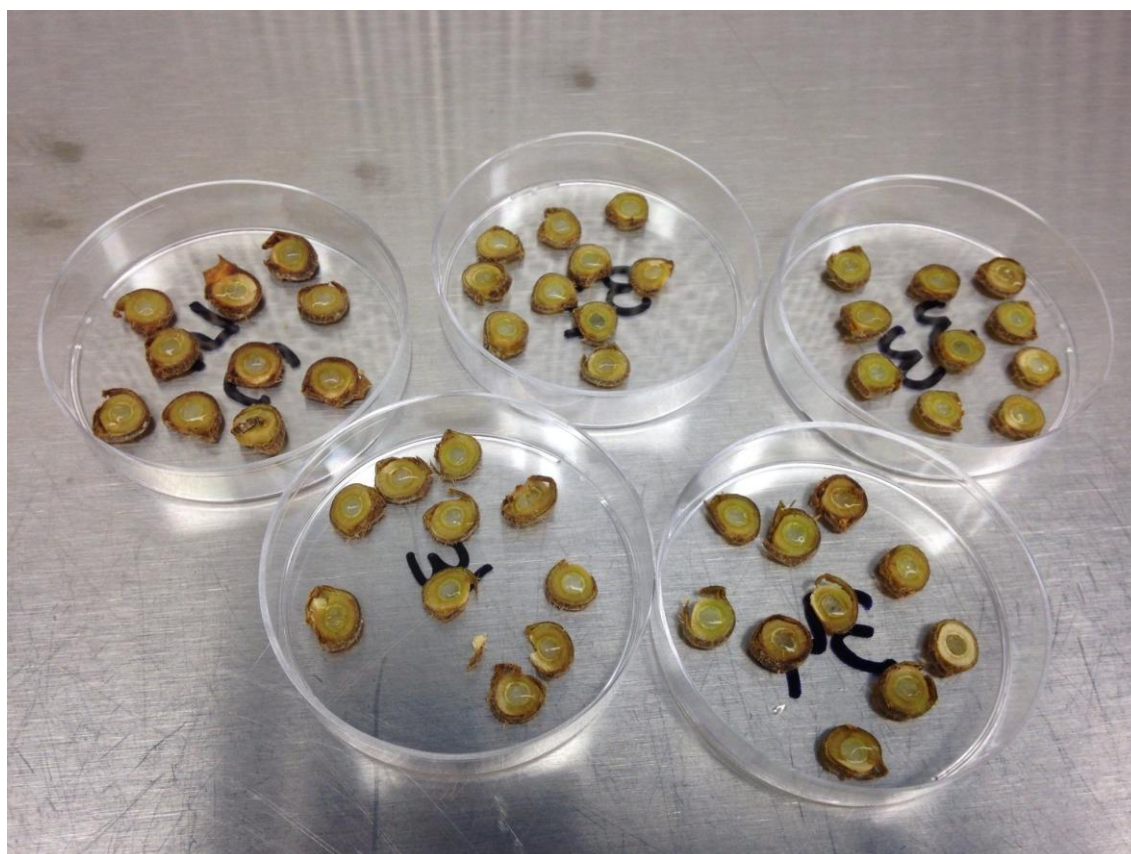
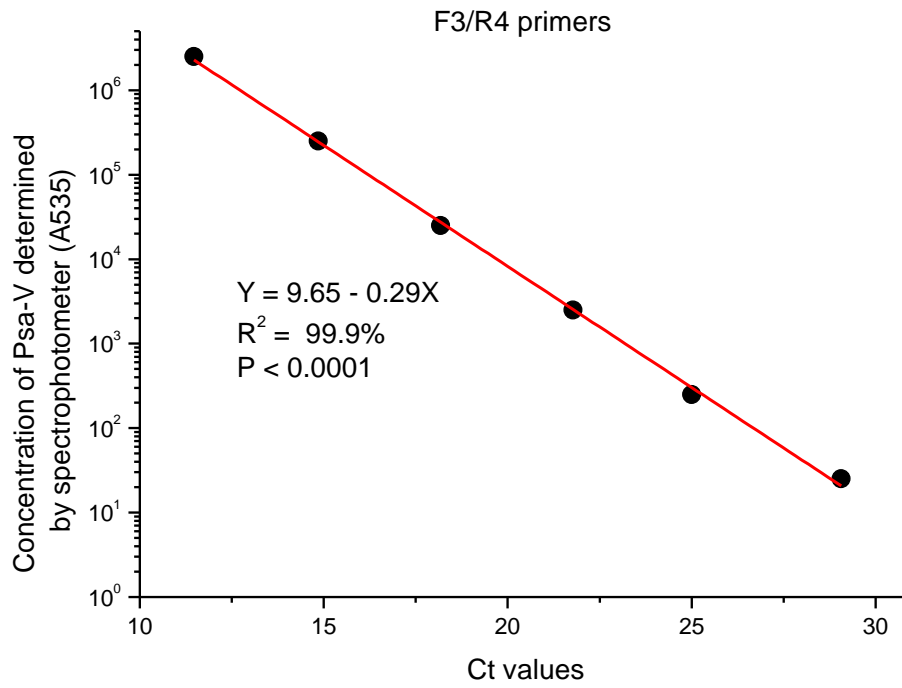


Figure 1: Cane discs in Petri plates contaminated with *Pseudomonas syringae* pv. *actinidiae* (Psa).

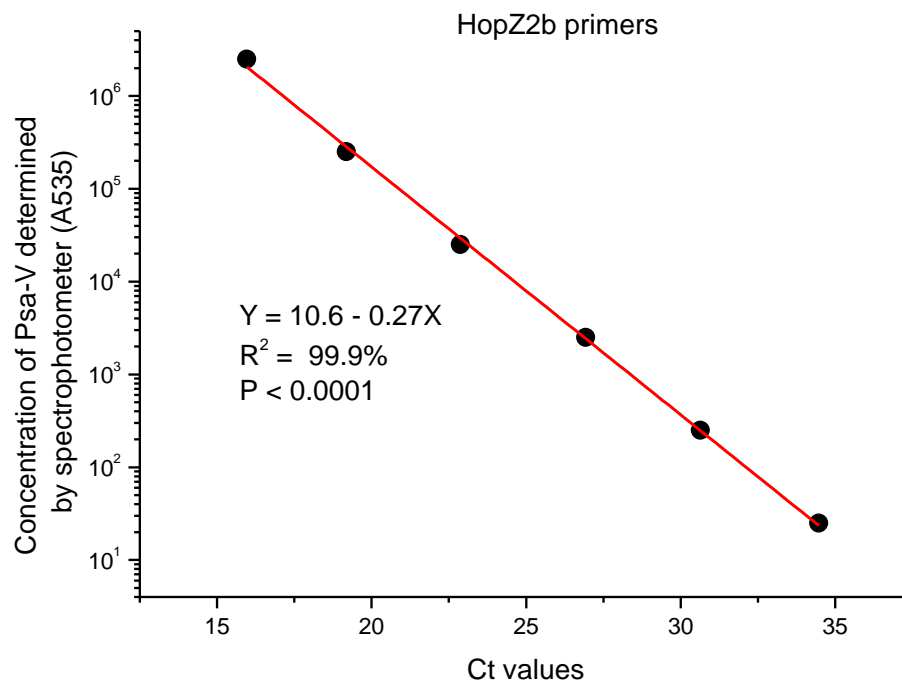
3 Results

3.1 Calibration curves

The amount of Psa-V in each qPCR reaction was plotted against the matching Ct value for the F3/R4 (Figure 2a) and the HopZ2b (Figure 2b) primers.



a)



b)

Figure 2: The amount of Psa-V (cfu) in each qPCR reaction versus Ct values. a) F3/R4 primers, b) HopZ2b primers.

3.2 Sampling kiwifruit vines

3.2.1 Artificially contaminated vines

Fewer Psa cfus were recovered than were applied, by a factor of 10, from artificially contaminated cane discs as determined by counting colonies on King's medium B (Figure 3). The Psa colonies were not able to be counted from field infected vines because of the large number of bacterial colonies.

Fewer Psa cfus were recovered than were applied, by a factor of 36.2, when the F3/R4 primers were used to quantify recovery, and by a factor of 15.8 when the HopZ2b primers were used (Figure 4). The dilution used in this experiment was not sufficient to remove the effect of contaminants inhibiting the PCR reaction at high concentrations of Psa-V.

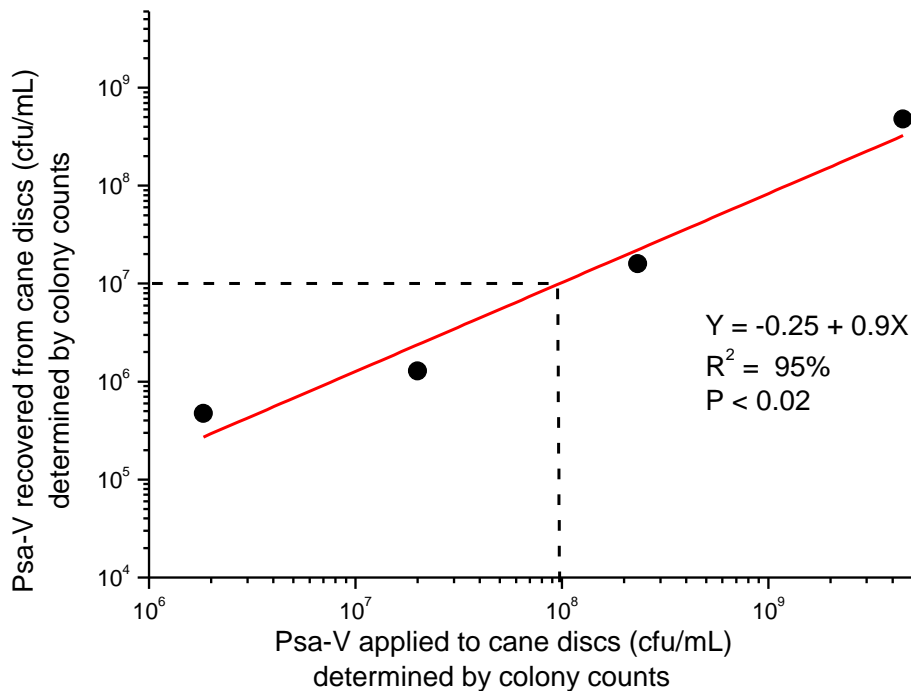
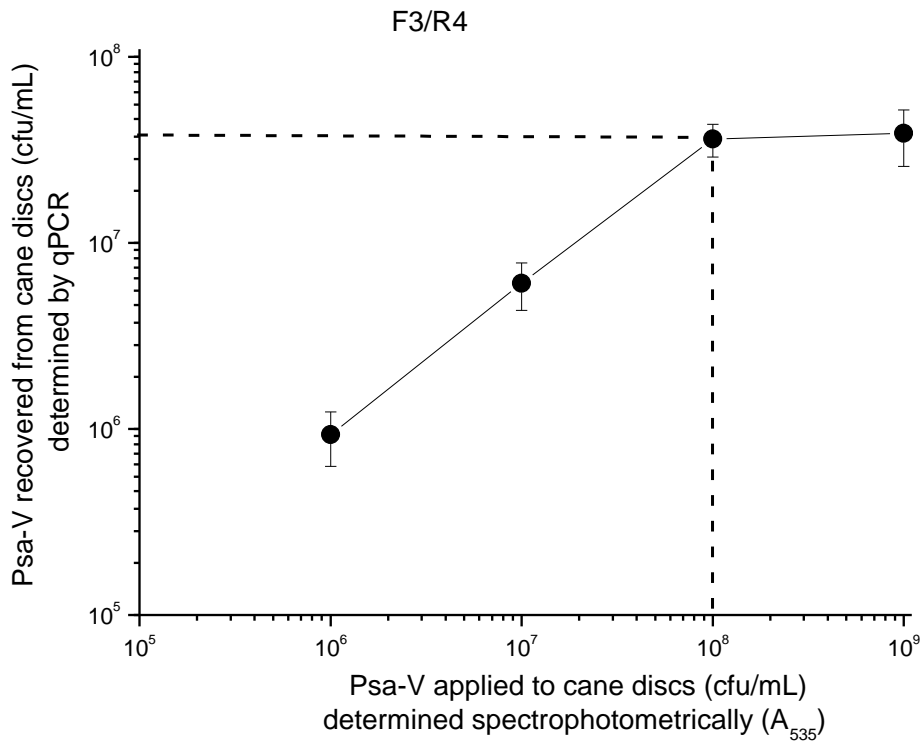
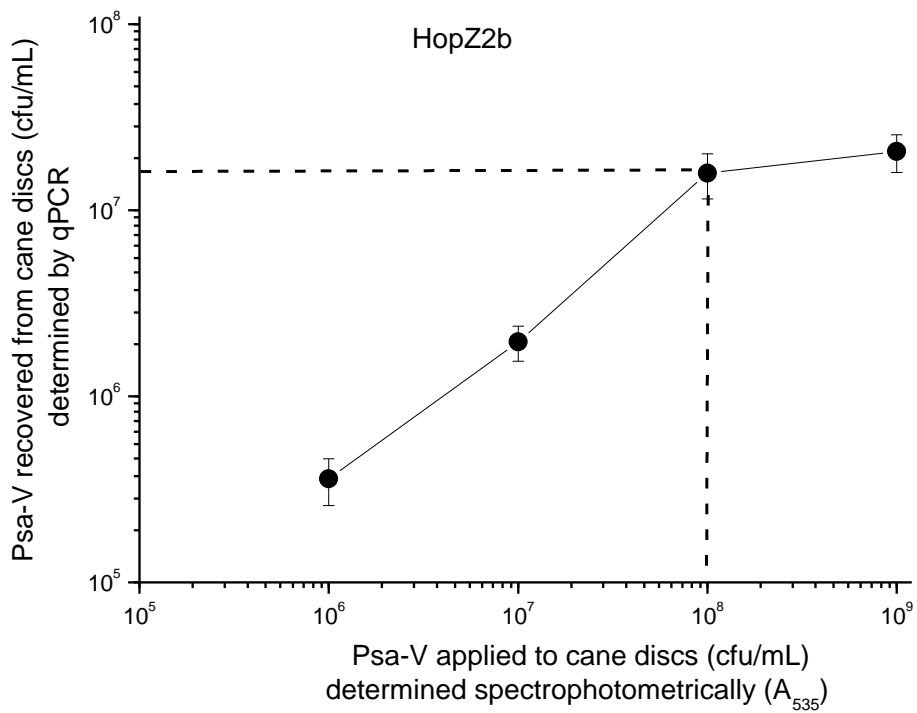


Figure 3: Recovery of Psa from artificially contaminated cane discs as determined by counting colonies on King's medium B.



a)



b)

Figure 4: Recovery of Psa-V from artificially contaminated cane discs as determined by qPCR. a) F3/R4 primers, b) HopZ2b primers.

3.3 Quantification of Psa-V in the kiwifruit orchard

The average Ct values per vine were all above 30 regardless of which primer set was used (Table 1), and, as such, were either in the weak positive (31-35) or negative (>35) categories applied to the qPCR results (Everett et al. 2012). This was despite pooling 10 cane discs per replicate.

The F3/R4 primers were more sensitive than the HopZ2b primers. The amount of Psa quantified by both sets of primers was equivalent, but bacterial numbers fewer than 9.53×10^2 cfu/mL were below the limit of detection of the HopZ2b primers.

When Ct values were converted to cfu/ml, Psa was apparently unequally distributed in the orchard. Variation between individual vines ranged from a mean of 4.52×10^1 cfu/mL to 7.53×10^6 cfu/mL, determined by the F3/R4 primers (Table 2), and from not detectable to 4.33×10^6 cfu/mL determined by the HopZ2b primers. The amount of Psa-V recovered from two of these vines was below the limit of detection of the HopZ2b primers (Table 1 and 2).

Psa-V was unequally distributed in the vines. Data obtained by the F3/R4 primers showed variation between replicate canes from the same vine of not detectable to 3.8×10^7 cfu/mL. Psa was not able to be detected in nine of the 50 sampled canes with the F3/R4 primers, and from 21/50 canes with the HopZ2b primers. One cane from vine 2 yielded 1.8×10^7 cfu/mL Psa, but the other four canes from this same vine yielded 4.5, 8.5, 111 and 150 cfu/mL.

Table 1: The Crossing threshold (Ct) values generated by extracting DNA from washings from cane discs taken from five infected 'Hort16A' kiwifruit vines and amplification by quantitative PCR.

Vine	PCR primers	
	F3/R4	HopZ2b
1	37.28± 1.67	39.69 ± 0.31
2	34.86 ± 4.70	36.35 ± 3.65
3	35.63 ± 1.16	BLOD
4	34.36 ± 1.66	39.52 ± 0.38
5	37.74 ± 1.03	BLOD
Average	35.97 ± 0.66	39.11 ± 0.70

BLOD = below the limit of detection.

Table 2: The amount of Psa (cfu/mL) detected from five naturally infected 'Hort16A' kiwifruit vines planted in an orchard at Te Puke Research Centre

Vine	PCR primers	
	F3/R4	HopZ2b
1	$3.76 \times 10^2 \pm 3.62 \times 10^2$	$3.33 \times 10^2 \pm 8.02 \times 10^1$
2	$7.53 \times 10^6 \pm 7.53 \times 10^6$	$4.33 \times 10^6 \pm 4.32 \times 10^6$
3	$1.69 \times 10^2 \pm 7.79 \times 10^1$	BLOD
4	$9.53 \times 10^2 \pm 7.27 \times 10^2$	$3.87 \times 10^2 \pm 1.16 \times 10^2$
5	$4.52 \times 10^1 \pm 2.19 \times 10^1$	BLOD
Average	$1.51 \times 10^6 \pm 1.51 \times 10^6$	$8.65 \times 10^5 \pm 8.65 \times 10^5$

BLOD = below the limit of detection.

4 Discussion

The most sensitive detection method was the use of the F3/R4 primers in a qPCR reaction. However, the amount of Psa-V detected in infected 'Hort16A' kiwifruit vines sampled in June was very low. Five canes from one of the five sampled vines yielded an average of only 45 cfu/mL Psa.

The amount of Psa-V from two of the infected vines was below the limit of detection of the HopZ2b primers. It was also below the limit of detection of the F3/R4 primers for nine of the 50 canes that were sampled. This was despite pooling 10 cane discs per replicate.

The amount of Psa-V was unevenly distributed between canes from the same vine, and between the vines which made detection unreliable.

The results of artificially contaminating cane discs with Psa-V showed that only 36.2% of the applied bacterial cells were able to be recovered using the washing method and the most sensitive detection method. Some improvement of detection would therefore be possible if canes were macerated instead of washed. If the numbers of Psa detected were increased by 36.2%, the Ct values remain in the 'negative' range (>35). Therefore, because of the low amount of Psa detected in infected vines, a 36.2% increase in sensitivity would not raise the Ct values into a 'positive' (<30) or 'weak positive' (30-35) range.

Psa-V was not able to be detected reliably in symptomatic 'Hort16A' kiwifruit vines planted in an orchard when sampled in late June using the methodology described here. This was because of the low numbers of Psa and the uneven distribution between individual canes from the same vine, and between vines.

Further dilutions were made of three of the DNA samples from symptomatic kiwifruit vines, up to 1:1000 v/v, but there was no improvement in detection of Psa. In addition, a different DNA extraction method was used (boiling method as described in Everett et al. (2012)). There was still no improvement in detection of Psa.

It is not clear why the titre of Psa in infected kiwifruit vines in winter was so low. However, it is possible that the oozing that is observed in spring is an indication of rapid multiplication and that Psa would be more easily detected in spring. This warrants further research to elucidate.

Because of the low numbers of Psa-V present in symptomatic kiwifruit vines, sampling canes during winter for a high health validation scheme cannot be recommended.

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