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Psa 2.4 Psa on asymptomatic leaf tissue

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A report prepared for:

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Plant & Food Research, Mt Albert

SPTS Client Report No. 5256

PLANT & FOOD RESEARCH *Pseudomonas syringae* pv. *actinidiae* (Psa) RESEARCH NOTE

KEY PROJECT DETAILS

Project Title	Psa on asymptomatic leaf tissue
Project Protocol No./ Objective No.	2.4
Project Leader	Kerry Everett
Research Requested / Contracted by	ZESPRI Group Ltd
Date (Month, Year)	March 2011
Based on information as at	March 2011

RESEARCH QUESTION AND AIM

Can Psa infect plants endophytically, i.e. inside the plant without causing symptoms, or epiphytically i.e. outside the plant without causing symptoms?

Aim: To provide evidence to answer that question.

METHODOLOGY

Experimental design

Kiwifruit leaf tissue was sampled from 10 orchards in the Bay of Plenty on 12 January 2011. Four of these orchards were infected with Psa-V and five with the Asian strain as reported by MAF Biosecurity. One orchard was negative for Psa. Each orchard was assessed visually for severity of symptoms on a scale of 0.5-10, where '0.5 ' was a few leaf spots that were difficult to find, and 10 was leaf spots that were common and secondary symptoms, that is of cane browning and death.The orchard that was negative had leaf spot symptoms that were indistinguishable from those caused by Psa, and the severity rating for that orchard was 7/10.

Five epicentres were sampled on each of the nine infected orchards, with sampling at each epicentre as follows:

1. Leaves with symptoms (infected control)
2. Asymptomatic leaf tissue immediately adjacent to symptomatic leaf tissue
3. Asymptomatic leaf tissue 1 m from symptomatic leaf tissue on the same cane
4. Asymptomatic leaf tissue 2 m from symptomatic leaf tissue on the same vine
5. Asymptomatic leaf tissue 5 m from symptomatic leaf tissue on a different vine

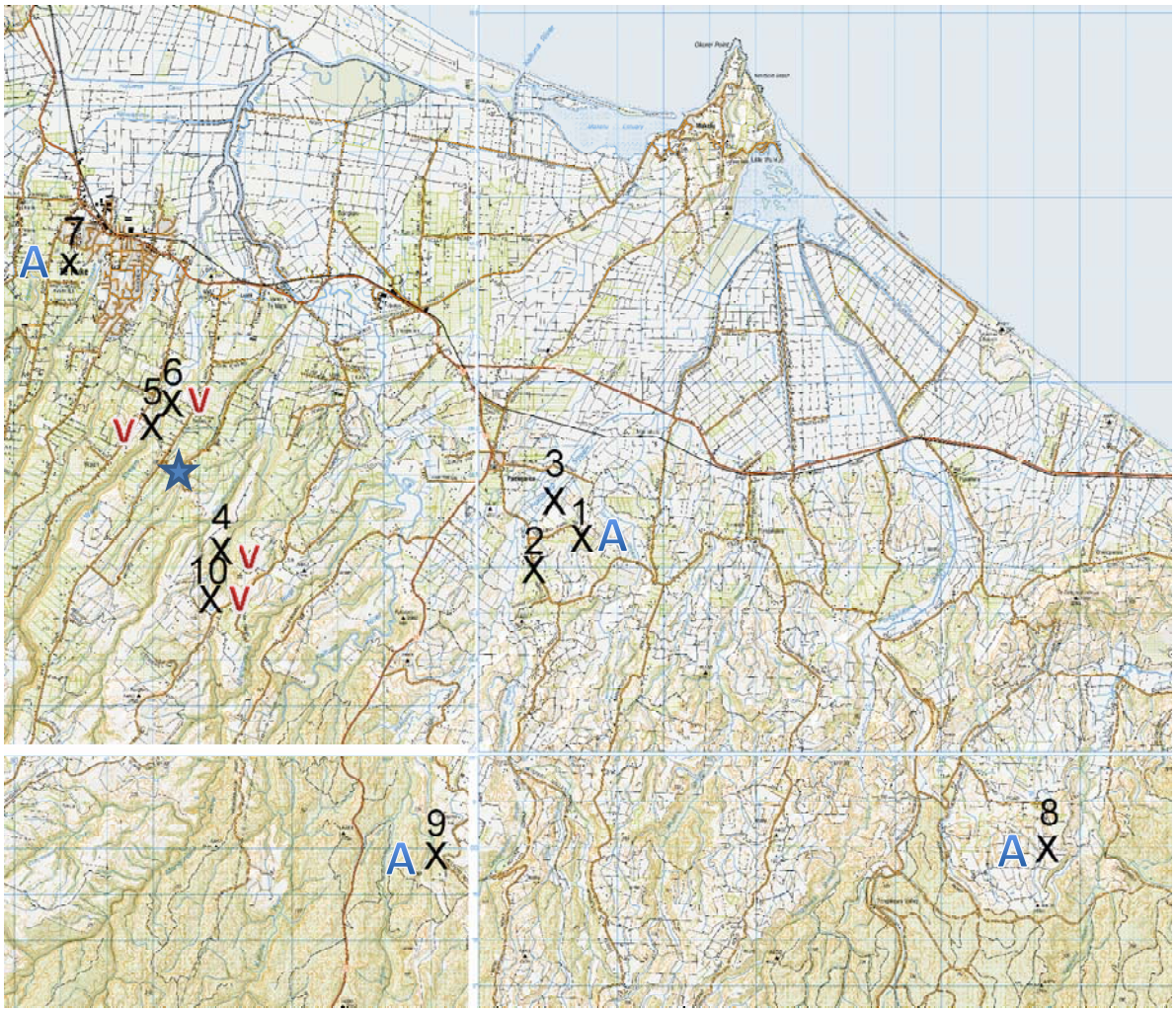

Figure 1. Location of the ten study orchards in the Bay of Plenty area. A = Psa Asian strain and V= Psa Virulent strain. Star is location of RP1, where Psa was first diagnosed in New Zealand.

Careful checks were made to ensure there were no symptomatic leaves between the chosen epicentres and the asymptomatic leaves sampled, but this possibility cannot be ruled out.

Kiwifruit tissue was placed in sealed plastic bags, double contained, and immediately transported to Mt Albert Research Centre.

Methods and protocols

Upon arrival at the laboratory, seven 1 cm diameter leaf discs were excised from every sample and were processed in one of three ways:

1. Tissue was surface sterilised with 70% ethanol and hypochlorite and by rinsing with sterile deionised water (SDW) following the protocol of Everett et al. (2003) (3 discs)
2. Tissue was not surface sterilised (3 discs)

3. Tissue was washed in 1 ml bacterial saline by placing in a plastic bag and gently massaging. A 100-ul aliquot was spread over a Petri plate containing King's medium B (King et al. 1954) (1 disc).

The discs from the leaf with symptoms were taken from tissue with leaf spots. Two surface sterilised and two non-surface sterilised discs were placed in an eppendorf tube and stored at -80C for future reference.

Isolations were made from the remaining two discs by crushing surface sterilised and non-surface sterilised tissue in 100 uL SDW and spreading on a Petri plate containing King's medium B.

DNA from washings from Petri plates incubated at 25°C for 24 hours was extracted using the boiling method .

DNA was tested by real-time Polymerase Chain Reaction (PCR) and the primers of Rees-George et al. (2010). Bacterial 23S primers (Rees-George et al. 2010) were used to check the quality of the DNA.

Results were reported as strong positive when the crossing threshold (CT) was <30 cycles, as weak positives when the CT was 30-35 cycles, and as negative when the CT was >35 cycles. The melting temperature was also used to confirm that the amplified product was Psa.

KEY RESULTS

Results of PCR tests from each of the sampled orchards are presented in Table 1, with analyses of orchard symptom severity versus PCR results presented in Figures 2 and 3.

- Samples from Orchards 2 and 8 were negative by qPCR for Psa, despite severity ratings by symptoms of, respectively, 7 and 8. Orchard 2 had previously tested negative for Psa, but Orchard 8 was positive.
- Blossom blight (*Pseudomonas* sp. or *Pseudomonas viridiflava*) causes leaf symptoms that are not able to be distinguished from leaf symptoms caused by Psa. It is therefore likely that at least one of these two orchards could be infected by blossom blight.
- Orchards 4 and 10 were worse than all other orchards by both severity ratings, and by number of positives by qPCR. These orchards have been diagnosed by MAF Biosecurity as being infected with the Virulent strain of Psa.
- Of the remaining six orchards two had strong qPCR positives (CT <30 cycles) from one sample each and had been diagnosed as infected with the Asian haplotype, and four orchards had only weak positives (CT 30-35 cycles) of which two were infected with Psa-V and two with the Asian haplotype.
- On one orchard (no. 5), two surface-sterilised samples from symptomless leaves on an adjacent vine yielded Psa. This is some evidence that in symptomless leaves Psa can infect

Table 1. Results of qPCR tests using Psa-specific primers.

Orchard		leaf samples															
Psa test		symptoms			no symptoms												Severity
KPIN	Epicentre				adjacent			1m same cane			2m different cane			5m different vine			rating/10
variety		ss	ns	bs	ss	ns	bs	ss	ns	bs	ss	ns	bs	ss	ns	bs	
1	1																4
Asian	2																
7253	3																
GK	4																
	5																
2	1																7
negative	2																
1712	3																
GK	4																
	5																
3	1																4
negative	2																
4369	3																
GK	4																
	5																
4	1																10
Psa-V	2																
1180	3																
GK	4																
	5																
5	1																2
Psa-V	2																
8714	3																
HW	4																
	5																
6	1																0.5
Psa-V	2																
9745	3																
HW	4																
	5																
7	1																1
Asian	2																
8337	3																
GK	4																
	5																
8	1																8
Asian	2																
2389	3																
GK	4																
	5																
9	1																2
Asian	2																
5970	3																
GK	4																
	5																
10	1																8
Psa-V	2																
2375	3																
HW	4																
	5																

Key:

Colour code	CT value
negative	>35
weak positive	30-35
positive	<30

ss	surface sterilised
ns	not surface sterilised
bs	washed with bacterial saline

HW	Hayward
GK	Hort16A

inside the tissue without causing symptoms (endophytic infection). It could also have been early stages of infection that were not noticed during sampling. If this was an endophytic infection it does not appear to be common, as Psa was not isolated from the remaining 198 surface-sterilised samples from symptomless leaves. This result was from testing leaves from an orchard that was infected with the virulent strain of Psa. Before conclusions are made this result needs to be confirmed by further testing or experimentation.

- It was difficult to isolate Psa from leaves with symptoms from orchards infected with the Asian strain. This suggests that the Asian strain of Psa may not have been causing new infections, or only a few new infections, when samples were collected in January. It could also be that the measures used by growers to control Psa A were effective.
- Psa was isolated from 4/5 leaves with symptoms on the two orchards infected with the V strain. It was also isolated from 3/5 and 2/5 adjacent symptomless leaves, from 5/5 and 3/5 symptomless leaves 1 m away on the same cane, from 2/5 and 3/5 symptomless leaves 2 m away on a different cane, and from 1/5 and 1/5 symptomless leaves on a different vine 5 m away.
- Because Psa V was isolated from symptomless leaves up to 5 m from the epicentres (leaves with symptoms), this suggests that Psa V was still active, spreading, and possibly causing new infections in January when these samples were taken. It is also possible that Psa V was able to survive for long periods of time on the outside of leaves without causing symptoms (epiphytically). Further research is required to determine the length of time that Psa V can survive on the outside of leaves in New Zealand kiwifruit orchards.

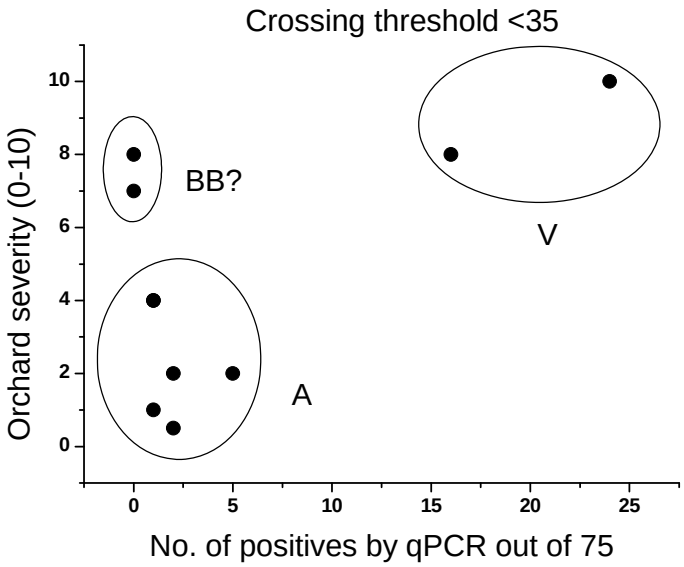

Figure 2. Severity of symptoms in the 10 sample orchards plotted against number of positive qPCR tests for which the Crossing threshold (CT) <35. Circles denote the orchards putatively infected with blossom blight (BB?), the Virulent strain of Psa (V) and the Asian strain of Psa (A).

- When orchard severity was plotted against the number of Psa postives (out of 75 possible) for every orchard, there appeared to be three distinct groups of orchards; those that were infected with Psa V, those infected with Psa A and a group that included the orchard that may be infected with blossom blight (BB?) (Fig. 2). The BB? group probably had one orchard that really was infected with blossom blight (No. 2), but the other orchard (No. 8) had no Psa positives when sampled in January, but when sampled earlier (November 2010) it was positive for Psa A. Orchard No. 8 had a high severity rating of 8/10, that means there was an abundance of leaves with symptoms, but the Psa could not be isolated from the symptoms. This could be explained by the microclimate of this particular orchard not being suitable for long term survival of Psa (Fig. 1). It could also be explained by the presence of a successful microbial competitor, or by grower applied products suppressing or killing Psa cells. Whatever the reason, further research could help to explain this result.
- When the putative blossom blight group was removed from the analysis (Fig. 3), there was a strong (r=0.9) and significant (P=0.002) relationship between orchard symptom severity and the number of times that Psa was detected by qPCR out of 75 possible samples.

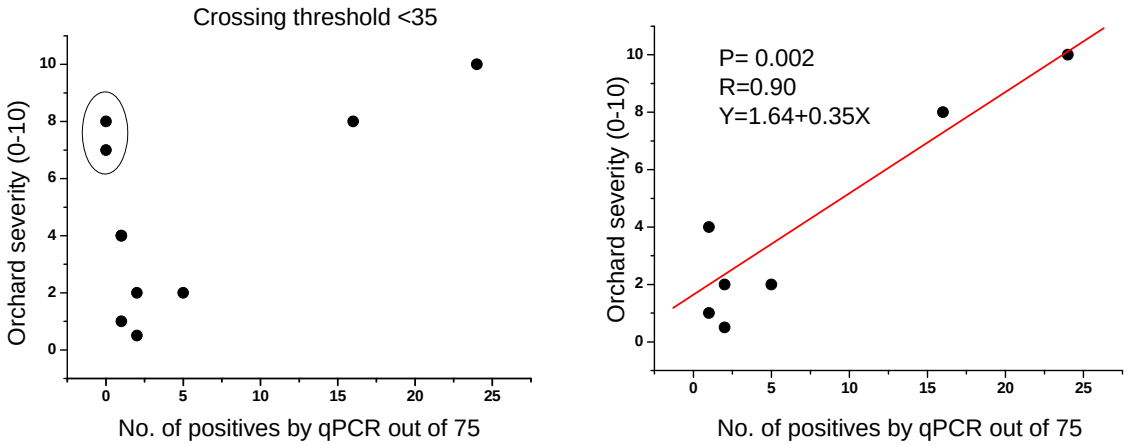

Figure 3. Severity of symptoms in the 10 sample orchards (left graph) and the 8 orchards that were positive for Psa by qPCR (right graph) plotted against number of positive qPCR tests for which the Crossing threshold (CT) <35. A linear regression analysis has been performed on both sets of data, and was significant for the right graph.

- Psa was not easily found on two orchards infected with Psa-V. Both orchards had low severity ratings, and it is possible that these orchards had become infected only a short time before these samples were taken. However, KPIN 8714 and KPIN 9745 were, respectively, RP 46 and RP 44. Although no dates were available for when these samples were tested, the other two Psa-V orchards with more severe symptoms were RP 13 (KPIN 1180) and RP 21 (KPIN 2375). Orchards KPIN 1180 and 2375 are south and inland to RP1 (Fig. 1), the orchard on which Psa was originally diagnosed in New Zealand (Everett et al. 2011). These orchards may have been infected earlier because symptoms were more severe and orchards may have been down wind of the prevailing off-sea breezes to the putative epicentre of the current epidemic. However, it could also be because the measures that the growers on KPIN

8714 and 9745 are using to control Psa are more effective than the measures taken by growers on KPIN 1180 and 2375.

RECOMMENDATIONS FOR INDUSTRY

- These results suggest that symptomless vines at least 5 m away from leaves with symptoms need to be removed, or sprayed with an effective bactericide, for successful elimination of Psa from orchards infected with the V strain.
- The measures used to control Psa by growers on KPIN 8714 and 9745 should be scrutinised and compared with those measures used by growers on KPIN 1180 and 2375. The KPINs of these orchards should be verified following revisitation of these properties to collect cane samples as part 2 of this project.
- The control measures used by growers on those orchards that had tested positive for Psa and for which all samples were negative in this study (Orchard 8), or on which Psa was difficult to find (Orchard 1, 3, 7 and 9) should also be scrutinised.

CONCLUSIONS

- For effective reduction of Psa V inoculum in an infected orchard, vines at least 5 m distant from leaves with symptoms need to be removed or treated with bactericides even if they do not show symptoms.
- There was some evidence for an endophytic growth habit of Psa in this study (that is, it resides inside symptomless leaves), but before any conclusions are made these results need to be verified by further experimentation.
- Both Psa V and Psa A have an epiphytic growth habit, and were thus able to be isolated from the surface of symptomless leaves.
- These results suggest that the Asian strain of Psa is either being managed well by growers, or that it is not very active during January in New Zealand. However, Psa V on two orchards from this study was very active when samples were taken.
- The methodology used in this study could be used to compare the effectiveness of control measures for Psa in the field.

FUTURE RESEARCH STEPS

- Canes now need to be sampled and tested from the same orchards, using the same sampling and sample preparation strategies.
- Further investigation of the survival time of Psa on leaf surfaces and whether it can reside inside symptomless leaves should be conducted.

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