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Survival of Psa in the orchard environment

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SPTS No. 6389

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Executive summary

Survival of Psa in the orchard environment

IJ Horner, KR Everett, MA Manning, JL Tyson, JL Vanneste, December 2011, SPTS No. 6389

The bacterial pathogen *Pseudomonas syringae* pv *actinidiae* (Psa) was first detected in New Zealand kiwifruit vines in November 2010, and a virulent strain of this bacterium is causing serious vine losses. Knowledge of the biology and ecology of this pathogen is limited. In order to develop effective orchard management strategies to minimise disease incidence and spread, it is important to understand how the pathogen responds to the environment and its kiwifruit host, and gain a full understanding of the biology and life cycle in New Zealand orchards.

The current programme focused on where the Psa bacterium survives in the orchard, particularly over the winter period. Various components of the kiwifruit orchard environment were investigated, including internal and external vine tissues, leaf litter and prunings, shelterbelts and weeds, soil and water.

In an investigation of various plant parts of 'Hort16A', 'Hayward' and 'Chieftain' vines, it was found that Psa could survive in just about all plant organs. Dormant buds, old flower stalks (on males), fruit stalks, canes, bark and wood could all harbour living Psa, either on the surface, internally, or both. In systemically infected vines, both symptomatic and asymptomatic tissues were sampled and found to contain live Psa. There was a high rate of Psa detection in tissues from 'Hort16A' and 'Chieftain' vines showing secondary symptoms, in comparison to lower recovery from 'Hayward'.

Studies were made of the internal (in wood) distribution of Psa in 'Hort16A', 'Hayward' and *Actinidia deliciosa* male vines showing secondary symptoms, by sampling wood every 10-20 cm from trunk base to near the cane tip, with samples taken in autumn, winter and spring. Live Psa was found in the canes, leaders, and trunks of 'Hort16A', 'Hayward' and *A. deliciosa* male vines, indicating that Psa can move systemically throughout kiwifruit vines. Psa was found below graft unions, indicating that the graft does not impede progress of the bacterium and that there is potential for resident populations to be present in the remaining portions of rootstocks and stumps, prior to re-grafting. Removal of obviously diseased canes does not necessarily remove the bacterium, as it was often present in asymptomatic tissues quite remote from diseased tissues. Psa appeared to have a discontinuous distribution in the trunks and an "advancing tide" of bacteria was not obvious. The distribution of the pathogen within the tissues across any cross-section of the plant was not regular. Thus, a conservative sampling system from trunks or canes of plants may fail to detect the organism. In general, there was a higher rate of isolation in spring, suggesting progression of the pathogen internally during winter.

Leaf disc assays were used to investigate potential epiphytic growth of Psa on kiwifruit leaves. Over the first few days following application of low doses of Psa to leaf discs, there was a rapid and massive increase in bacterial numbers on the leaf surface, in advance of any evidence of leaf spots or other symptoms. Although experimental conditions would certainly have favoured the pathogen, it shows that Psa is capable of living and multiplying on the kiwifruit leaf surface.

To determine overwintering potential on kiwifruit plant debris, leaves naturally infected with Psa were collected during autumn and cane prunings from systemically infected vines were

collected in July, and incubated either on the orchard floor or in the laboratory. Samples were tested weekly for 15 weeks (leaves) or 13 weeks (cane prunings), to determine the presence of living Psa. Soon after collection, Psa was readily detected in both leaves and cane prunings. Although there was a decline in detection rates as the winter progressed, living Psa was still detected in 25-50% of samples at the end of the trial (early September or early October for leaves and prunings, respectively). Similar results were obtained in both the laboratory and the field, indicating that the result was not from re-colonisation of material on the orchard floor during the winter. These results conclusively demonstrate that leaf litter and cane prunings are potential reservoirs for Psa survival.

A range of shelterbelt (*Casuarina*, pine, *Cryptomeria*, poplar and willow) and weed species (*Carex* sp. and *Crepis* sp.) were tested in the laboratory to determine if Psa was able to survive or multiply on them. All species tested gave similar results; populations declined over a few days, and there was no evidence of long-term survival (over a few weeks) or of multiplication of Psa on any of these plants. In samples collected from shelterbelts and weeds in Psa-infected orchards, no Psa was detected, except from one pine sample taken immediately adjacent to an infected vine. It is likely that Psa presence on non-host plants is only temporary.

In laboratory tests with soil, Psa was able to survive in sterilised soil for at least 3 months. However, Psa could not be detected from unsterilised soil. This could reflect the pathogen's inability to survive in natural soil, or alternatively it could simply reflect an inability of current techniques to detect or isolate Psa successfully from the microbiologically complex environment of the soil.

Various water sources were tested for the ability of Psa to survive. Psa populations declined very rapidly in tap water, probably a result of water treatment. In rainwater, Psa could survive for very long periods (at least 6 months) if the water had first been sterilised to remove microorganisms. This suggests that rainwater has sufficient nutrients to sustain Psa. However, in unsterilised rainwater, Psa populations declined rapidly in two out of three experiments, probably reflecting the impact of other microorganisms better adapted to survival in this environment. Psa could not be detected in ponds tested on a heavily Psa-infected orchard.

The various trials and studies discussed in this report have demonstrated that Psa is able to survive on or in most parts of the kiwifruit vine, including vine debris on the orchard floor. The pathogen can be extensively distributed within the vine, well beyond obvious signs of infection. Non kiwifruit sites such as shelterbelts, water and soil could potentially harbour the pathogen, at least for short periods, but further investigation is required to confirm this. No attempt has been made to quantify the relative importance of the various potential Psa overwintering sites; the first step was to identify where the pathogen can survive, particularly throughout the winter. The information generated should help growers to assess the sources of risk within their orchards, and help in development of modified management practices to reduce new infections or re-infections, reduce the spread of systemic infections, and feed into future research on Psa epidemiology and control.

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1 Introduction

The bacterial pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa) was first detected in New Zealand kiwifruit vines in November 2010. A virulent strain of this bacterium has recently caused serious vine losses in Italy, and is now causing similar losses in New Zealand. Knowledge of the biology and ecology of this pathogen is limited, with a few studies on less virulent strains in Asia and Italy (Balestra & Varvaro 1999; Koh et al. 1996; Scortichini 1994; Scortichini et al. 2002; Serizawa & Ichikawa 1993a, b, c, d; Serizawa et al. 1994; Serizawa et al. 1989; Takikawa et al. 1989) and more recently some preliminary work on the virulent strain causing problems in Italy since 2008 (Balestra et al. 2009a; Balestra et al. 2009b; Ferrante & Scortichini 2009, 2010, Vanneste et al. 2011).

In order to develop effective orchard management strategies, it is important to understand how the pathogen responds to the environment and its kiwifruit host, and gain a full understanding of the biology and life cycle in New Zealand orchards. Studies underway at present include investigation of infection processes and timing, influences of environmental parameters, pathogen spread in the environment, and development of disease prediction protocols. The current study focuses on where the Psa bacterium survives in the orchard, particularly over the winter period. Elucidation of the important sites of pathogen survival could potentially influence management practices employed to minimise disease incidence and spread.

The programme has been divided into seven research objectives, based around different components of the kiwifruit orchard environment. No attempt has been made to quantify the relative importance of the various potential Psa overwintering sites; the first step is to identify where the pathogen can survive, particularly throughout the winter. Information generated will help growers to assess the sources of risk within their orchards, and help in development of modified management practices that could reduce new infections or re-infections, reduce the spread of systemic infections, and feed into future research on Psa epidemiology and control.

The research objectives are:

- Survival on the vine
- Survival and spread in the vine
- Psa as an epiphyte
- Survival in leaf litter and prunings
- Survival on shelter and weeds
- Survival in soil
- Survival in water.

This report summarises research carried out from May to December 2011.

2 Survival of Psa on and in various vine parts

Kerry Everett, Shamini Pushparajah, Michelle Vergara, Ngaire Larsen, Carol Curtis, Mike Manning, Jonathan Rees-George.

2.1 Research questions and aims

What plant parts are infected in vines showing systemic symptoms?

What plant parts are infected in vines that only showed leaf symptoms or no symptoms?

To determine the distribution of Psa on and in kiwifruit vines in autumn, late-winter and early spring.

2.2 Methods

Orchards and tissues sampled

Samples were taken from orchards in the wider Te Puke region (Figure 2.1). Wherever possible, vines from at least three of the same orchards were sampled at each sampling time.

2.2.1 Autumn

In autumn (May 15 and 16) some of the same orchards sampled in January by Everett et al. (2011a) were used. Several of these orchards were no longer available because they had been cut out, or there were problems with access. There were no visible systemic cane symptoms on these orchards at this time, so instead the place where the symptomatic leaf had been sampled in January was selected as the epicentre. Five epicentres were sampled on each of the orchards, with sampling at each epicentre as follows:

- Cane tissue immediately adjacent to where the leaf showed symptoms in January
- Asymptomatic cane tissue 1 m from symptomatic leaf tissue on the same cane
- Asymptomatic cane tissue 2 m from symptomatic leaf tissue on the same vine
- Asymptomatic cane tissue 5 m from symptomatic leaf tissue on a different vine.

Two orchards that were not infected by Psa were included as negative controls. The negative control orchard of choice (orchard no. 8 in the previous study) was not accessible at this time.

2.2.2 Late-winter (pre-bud emergence)

The late-winter (dormant) samples were taken on 28 and 29 September, just before budburst on 'Hayward'. Because it was extremely difficult to find vines with systemic symptoms that had not been cut out, we sampled orchards that we knew had these types of symptoms and still had vines. 'Hayward' orchards from which systemic symptoms (cankers) had been reported were targeted. However, on only one of those orchards did we find a single 'Hayward' vine that had systemic symptoms; all other systemic symptoms that had been reported from 'Hayward' blocks were on green males. Two of the orchards sampled in January and May were sampled again in September. The original protocol was modified slightly to include more sample types, and to do more intensive sampling from the isolated uninfected orchard. Some tissue types were rare

or absent on some orchards (e.g. fruit stalks on male vines), and some samples were not taken because of grower concerns (e.g. trunk and leader bark samples).

Tissues sampled were:

1 Asymptomatic cane material from an isolated uninfected orchard
2 Pruned terminus of a cane
3 Terminated cane
4 Secondary cane
5 Dormant buds
6 Fruit stalks
7 Cane with symptoms
8 Trunk or main leader bark
9 Bud with symptoms
10 Swollen bud
11 Bud no symptoms (pruned cane)
12 Bud no symptoms (terminated cane)
13 Spur
14 Flower stalks.

Tissue types 2 to 14 were all collected from Psa-positive orchards.

2.2.3 Early spring

The early spring samples were taken from 'Hort16A' vines with systemic symptoms (cankers, wilting, failure of buds to break and oozing) about two weeks after shoot emergence. As before, the number of sample types was increased, and some sample types were rare on some orchards.

Tissue types from Psa-positive orchards were:

1 Terminus of a newly emerged cane
2 Dormant buds
3 Swollen buds
4 Cane with symptoms
5 Trunk or main leader bark
6 Bud with symptoms
7 New bud
8 Bud no symptoms
9 Cane no symptoms
10 Fruit stalks.

Samples taken from the asymptomatic isolated orchard were:

1 Asymptomatic cane tissue
2 Terminus of a newly emerged cane
3 Dormant buds
4 Swollen buds
5 New buds
6 Trunk or main leader bark.

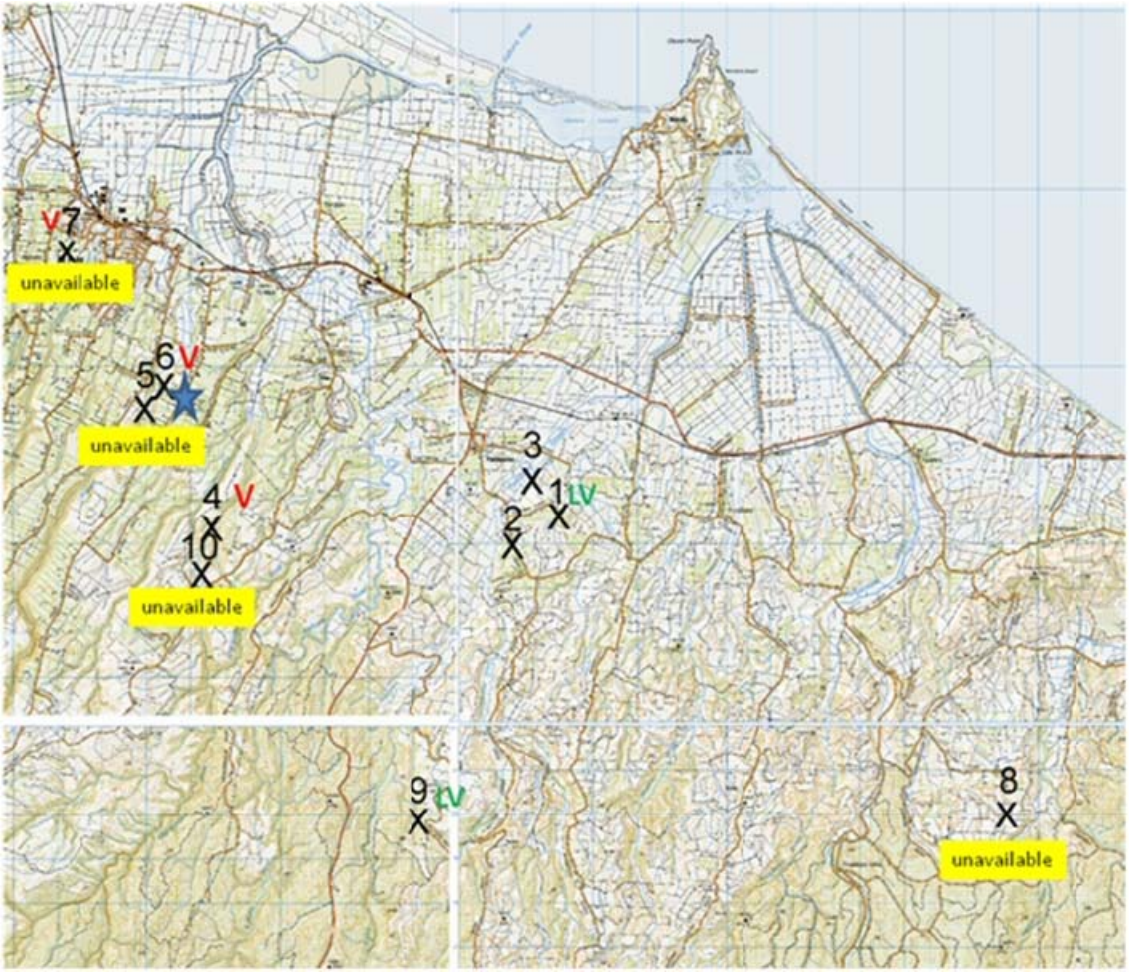


Figure 2.1. Location of the study orchards in the Bay of Plenty area sampled in May 2011. *Pseudomonas syringae* pv. *actinidiae* (Psa) status at the time of sampling is indicated for each orchard. LV = Psa low virulence strain and V= Psa Virulent strain. Star is location of RP1, where Psa was first diagnosed in New Zealand (Everett et al. 2011b).

Testing methods and protocols

Kiwifruit tissue was placed in sealed plastic bags, double contained, and immediately transported to Mt Albert Research Centre. Upon arrival at the laboratory, every sample was processed in one of two 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). Tissue was then dissected and placed in bacterial saline.
2. Tissue was dissected and placed in bacterial saline, without surface sterilisation.

A 100-ul aliquot from each sample was then spread over King's medium B (King et al. 1954) in a Petri plate, and incubated.

DNA was then extracted using the protocol recommended by MAF (Diagnostic protocol for detection of *Pseudomonas syringae* pv. *actinidiae* in Leaf Samples) 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.

2.3 Results

2.3.1 Autumn

Results of Polymerase Chain Reaction (PCR) tests from each of the orchards sampled in autumn are presented in Table 2.2. For ease of comparison, the results from the samples from the same orchards taken in January are presented in Table 2.1.

There were only three positive samples (Ct value < 30) from canes collected in autumn. Two were from a 'Hort16A' orchard diagnosed as infected with Psa-V before January, with severe (10/10) leaf symptoms and 10 positive leaf samples at that time. The other Psa-positive sample collected in May was from an orchard infected with Psa-LV in January, which had one Psa positive leaf sample at that time.

Almost all other samples from these orchards were weakly positive for Psa (Ct 30≤35) (Orchard 1; 30/40, Orchard 2; 40/40, Orchard 3; 24/40, Orchard 6; 29/40, Orchard 9; 27/40) except for Orchard 4 (3/40).

The total number of negative samples was 47/200 from orchards 1, 2, 3, 6 and 9, and 76.5% of these samples were positive or weakly positive.

Table 2.1. Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) for leaves sampled in January 2011.

Orchard		leaf samples															
Psa test		symptoms			no symptoms												Severity
	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
Psa-LV	2																
	3																
GK	4																
	5																
2	1																7
negative	2																
	3																
GK	4																
	5																
3	1																4
negative	2																
	3																
GK	4																
	5																
4	1																10
Psa-V	2																
	3																
GK	4																
	5																
6	1																0.5
Psa-V	2																
	3																
HW	4																
	5																
9	1																2
Psa-LV	2																
	3																
GK	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

Table 2.2. Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) for canes sampled from these same orchards in May 2011.

Orchard		cane samples								
Psa test		leaf symptoms		no symptoms						Date of Psa-V diagnosis for orchards negative in January 2011
variety	Epicentre			1m same cane		2m difft cane		5m difft vine		
		ss	ns	ss	ns	ss	ns	ss	ns	
1	1									16/09/2011
Psa-LV	2									
	3									
GK	4									
	5									
2	1									7/09/2011
negative	2									
	3									
GK	4									
	5									
3	1									12/08/2011
negative	2									
	3									
GK	4									
	5									
4	1									
Psa-V	2									
	3									
GK	4									
	5									
6	1									
Psa-V	2									
	3									
HW	4									
	5									
9	1									1/11/2011
Psa-LV	2									
	3									
GK	4									
	5									

Key:

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

ss	surface sterilised
ns	not surface sterilised

HW	Hayward
GK	Hort16A

2.3.2 Late-winter (pre bud emergence)

Results of PCR tests from the single 'Hayward' female vine with secondary symptoms are presented in Table 2.3, and of systemically infected males in each of the 'Hayward' orchards sampled pre-budburst in September are presented in Table 2.4.

Fruit stalks on the 'Hayward' vine were systemically infected, as demonstrated by the positive result in surface-sterilised samples (Table 2.3). However, the cane tissue with symptoms appeared only to be surface contaminated with Psa, as surface-sterilised tissue tested negative. There were several weakly positive samples that indicated systemic infections, including pruned canes with no symptoms, spurs with no symptoms, and buds with no symptoms. Parts of this vine were only infected on the surface, as indicated by negative test results following surface sterilisation. These parts were symptomless terminated canes, the trunk, and symptomless buds on pruned and terminated canes. More systemically infected 'Hayward' vines would need to be tested before any conclusions can be made from these results.

In 'Chieftain' (green males) sampled at the same time from the 'Hayward' orchard above, canes with symptoms were systemically infected, as were symptomless buds from pruned and terminated canes, and one of three flower stalks (Table 2.4). On the 'Chieftain' vines with cankers on a second 'Hayward' orchard sampled at the same time, three out of four flower stalks were systemically infected. Other samples on this second orchard that were positive for Psa by PCR were one bud without symptoms on a terminated cane, two dormant buds, two buds with symptoms, spurs, and one symptomless pruned cane.

Table 2.3. Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) for the same tissue surface sterilised or not from a single dormant 'Hayward' vine showing systemic symptoms.

	cane				spur	trunk	fruit	bud		
	symptoms	no symptoms pruned	no symptoms terminated	second year	no symptoms		stalks	symptoms	no symptoms pruned	no symptoms terminated
ns										
ss										

Key:

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

ss	surface sterilised
ns	not surface sterilised

Table 2.4. Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) for the same tissue surface sterilised or not from dormant 'Chieftain' vines showing systemic symptoms.

Orchard		Sample type																									
Psa test		cane								spur		leader or trunk		bud												flower stalks	
		symptoms		no symptoms pruned		no symptoms terminated		second year		no symptoms		bark		symptoms		dormant		swollen		no symptoms pruned		no symptoms terminated			
variety	Vine	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns
12	1							md	md			md	md			md	md	md	md								
Psa-V	2							md	md			md	md			md	md	md	md					md	md		
	3							md	md			md	md			md	md	md	md					md	md		
HW	5							md	md			md	md			md	md	md	md								
11	1											md	md	md	md	md	md	md	md					md	md		
Psa-V	2							md	md			md	md			md	md	md	md								
	3					md	md	md	md			md	md	md	md							md	md				
HW	4					md	md	md	md													md	md				
	5			md		md	md	md	md											md							

Key:

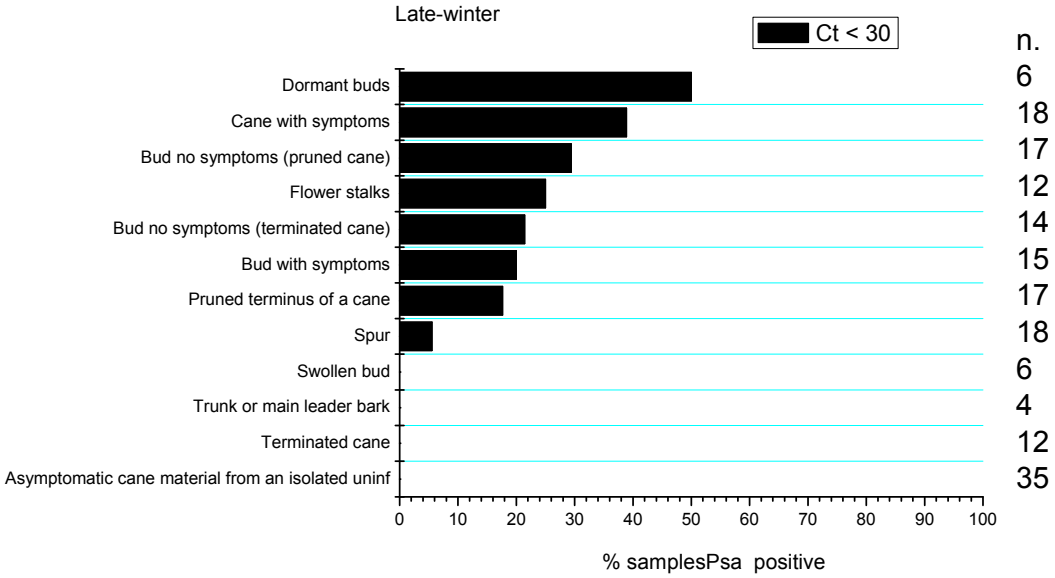
Colour code	CT value
negative	>35
weak positive	30-35
positive	<30

ss	surface sterilised
ns	not surface sterilised
md	missing data

HW	Hayward
GK	Hort16A

When all samples from the 'Chieftain' vines were pooled, (Figure 2.2), all tissue types sampled tested positive for Psa. Those with strong positive results (Ct<30) were dormant buds, canes with symptoms, asymptomatic buds from pruned canes and terminated canes, flowers stalks, buds with symptoms, pruned terminus of a cane, and spurs.

A.



B.

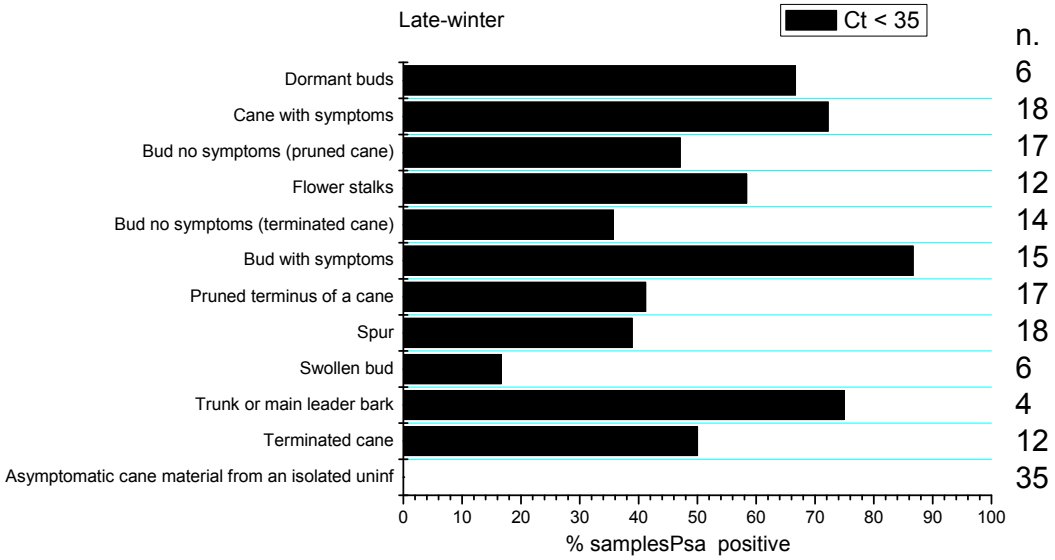


Figure 2.2. *Pseudomonas syringae* pv. *actinidiae* (Psa)-positive results from each tissue type sampled from dormant 'Chieftain' vines showing systemic symptoms in September. Results for samples that were surface sterilised and those that were not were combined. A. Positive (Ct <30), B. Positive or weak positive (Ct<35).

Figure 2.3. Systemic *Pseudomonas syringae* pv. *actinidiae* (Psa) symptom (canker) on a 'Hayward' vine.

Figure 2.4. Oozing *Pseudomonas syringae* pv. *actinidiae* (Psa) cankers on systemically infected dormant 'Chieftain' vines.

2.3.3 Early spring

'Hort16A' vines on the three orchards that were sampled in spring all showed systemic symptoms, and the orchards had all been diagnosed as Psa-V positive by commercial laboratories. Two of the three orchards were those that had been sampled previously in January and in May 2011. Results of qPCR testing samples from individual vines are shown in Table 2.5, with pooled results shown in Figure 2.5.

Almost all (12/15) canes with symptoms tested positive for Psa, and all positives were systemically infected.

Almost all (13/15) buds with symptoms tested positive for Psa, and of these all but one were systemically infected. Buds with no symptoms were positive or weakly positive (5/15 new buds, 8/13 swollen buds, 8/15 dormant buds and 2/9 buds with no symptoms) and of these 11/52 were systemically infected. Of the fruit stalks that were sampled, 3/5 were positive or weakly positive, and 2/5 were systemically infected. Newly emerged canes were positive or weakly positive for Psa (10/15) and of these, three were systemically infected. Only 2/8 symptomless canes were positive or weakly positive for Psa, and none were systemically infected.

When all samples were pooled, at least some of all the tissue types were positive for Psa, and all except buds with no symptoms were strongly positive (Ct <30) (Figure 2.5A and B).

Table 2.5: Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) for the same tissue surface sterilised or not from 'Hort16A' vines showing systemic symptoms in early spring.

Orchard		Sample type																					
Psa test		cane						leader		trunk		fruit stalks		bud									
KPIN		symptoms		no symptoms		newly emerged								symptoms		no symptoms		dormant		swollen		new	
variety	Vine	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns
2	1			md	md																		
Psa-V	2																						md
	3																						md
GK	4																						
	5																						
11	1			md	md			md	md			md	md			md	md						
Psa-V	2							md	md			md	md							md	md		
	3							md	md			md	md							md	md		
GK	4							md	md			md	md										
	5							md	md			md	md										
4	1			md	md							md	md			md	md						
Psa-V	2			md	md							md	md			md	md						
	3			md	md							md	md			md	md						
GK	4			md	md							md	md			md	md						
	5			md	md							md	md			md	md						

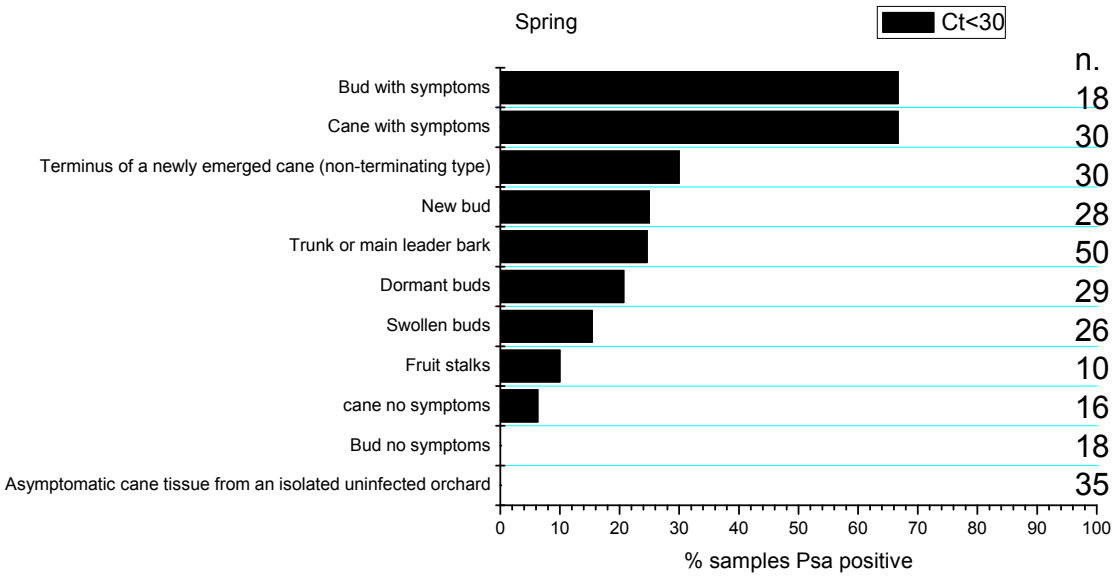
Key:

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

ss	surface sterilised
ns	not surface sterilised
md	missing data

HW	Hayward
GK	Hort16A

A.



B.

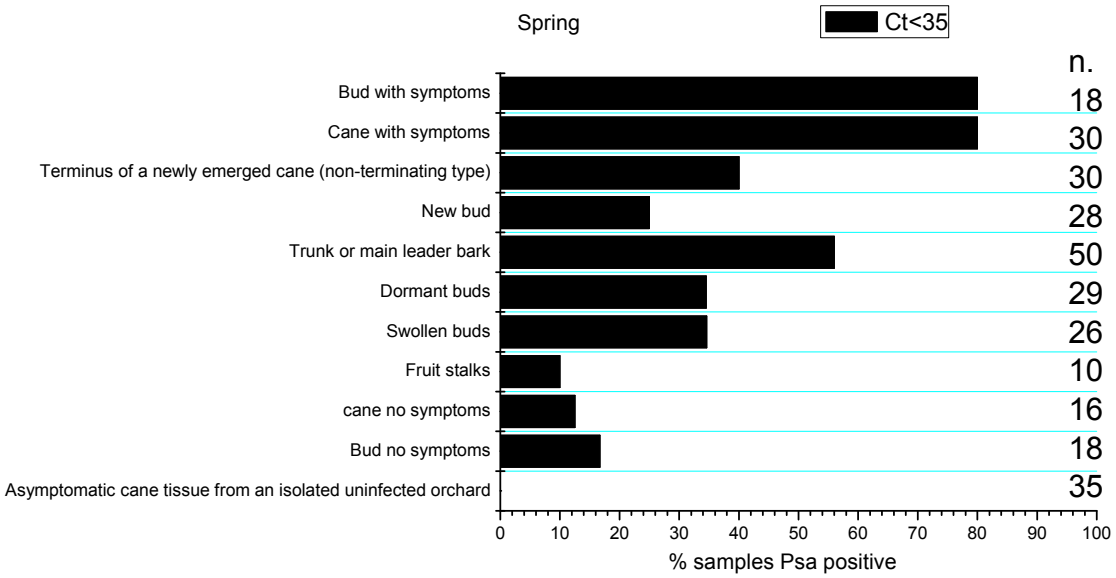


Figure 2.5. *Pseudomonas syringae* pv. *actinidiae* (Psa)-positive results from each tissue type sampled from 'Hort16A' vines showing systemic symptoms (cankers, wilting, failure of buds to break, oozing) in early spring. Results for samples that were surface sterilised and those that were not were combined. A: Positive (Ct <30), B: Positive or weak positive (Ct<35).

Figure 2.6. Canker on 'Hort16A', *Pseudomonas syringae* pv. *actinidiae* (Psa) symptoms on the outside (left) and underneath the bark (right).

Figure 2.7. 'Hort16A' showing wilting, vascular staining, and inhibited budburst (right top).

2.3.4 Asymptomatic orchard

When the asymptomatic 'Hort16A' orchard was sampled in January 2011, no samples were positive for Psa (Table 2.6).

However, when the same orchard was sampled after budburst on 29 September 2011, there was one positive and one weakly positive result. These were both of new buds, and were both surface contaminations. This orchard was diagnosed positive for Psa-V on 11 October 2011.

Table 2.6. Results of qPCR (Polymerase Chain Reaction) tests for *Pseudomonas syringae* pv. *actinidiae* (Psa) from the same asymptomatic orchard (Psa-negative when tested in January 2011 and 29 September 2011, but Psa-positive on 11 October 2011).

Orchard 8

GK

		leaf samples															
		symptoms			no symptoms												Severity
sampling	Epicentre				adjacent			1m same cane			2m different cane			5m different vine			rating/10
date		ss	ns	bs	ss	ns	bs	ss	ns	bs	ss	ns	bs	ss	ns	bs	
January	1																8
2011	2																
	3																
	4																
	5																

		cane				bark				bud					
		no symptoms		newly emerged		leader		trunk		dormant		swollen		new	
	Vine	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns	ss	ns
September	1														
2011	2														
	3														
	4														
	5														

Key:

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

ss	surface sterilised
ns	not surface sterilised
GK	Hort16A

2.4 Discussion

Vines from two orchards that had leaf spot symptoms in spring 2010 diagnosed with Psa-V were sampled in summer (January 2011) and then in autumn (May 2011) and finally in September 2011. One of these orchards (Orchard 2) was negative when sampled in January 2011, but all samples were weakly positive in May 2011. From these results, Orchard 2 became infected between January and May, but symptoms did not express until 7 September when it was diagnosed as infected with Psa-V. The most likely means of infection during this time period would be via pruning wounds made in late summer and early winter.

The other of these orchards (Orchard 4) was heavily infected in January 2011, but when sampled in May was only slightly infected. In September 2011, the vines sampled from this orchard were again heavily infected, and it was difficult to find canes or buds without symptoms. This result is difficult to explain, although it possibly reflects the erratic distribution of Psa within

kiwifruit tissue. Further testing would be required to determine this, or to formulate another hypothesis.

The one ‘Hayward’ vine with a canker seemed to be less infected than the ‘Chieftain’ or the ‘Hort16A’ vines that were sampled. However, more vines should be sampled before conclusions can be made.

The two infections on the ‘symptomless negative orchard (Orchard 8) that was negative when sampled in January 2011, were of new buds. This suggests that new buds may be more susceptible to new infections than any of the other tissue types that were sampled, and that this orchard may have become infected by inoculum that was wind and rain dispersed from adjacent infected orchards during early spring.

When ‘Hort16A’ or ‘Chieftain’ show systemic symptoms (cankers, ooze, failure of buds to break), it would be prudent to remove the vines and destroy them, to reduce the amount of inoculum available to infect other vines

Removal of fruit stalks from ‘Hayward’ vines after picking followed by immediate application of a protectant spray may reduce infections.

2.5 Conclusions

- When sampled in May, most ‘Hort16A’ cane tissue sampled was weakly positive for Psa whether the orchards from which the cane had been sourced had been reported to be Psa-positive or -negative in January.

- When the same orchards were sampled again in September, the vines had cankers and almost all plant parts sampled were infected with Psa, including the orchard that was negative in January.

- These results suggested that new infections of ‘Hort16A’ took place between January and May, Psa invaded and spread within the vines without causing symptoms, and that symptoms were expressed in spring.

- Not all plant parts sampled of the one ‘Hayward’ vine with canker symptoms were infected, in contrast to ‘Hort16A’, of which nearly all plant parts sampled were infected.

2.6 Future research

Systematic sampling of ‘Hayward’ vines with cankers should be made and compared with the same types of samples from ‘Hort16A’ vines with cankers to ascertain if ‘Hayward’ vines are less systemically infected than ‘Hort16A’, which could explain why this cultivar is more tolerant to Psa. If a difference is reliably detected, then this sampling technique could be used to test the new ‘tolerant’ cultivars (G3, G9, G14), and then used for testing other advanced selections. These results could inform research examining underpinning mechanisms for tolerance, leading to more rapid screening of germplasm.

3 Survival and spread of Psa within the vine

Joy Tyson, Mike Manning, Carol Curtis, Jonathan Rees-George

3.1 Key question and aim

At present there is very little knowledge of the spread of *Pseudomonas syringae* pv. *actinidiae* (Psa) within the kiwifruit vine in New Zealand; this is especially true for woody tissues. It may be possible to eradicate Psa from a vine by identifying the extent of infection and removing the affected tissue. The information gained by this project should help growers to assess the extent of pruning required to remove diseased vine tissue from vines, ideally halting the spread of systemic infections and reducing the risk of re-infection.

3.2 Methods

3.2.1 Orchards

Three 'Hort16A' kiwifruit orchards (*Actinidia chinensis* 'Hort16A') with virulent Psa (Psa-V) were chosen to allow vines of various ages to be tested (Figure 3.1). Five female vines were sampled in each orchard and samples were taken at three times: late autumn (leaf fall), mid-winter and early spring (budburst). Vines were clearly marked to allow the same vines to be sampled at each sampling date. All the orchards were in the Te Puke district.

In addition to the three 'Hort16A' orchards, one 'Hayward' orchard (*Actinidia deliciosa* 'Hayward') confirmed with Psa-V was also sampled. Five male and five female vines were sampled in this orchard at two times: mid-winter and early spring (budburst).

Orchard 1: This orchard contained the earliest known infected vines. The vines have large rootstocks and leader wood, grafted to 'Hort16A' in the late 1990s. The variety of the rootstock is unknown and dates from ~1967. The vines in this orchard were decapitated over the summer of 2010-11 and at the first sampling all canes present were summer regrowth.

Orchard 2: Orchard 2 had the largest infected vines in this study. The vines have very large rootstocks and leader wood. The rootstocks were *A. deliciosa*, grafted to 'Hort16A'.

Orchard 3: This orchard contains the youngest and most recently grafted vines. Vines have 'Bruno' rootstock, 'Hayward' interstock and were grafted to 'Hort16A' in ~2002. As with Orchard 1, the canes in this orchard were removed over the summer of 2010-11 and at the first sampling all canes present were summer regrowth.

Orchard 4: Orchard 4 ('Hayward') had medium-large vines based on 'Bruno' rootstock grafted to 'Hayward' – Kramer wood and had previously tested positive for Psa-V in one systemically infected cane. That vine was included in this trial (vine 1, Orchard 4). Only the trunks and leaders were sampled. Both male and female vines were sampled on this orchard.

3.2.2 Sampling sites on vines:

Samples were taken at up to 31 planes in the vine starting from just above the roots to well within an obvious 'dieback' cane. The sampling planes were either 10 cm apart (Orchards 1 and 3) or 20 cm apart (Orchards 2 and 4), depending upon the size of the vines.

At each plane on the trunk and leader, four individual samples were taken from around the girth of the trunk. Because of the smaller diameter of the canes, at each sampling plane on a cane, only a single sample, extending across the entire diameter, was taken.

Figure 3.1 Clockwise from top left: representative sample kiwifruit vines in Orchard 1, 2, 3 and 4. Paint marks indicate tissue sampling points.

3.2.3 Sample method

The top layer of bark was removed and the wound site was sterilised with 95% ethanol. This area was drilled through with a sterilised 3.2-mm wood drill to a depth of 1.5 cm. The shavings adhering to the drill bit were then placed in a sterile Eppendorf tube containing 1 ml sterile bacteriological saline (0.85% NaCl in sterile distilled water). The four samples taken from each plane were bulked. The wounds on the vine were sealed with pruning paste.

3.2.4 Isolation and identification

Samples were agitated and a 200-µL aliquot of the resulting suspension was then streaked onto King's B medium (King et al. 1954) with a series of sterile loops. Plates were incubated at room temperature (c. 20°) for two days.

DNA extractions were made from the mixed-colony agar plates using the boiling method of R.K. Taylor (Rob Taylor, MAF, pers. comm., March 2011). Subsequent identification used the method of Rees-George et al. (2010), modified for use with qPCR.

3.3 Results

3.3.1 Orchard 1 ('Hort16A')

Figure 3.2 shows the results of seasonal sampling (autumn, winter, spring) of a representative vine in Orchard 1. Full results are presented in Appendix 3.1.

At the autumn sampling time, *Pseudomonas syringae* pv. *actinidiae* was found in all canes sampled, frequently well below the zone of cane dieback. It was also found in three of the five trunks sampled.

By the time of winter sampling, all the canes with dieback that had been sampled in autumn had been removed, and two of the five vines sampled had also had a portion of the trunk removed. All the new canes sampled were positive for Psa, as were two of the five trunks.

At the spring sampling time, all the vines were in the process of being further cut back, with the aim of leaving only stumps. At this point, the tallest trunk was 1 m high; the shortest 15 cm. At that sampling, only one of the stumps tested positive for Psa (Figure 3.2).

Vine 3 - Orchard 1					
		cane 2	positive		
		cane 2	positive		
cane	positive	cane 2	positive		
cane	positive	cane 2	positive		
cane	positive	cane 2	positive		
cane	positive	cane 2	positive		
cane	positive	cane 2	positive		
cane	positive	cane 2	positive		
cane	negative	cane 2	positive		
cane	negative	cane 2	negative		
cane	positive	cane 2	weak +ve		
cane	weak +ve	cane 2	negative		
cane	negative	cane 2	negative		
cane	weak +ve	cane 2	negative		
cane	weak +ve	cane 2	negative		
trunk	negative	Trunk	negative	trunk	negative
trunk	negative	Trunk	negative	trunk	negative
trunk	negative	Trunk	negative	trunk	weak +ve
trunk	weak +ve	Trunk	negative	trunk	weak +ve
trunk	weak +ve	Trunk	negative	trunk	negative
trunk	weak +ve	Trunk	positive	trunk	positive
trunk	weak +ve	Trunk	negative	trunk	positive
	Autumn		**Winter**		**Spring**

Figure 3.2 Results of seasonal sampling (autumn, winter, spring) of a representative kiwifruit vine in Orchard 1, 2011. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 10-cm intervals. Red = *Pseudomonas syringae* pv. *actinidiae* (Psa) positive (Ct value <30) Yellow = Psa weak positive (Ct value from 30 to 35), Green = negative (Ct value > 35).

3.3.2 Orchard 2 ('Hort16A')

Figure 3.3 shows the results of seasonal sampling (autumn, winter, spring) of a representative vine in Orchard 2. Full results are presented in Appendix 3.1. Figure 3.4 shows the average percentage of trunk and leader samples in Orchard 2 that tested positive for live Psa at each sampling time.

In autumn, each of the sampled vines in Orchard 2 had many canes with symptoms of dieback. In most cases the dieback extended down to the point of attachment to the leader. Many of these canes were shrivelled and very dry. Two out of five canes were positive. The dry canes did not test positive for live Psa. Only one of the trunks tested positive in the autumn sampling.

At the winter sampling time, all the die-back canes that were sampled in the autumn had been removed, some with a 5-cm 'stub' remaining. Four out of five trunks/leaders were positive at that time.

At the spring sampling time, all the trunks/leaders tested positive for Psa. Four of the five vines had developed oozing cankers on the leader.

Vine 2 - Orchard 2					
cane	negative				
cane	negative				
cane	negative	cane	negative	cane	negative
leader	negative	leader	weak +ve	leader	positive
leader	negative	leader	weak +ve	leader	positive
leader	negative	leader	weak +ve	leader	weak +ve
leader	no growth	leader	negative	leader	positive
leader	negative	leader	negative	leader	positive
leader	negative	leader	negative	leader	positive
leader	negative	leader	negative	leader	positive
leader	negative	leader	negative	leader	positive
leader	negative	leader	weak +ve	leader	positive
trunk	negative	trunk	negative	trunk	positive
trunk	positive	trunk	negative	trunk	positive
trunk	no growth	trunk	negative	trunk	positive
trunk	negative	trunk	negative	trunk	positive
	Autumn		**Winter**		**Spring**

Figure 3.3 Results of seasonal sampling (autumn, winter, spring) of a representative kiwifruit vine in Orchard 2, 2011. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 20-cm intervals. Red = *Pseudomonas syringae* pv. *actinidiae* (Psa) positive (Ct value <30) Yellow = Psa weak positive (Ct value from 30 to 35), Green = negative (Ct value > 35).

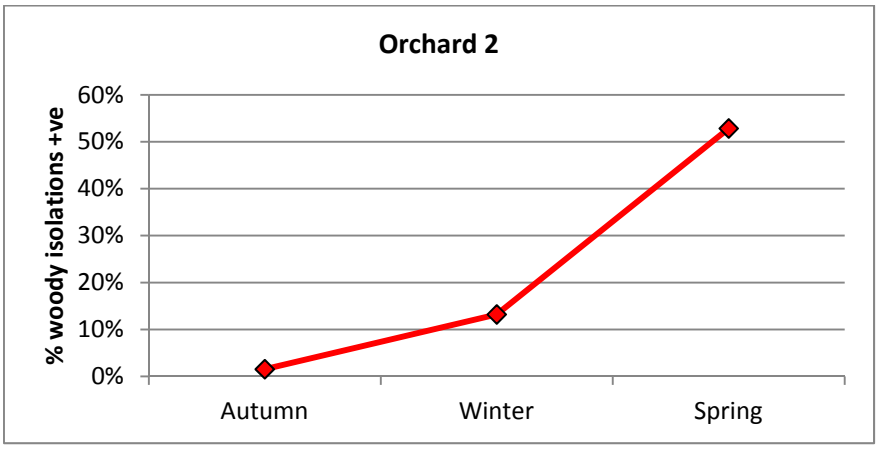


Figure 3.4 Percent kiwifruit trunk and leader isolations positive for *Pseudomonas syringae* pv. *actinidiae* (Psa) at each sampling time in Orchard 2, 2011 (average of five vines).

3.3.3 Orchard 3 ('Hort16A')

Figure 3.5 shows the results of seasonal sampling (autumn, winter, spring) of a representative vine in Orchard 3. Full results are presented in Appendix 3.1.

In autumn, the new canes on vines within Orchard 3 were showing 'tip dieback'; Psa was not confirmed in any of the samples taken at that sampling. At the winter sampling, Psa was confirmed in tip dieback tissue and in the trunk of one vine. None of the remaining four vines tested positive. At the spring sampling time, Psa was confirmed from 60% of the canes tested but not from the trunks.

Vine 5 - Orchard 3					
dieback	negative	dieback	negative		
dieback	negative	dieback	negative		
dieback	negative	dieback	negative	dieback	Positive
cane	no growth	cane 2	-	dieback	Negative
cane	negative	cane 2	-	cane 3	weak +ve
cane	no growth	cane 2	-	cane 3	no growth
cane	no growth	cane 2	-	cane 3	no growth
cane	no growth	cane 2	-	cane 3	no growth
cane	no growth	cane 2	-	cane 3	weak +ve
cane	no growth	cane 2	-	cane 3	no growth
cane	no growth	cane 2	-	cane 3	no growth
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	weak +ve
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
trunk	negative	trunk	negative	trunk	Negative
	Autumn		**Winter**		**Spring**

Figure 3.5 Results of seasonal sampling (autumn, winter, spring) of a representative kiwifruit vine in Orchard 3, 2011. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 10-cm intervals. Red = *Pseudomonas syringae* pv. *actinidiae* (Psa) positive (Ct value <30) Yellow = Psa weak positive (Ct value from 30 to 35), Green = negative (Ct value > 35).

3.3.4 Orchard 4 ('Hayward')

Figure 3.6 shows the results of seasonal sampling (winter and spring) of representative male and female vines in Orchard 4. Full results are presented in Appendix 3.2. Figure 3.7 shows the average percentage of trunk and leader samples in Orchard 4 that tested positive for live Psa at each sampling time.

During winter, Psa was found in the leaders of one each of the male and female vines. At the spring sampling time, Psa was confirmed in all the male vines and in three out of five of the female vines.

Vine 1M - Orchard 4 (male)				Vine 1F - Orchard 4 (female)			
				leader	no growth	leader	negative
				leader	no growth	leader	negative
cane	positive	cane	positive	leader	no growth	leader	positive
leader	negative	leader	no growth	leader	no growth	leader	no growth
leader	negative	leader	positive	leader	no growth	leader	positive
leader	no growth	leader	no growth	leader	negative	leader	no growth
trunk	no growth	trunk	no growth	leader	negative	leader	negative
trunk	negative	trunk	no growth	leader	negative	leader	positive
trunk	no growth	trunk	positive	leader	no growth	leader	weak +ve
trunk	no growth	trunk	negative	trunk	no growth	trunk	negative
trunk	no growth	trunk	weak +ve	trunk	negative	trunk	weak +ve
trunk	no growth	trunk	negative	trunk	no growth	trunk	positive
trunk	no growth	trunk	no growth	trunk	negative	trunk	positive
	Winter		**Spring**		**Winter**		**Spring**

Figure 3.6 Results of seasonal sampling (winter and spring) of representative *Actinidia deliciosa* male and female 'Hayward' vines in Orchard 4, 2011. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 20-cm intervals. Red = *Pseudomonas syringae* pv. *actinidiae* (Psa) positive (Ct value <30) Yellow = Psa weak positive (Ct value from 30 to 35), Green = negative (Ct value > 35).

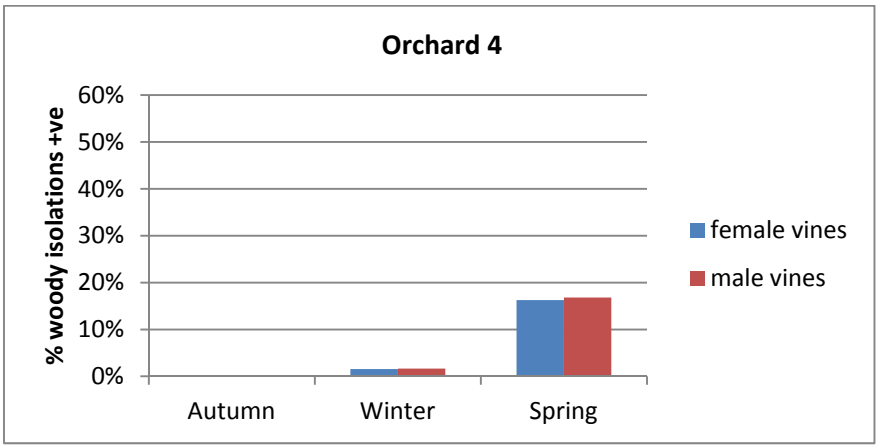


Figure 3.7 Percent trunk and leader isolations positive for *Pseudomonas syringae* pv. *actinidiae* (Psa) at each sampling time in Orchard 4 'Hayward', 2011 (average of five vines).

3.4 Discussion

In Orchard 1, the removal of obviously diseased canes and the progressive removal of sections of the trunk did not contain the bacterium. This suggests that the pathogen had progressed very rapidly down the vines on that orchard and was already well established beyond the point of cutting.

Although Orchard 2 had many dead canes present in autumn, Psa was found in only two canes, indicating that the bacterium may have died out in the very dry canes. Over the three sampling times, there was obvious progression of the disease in all the monitored vines in Orchard 2, with four of the five vines developing oozing cankers on the leader by spring.

Orchard 3 had been pruned back to the trunks and was showing 'tip dieback' of the new canes at all sampling times. Autumn results indicated that there were no bacteria present in the 'clean' parts of the canes and only weak positives were returned in some of the tip dieback samples and one of the trunks. There were no positive samples at this time. This was unexpected, as samples of tip dieback from this orchard had tested positive for live Psa a month earlier.

As in Orchard 2, there was obvious progression of the bacterium in all the vines in Orchard 4. Male and female 'Hayward' vines were similar, with few positive isolations during winter (~2%), increasing in spring to 16-17%.

In this project we used the bacterial growth medium KB during the enrichment step. Since starting this work, we have found that a semi-selective media allows greater recovery of Psa (Tyson & Curtis, unpub.). This is a semi-selective agar medium for *Pseudomonas syringae*, and is a modification of KBC medium (Mohan & Schaad 1987). It is thought that the work presented here underestimates Psa presence in samples and that if the semi-selective media had been used, significantly more Psa would have been recovered.

3.5 Key findings

These data indicate that Psa can move systemically throughout both 'Hort16A' and 'Hayward' cultivars of kiwifruit.

Live Psa has been found in the canes, leaders, trunks and roots (data not shown) of female 'Hort16A' vines, and in the canes, leaders and trunks of 'Hayward' and *A. deliciosa* male vines. Psa was found in trunks down to 10 cm above the ground, indicating that the graft does not impede progress of the bacterium and that there is potential for resident populations to be present in the remaining portions of rootstocks and stumps, prior to re-grafting.

Psa was detected in parts of the vine with no visible symptoms. Therefore, removal of obviously diseased canes does not necessarily remove the bacterium, as it can be present in asymptomatic tissues quite remote from diseased tissues.

The bacterium appears to be able to colonise the entire vine relatively rapidly (between sampling dates). Alternatively, a bacterial population below the limits of detection may have become established in asymptomatic tissues, resulting in a rapid increase in bacteria after some pre-disposing event or plant growth stage, or when a critical population size is reached.

The bacterium appeared to have a discontinuous distribution in the trunks and an "advancing tide" of bacteria was not obvious (such as that seen in fire blight, where the bacterium is

recoverable just in advance of developing necrosis). The distribution of the pathogen within the tissues across any cross-section of the plant is not regular and it may be isolated from some samples and not others taken from the same cross section. This means that a conservative sampling system from trunks or canes of plants may fail to detect the organism.

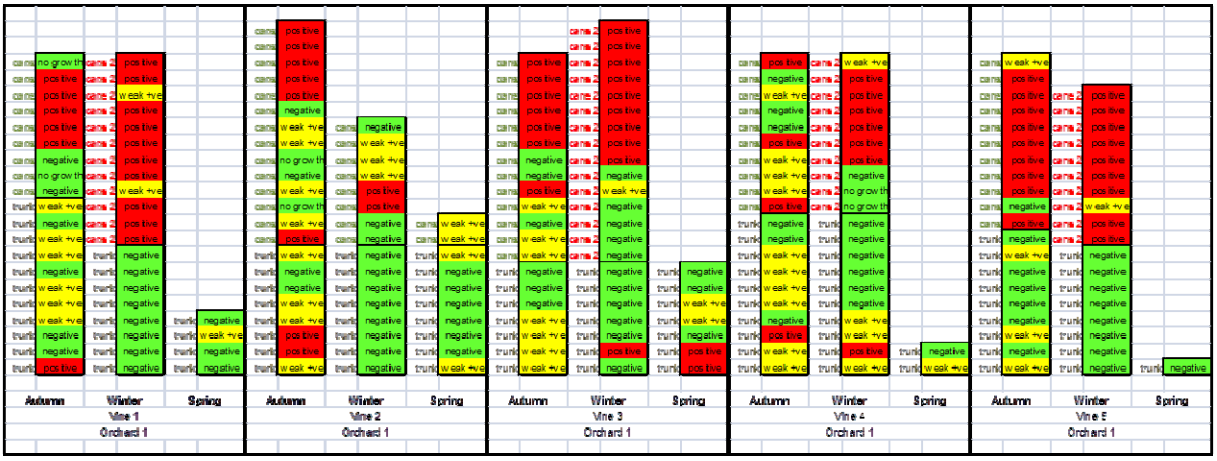
3.6 Recommendations for future work

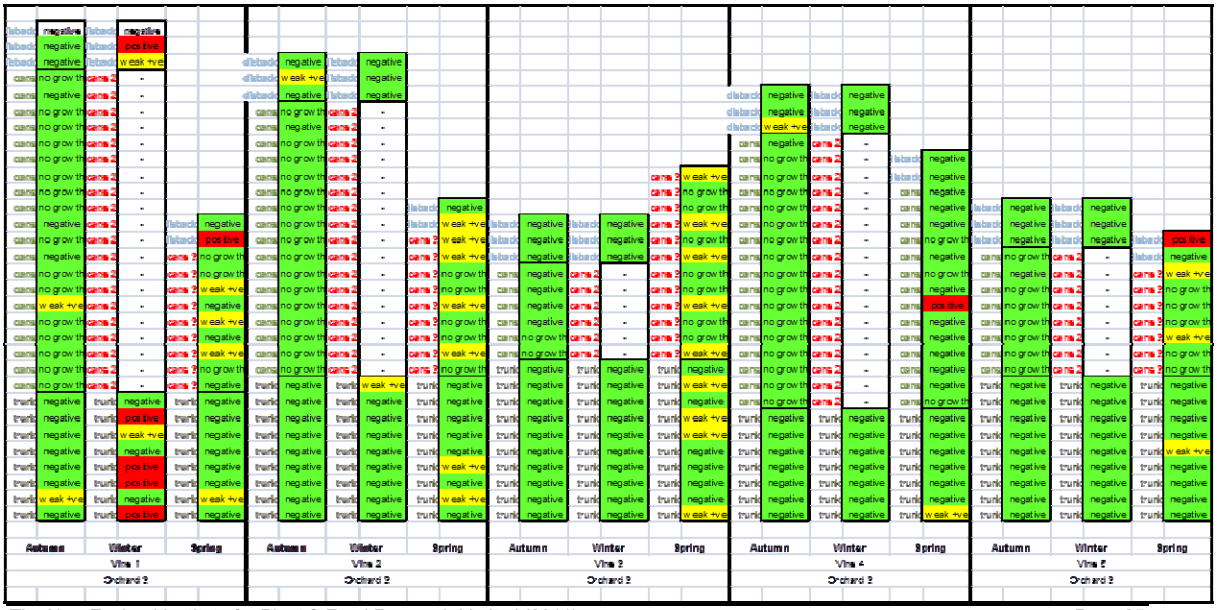
Questions that require further research include:

- What is the fate of systemically infected 'Hayward' vines? Following the five sample vines in Orchard 4 through summer and autumn would show the behaviour of systemic populations of Psa throughout an entire year.
- Is it feasible to graft new varieties on to previously infected stumps? What is the outcome when grafting on to "known status" stumps?

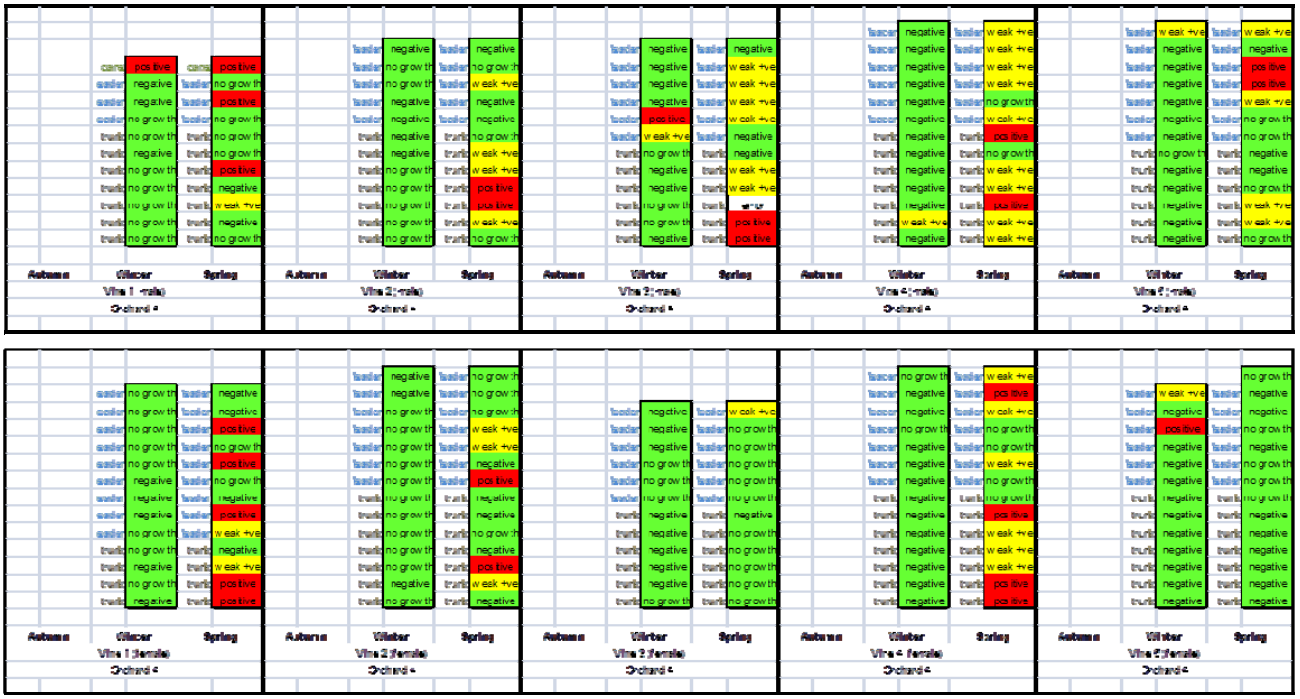
3.7 Appendices

Appendix 3.1 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) testing of all kiwifruit vines: autumn, winter and spring sampling. 'Hort16A' orchards #1 (top), #2 (middle), and #3 (bottom), 2011. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 10- or 20-cm intervals. Red = Psa positive (Ct value<30) Yellow = Psa weak positive (Ct from 30 to 35), Green = negative (Ct > 35).





Appendix 3.2 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) testing of all kiwifruit vines: winter and spring sampling, Orchard #4 ('Hayward'), 2011. Top = male vines, bottom = 'Hayward' vines. For each column, the top position represents a distal part of the cane, and the bottom square represents the sample taken near the bottom of the trunk. Positions in between represent samples taken at 20-cm intervals. Red = Psa positive (Ct value <30) Yellow = Psa weak positive (Ct value from 30 to 35), Green = negative (Ct value > 35).



4 The status of Psa as an epiphyte

Joy Tyson and Carol Curtis

4.1 Key question and aim

An epiphytic organism is an organism that is able to exist and multiply on the surface of a plant without causing infection.

Leben (1965) characterised microbial epiphytes into 'resident' and 'casual' epiphytes. Resident epiphytes are able to multiply on the surface of healthy, living plants, without causing disease. Casual epiphytes may be inactive, on the plant accidentally, or living saprophytically on foreign debris on the plant.

Based on the knowledge that many *Pseudomonas syringae* pathovars can survive epiphytically, it has been assumed that Psa is an epiphyte, presumably forming micro-colonies on asymptomatic leaves. However, there seems to be no published evidence for this. For example, Balestra & Varvaro (1999), in an article discussing bacterial diseases of kiwifruit in Italy, give a brief life-cycle for Psa but do not record Psa as an epiphyte. They do note that *P. viridiflava* is able to survive as an epiphytic micro-organism during unfavourable periods. A later article states that *P. s.* pv. *syringae* and *P. viridiflava* are characterised to be epiphytic microorganisms with a 'resident' phase on the host (Balestra & Bovo 2003); however, they again fail to characterise Psa in this way.

Currently, a significant amount of effort is being put in to the control of epiphytic populations of Psa, without clear evidence as to whether Psa is, or is not, able to multiply on asymptomatic leaves. Knowledge of the status of Psa as a potential epiphyte will allow more informed management decisions.

4.2 General methods

4.2.1 Strains of Psa

Psa isolate 3.2.3 (cc691) was isolated from kiwifruit leaf spots at Te Puke Research Orchard in February 2011. Psa 3.2.3 was previously determined to be haplotype NZ-V by J Vanneste (Plant & Food Research) and has been shown to be pathogenic in leaf disc assays (Tyson, Curtis & Fullerton unpub.).

Spontaneous rifampicin-resistant mutants of Psa were acquired by streaking isolates onto King's B medium (King et al. 1954) amended with 50 ppm rifampicin. Colonies that grew normally on this medium were then transferred to plates containing increasing concentrations of rifampicin (100, 150 and 200 ppm). The mutants have been maintained on KB containing 100 ppm of rifampicin.

The pathogenicity of the rifampicin-resistant strain used in this trial (Psa 3.2.3/rif) was compared with that of the wild strain in leaf disc assays and was shown to produce the same symptoms over the same time frame. Therefore it was concluded that the resistance to rifampicin was not detrimental to its pathogenicity (data not shown).

4.2.2 Inoculum

Two-day-old cultures of each isolate were suspended in bacteriological saline (0.85% NaCl in sterile distilled water). The appropriate concentrations of colony forming units per ml (cfu/ml) were achieved by measuring the turbidity with a spectrophotometer.

4.2.3 Leaf disc assay:

Plantlets of *Actinidia chinensis* 'Hort16A', grown from tissue culture in a greenhouse at Mt Albert Research Centre, were used for this work. The second youngest fully opened leaf was used. Leaves were surface-sterilised in 0.1% NaOCl for 15 minutes, then rinsed twice in sterile reverse-osmosis (RO) water.

The tests were carried out in Petri dishes using an agar-based medium (6 g agar/400 ml water) amended with 150 ppm benzimidazole (Aldrich) to prevent senescence of the leaf tissue. Six 15-mm diameter wells were made within the agar of each plate.

Six leaf discs (15 mm diam.) were excised from each leaf and placed abaxial side up in the agar wells (Figure 4.1). Five of the leaf discs were inoculated in the centre with a droplet of a bacterial suspension, the sixth with a drop of bacteriological saline (BS) as a control. Discs were incubated at 15°C in the dark.

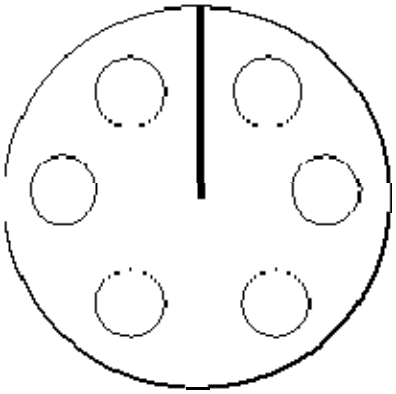


Figure 4.1. Layout of leaf disc assays in Petri dishes.

4.2.4 Experimental design

Leaf discs of 'Hort16A' were inoculated with known suspensions of either Psa 3.2.3 or the rifampicin-resistant strain Psa 3.2.3/rif. Bacterial populations were washed off the leaf discs at set times after inoculation, and serially diluted onto KB or rifampicin-amended KB. Colony counts were made on the plates to ascertain whether bacterial populations on the leaf discs were increasing, static or declining.

4.3 Methodology and results

4.3.1 Experiment 1

Five plates of six leaf discs each were inoculated on five of the leaf discs with 40-µL aliquots of a suspension of Psa 3.2.3 at 10^2 cfu/ml, delivering approximately 40 cfu of Psa per leaf disc. The sixth leaf disc in each plate was inoculated with the BS control.

Serial dilutions were made from five leaf discs (only one taken from each plate at each time) after 0, 2 and 4 days. At each time, the leaf discs were vortexed in 1 ml BS for 20 s and serial dilutions made. 0.1-ml aliquots were spread onto KB and colony counts were made after three days.

Figure 4.2 shows the increase in Psa 3.2.3 on the leaf discs over four days.

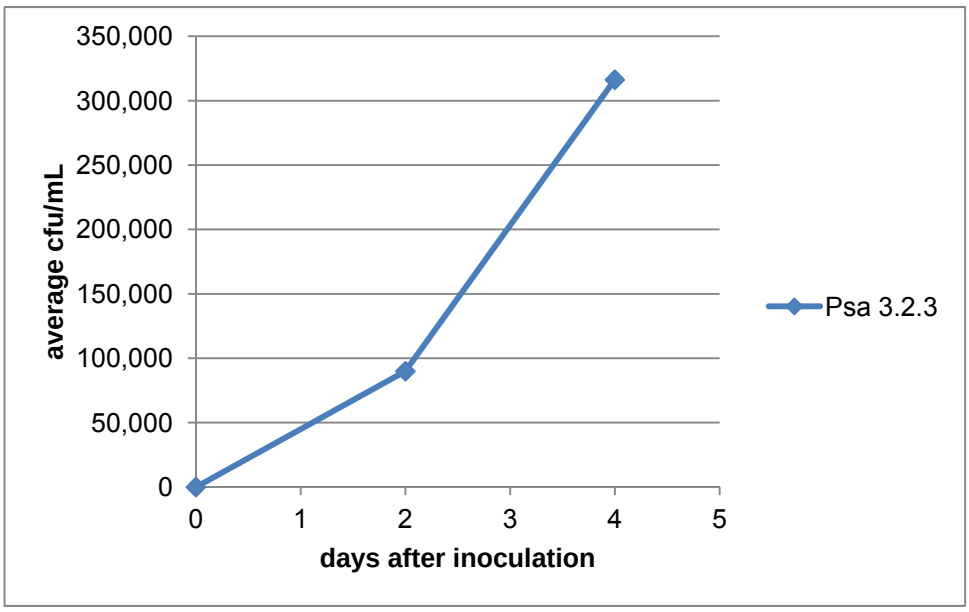


Figure 4.2 Average increase in populations of *Pseudomonas syringae* pv. *actinidiae* (Psa) 3.2.3 on kiwifruit leaf discs in experiment 1 over four days (day 0 inoculation ~40 cfu).

In experiment 1, initial populations of 40 cfu increased over four days to over 300,000 cfu. No symptoms (flecks, necrosis or bacterial ooze) were seen on the leaf discs over this time.

4.3.2 Experiment 2

Sets of ten plates containing six leaf discs were set up with each of four treatments as shown in Table 4.1. Leaf discs of tobacco were included as a non-host. The Psa isolates 3.2.3 and 3.2.3/rif were both tested, to ensure that the rifampicin-resistant mutant behaved similarly to the wild strain.

Table 4.1 Treatment numbers, *Pseudomonas syringae* pv. *actinidiae* (Psa) isolate and host in the second leaf disc experiment.

Treatment #	Isolate	Host
1	Psa 3.2.3	*Actinidia chinensis* 'Hort16A'
2	Psa 3.2.3/rif	*Actinidia chinensis* 'Hort16A'
3	Psa 3.2.3	*Nicotiana tabacum* 'White Burley'
4	Psa 3.2.3/rif	*Nicotiana tabacum* 'White Burley'

Leaf discs were inoculated with 10-µL aliquots of a suspension of Psa at approximately10^5 cfu/ml; approximately 1000 cfu per leaf disc. Serial dilutions and colony counts of the initial inoculum confirmed the inoculum rate of Psa 3.2.3 to be 950 cfu per leaf disc (9.5 x 10^4 cfu/ml) and that of Psa 3.2.3/rif to be 550 cfu per leaf disc (9.5 x 10^4 cfu/ml).

Serial dilutions were made from five leaf discs of each treatment (taken individually from five separate plates) after 0, 1, 2, 3, 4, 5 and 7 days. At each sampling time, the individual leaf discs were vortexed in 1 ml BS for 20s and serial dilutions made. 0.1-ml aliquots were spread onto KB (Psa 3.2.3 treatments) or KB amended with 100 ppm rifampicin (Psa 3.2.3/rif treatments). Colony counts were made after three days.

Leaf discs were also marked under a dissecting microscope for any developing symptoms, such as flecks, each day.

Figure 4.3 shows the increase in populations of Psa 3.2.3 and Psa/rif on leaf discs of 'Hort16A' or tobacco in experiment 2 over seven days. Figure 4.4 shows the average percent leaf necrosis on 'Hort16A' or tobacco inoculated with Psa 3.2.3 or 3.2.3/rif over seven days.

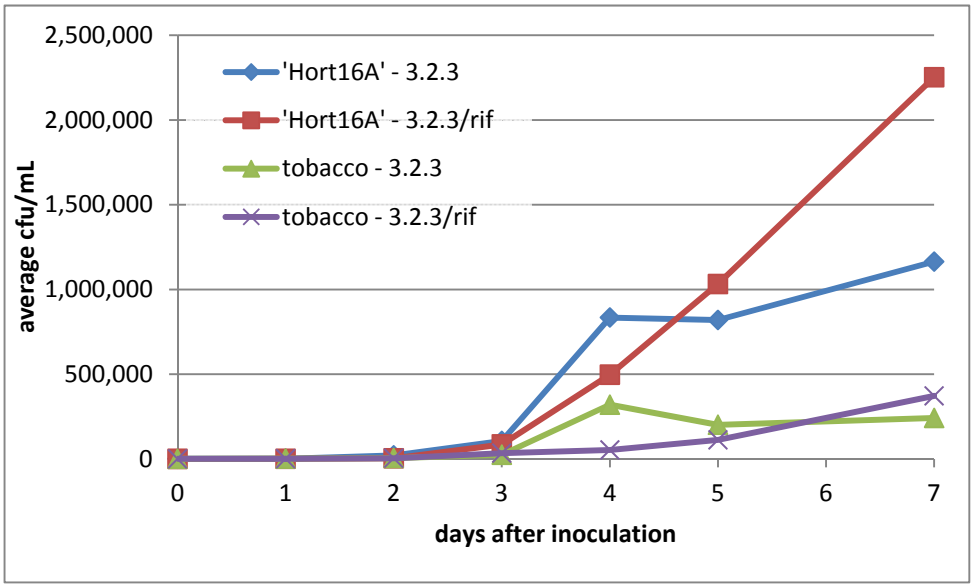


Figure 4.3 Average increase in populations of *Pseudomonas syringae* pv. *actinidiae* (Psa) 3.2.3 and Psa/rif on leaf discs of 'Hort16A' kiwifruit or tobacco in experiment 2 over seven days.

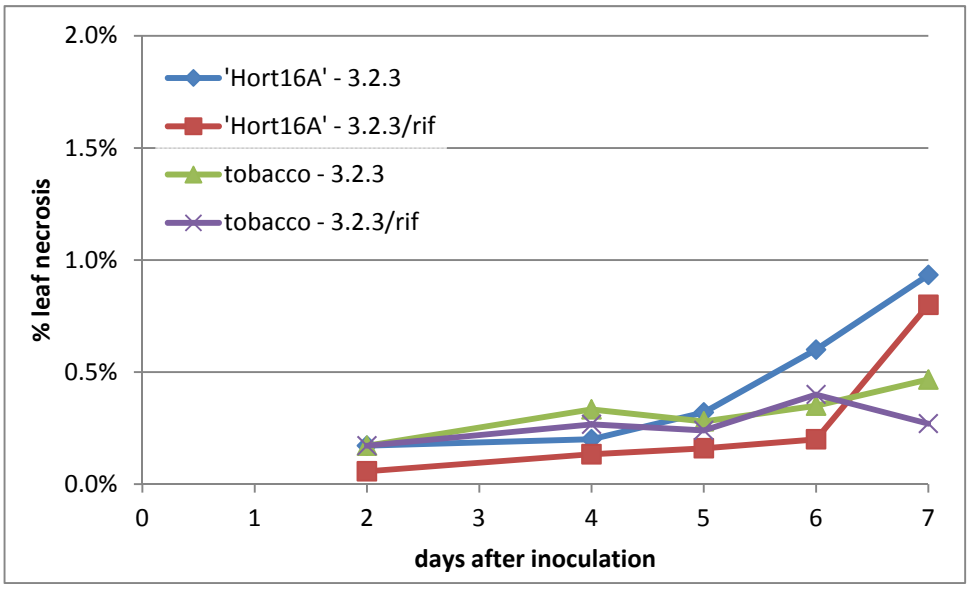


Figure 4.4 Average percent leaf necrosis on 'Hort16A' kiwifruit or tobacco inoculated with *Pseudomonas syringae* pv. *actinidiae* (Psa) 3.2.3 or 3.2.3/rif over seven days.

In experiment 2, initial populations of 950 cfu of Psa 3.2.3 increased over the seven-day observation period to over 1,100,000 cfu on 'Hort16A' and to over 240,000 on tobacco leaves. Typical flecks appeared on the 'Hort16A' leaf discs over this time and atypical HR-like flecks appeared on the tobacco leaves.

Similarly, initial populations of 550 cfu of Psa 3.2.3/rif increased over seven days to over 2,000,000 cfu on 'Hort16A' and to over 300,000 on tobacco leaves. Typical flecks appeared on the 'Hort16A' leaf discs over this time and atypical HR-like flecks appeared on the tobacco leaves.

4.4 Discussion

The rifampicin-resistant strain of Psa was shown to respond in a similar manner to the wild strain. It was easily recoverable by plating on to rifampicin-amended KB and as such is a good tool for studies of the behaviour of Psa.

The leaf disc assay has the advantage of allowing standardised incubation conditions, particularly temperature. However, it is likely that the conditions in these experiments were too favourable for *Pseudomonas syringae* pv. *actinidiae* to show if the pathogen was capable of living as an epiphyte under natural conditions.

Incubation at high humidity and at a temperature of 15°C, which was previously found to be the best temperature for leaf infection in leaf disc assays (data not shown), may have led to the formation of micro-colonies that reached the threshold for infection.

These experiments have shown that under laboratory conditions and the bacterium was able to multiply rapidly on the leaf surface of both its kiwifruit host ('Hort16A') and a non-host plant (tobacco). Further work will be necessary to show that it can exist as an epiphyte under natural conditions.

4.5 Recommendations for future work

Questions that require further research include:

- Is Psa truly an epiphyte? This work should be repeated using conditions less conducive for infection and using a variety of non-host species, preferably under glasshouse or field conditions.
- What is the threshold for infection by Psa? It may be possible to determine this using a faster and easier method than serial dilutions and colony counts.

5 Psa survival in leaf litter and prunings

Joy Tyson, Jonathan Rees-George, Carol Curtis, Mike Manning, Bob Fullerton

5.1 Key question and aim

The role of fallen leaves and cane prunings on the orchard floor in the life-cycle of *Pseudomonas syringae* pv. *actinidiae* (Psa) in New Zealand is not known. This work was designed to determine the inoculum potential of fallen leaves and cane prunings over winter, which will allow more informed management decisions by growers.

5.2 Methods

5.2.1 Leaves (leaf fall)

Heavily Psa-infected leaves were harvested from a population of *Actinidia chinensis* seedlings in Block 52 of the Te Puke Research Orchard (TPRO) during leaf-fall (25 May 2011). A large proportion of the leaves had conspicuous bacterial exudate on the lower surface.

The leaves were set up in four gauze-enclosed frames on the orchard floor, under 'Hayward' kiwifruit vines in Block 4, TPRO (Figure 5.1). No symptoms of Psa were visible in the block at that time. Four sets of leaf material were also held at room temperature in the laboratory, under similar conditions of alternating wet and dry. Sixteen sets of weekly leaf samples were taken, from day 0 through to 15 weeks (25 May - 9 September 2011).

5.2.2 Canes (winter pruning)

Prunings from 'die-back' canes were cut into 10-cm lengths during winter pruning (14 July 2011). These were set up in four frames on the orchard floor, under kiwifruit vines. Four sets of cane material were also held in the laboratory, under similar conditions of alternating wet and dry. Thirteen sets of approximately weekly cane samples were taken, from day 0 through to 13 weeks (15 September – 17 October 2011).

5.2.3 Isolation and identification

At each weekly sampling, isolations were made from a single piece of plant material in each frame/box, giving four replicates of each treatment.

Bacterial isolations were made as follows: Pieces of plant tissue (4 x 4-cm areas of leaf or 5-mm lengths of cane) were aseptically excised and macerated in 2 ml of bacteriological saline (0.85% NaCl in sterile distilled water), and left for at least five minutes. A 200-µL aliquot of the resulting suspension was then streaked onto Kings B medium (King et al. 1954) with a series of sterile loops. Plates were incubated at room temperature (ca. 20°) for 2 days.

DNA extractions were done on the mixed-colony agar plates using the boiling method of R.K. Taylor (Rob Taylor, MAF, pers. comm., March 2011). Subsequent identification used the method of Rees-George et al. (2010), modified for use with qPCR.

Figure 5.1 Frames on orchard floor, Te Puke Research Orchard.

5.3 Results

5.3.1 Leaves (leaf fall)

The results show that the bacterium was still alive in the orchard leaf litter and in the laboratory boxes on 9 September 2011, after fifteen weeks (Table 5.1). At the time of leaf-fall, all leaves tested were positive for live Psa. Although the frequency of detection declined over time, most markedly after 5-6 weeks, it was still possible to isolate live Psa from the leaf litter fifteen weeks after leaf-fall (Figure 5. and 5.3).

Table 5.1 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) detection in qPCR (Polymerase Chain Reaction) analysis of kiwifruit leaf samples incubated in the laboratory or the orchard floor over 15 weeks. Four leaves were sampled from lab boxes and orchard frames each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

		weeks after leaf collection (25 May 2011)															
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
lab. boxes	positive	4	2	4	1	4	2	2	0	0	1	0	0	0	1	0	2
	weak positive	0	1	0	1	0	1	2	0	0	1	0	4	4	3	0	2
	negative	0	1	0	2	0	1	0	4	4	2	4	0	0	0	4	0
field frames	positive	4	4	3	1	3	2	1	1	0	0	0	1	1	0	1	1
	weak positive	0	0	1	2	1	2	3	3	2	0	0	1	3	2	1	2
	negative	0	0	0	1	0	0	0	0	2	4	4	2	0	2	2	1

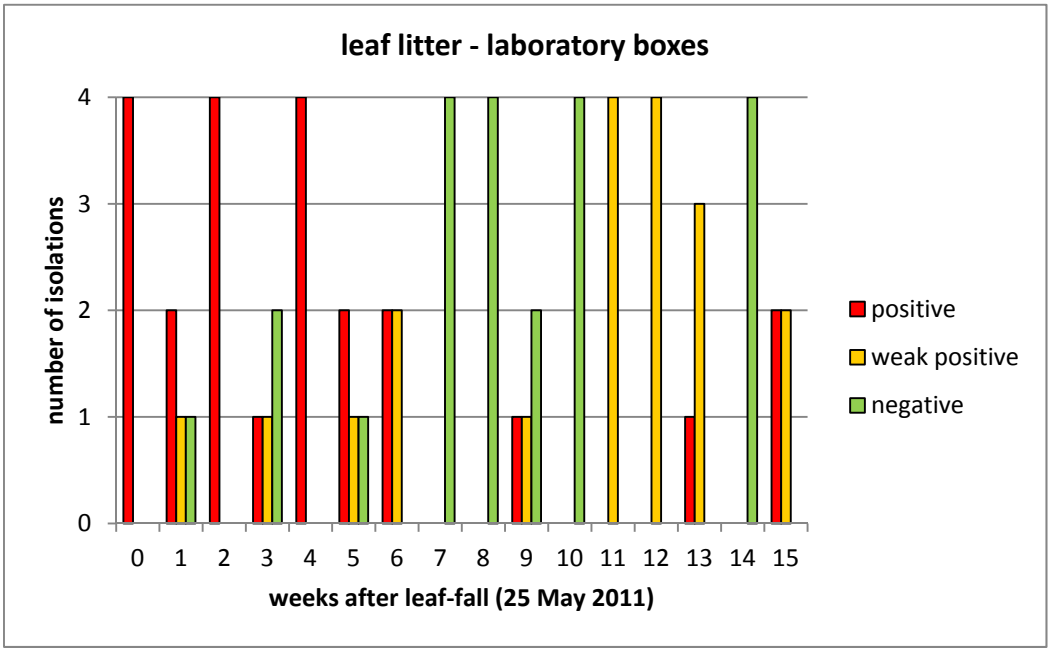


Figure 5.2 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolations and detection in qPCR (Polymerase Chain Reaction) analysis of kiwifruit leaf samples incubated in the laboratory for 15 weeks. Four leaves were sampled each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

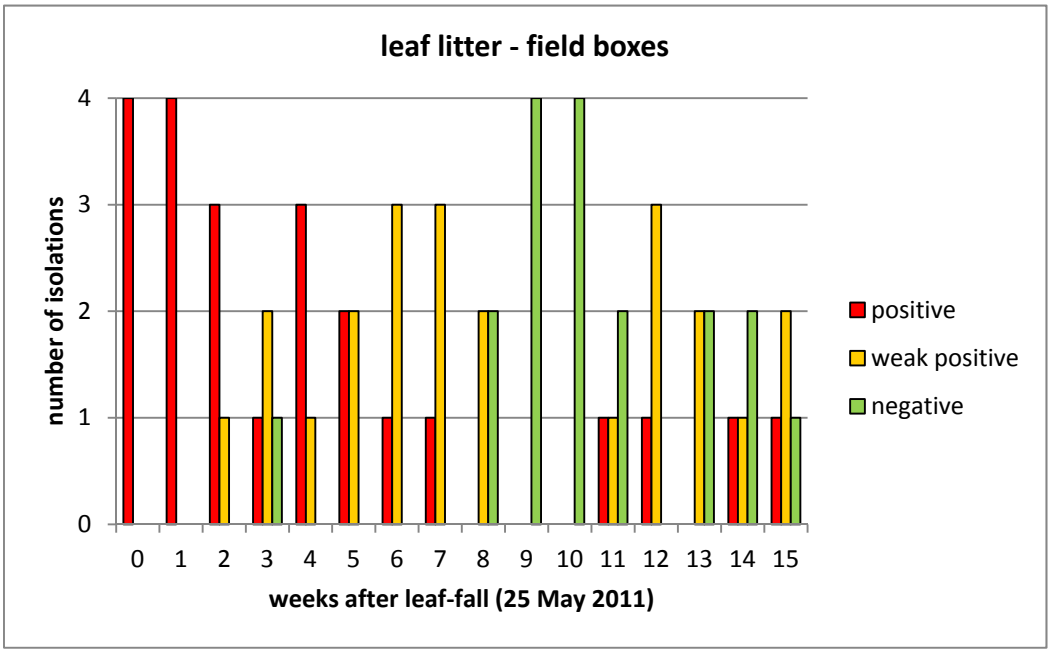


Figure 5.3 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolations and detection in qPCR (Polymerase Chain Reaction) analysis of leaf samples incubated in a kiwifruit orchard for 15 weeks. Four leaves were sampled each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

5.3.2 Canes (winter pruning)

The results show that Psa could be recovered intermittently from the cane prunings over time, but that it was still alive in the orchard canes and in the laboratory boxes on 3 October 2011, after eleven weeks (Table 5.2). At the time of pruning, half the canes tested positive for live Psa. This rate of retrieval declined over time; however, it was still possible to isolate live Psa from the prunings after eleven weeks (Figures 5.4 and 5.5).

Table 5.2 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) detection in qPCR (Polymerase Chain Reaction) analysis of kiwifruit cane samples incubated in the laboratory or on the orchard floor over 11 weeks. Four canes were sampled from laboratory boxes and orchard frames each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

		weeks after cane collection (15 July 2011)												
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**13**
lab boxes	positive	3	3	0	1	0	2	0	0	1	1	1	1	0
	weak positive	0	1	1	0	2	2	1	1	3	2	2	0	1
	negative	1	0	3	3	2	0	3	3	0	1	1	3	3
field boxes	positive	1	1	0	2	0	0	0	0	3	1	1	1	0
	weak positive	2	1	0	0	2	2	2	2	1	0	1	1	0
	negative	1	2	0	2	2	2	2	2	0	3	2	2	4

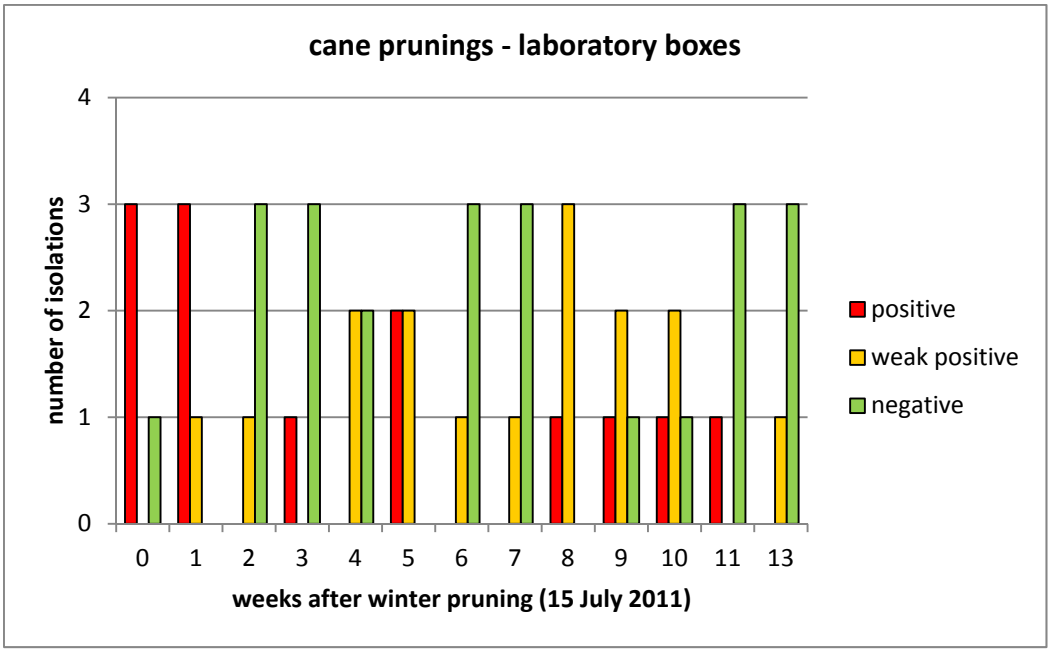


Figure 5.4 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolations and detection in qPCR (Polymerase Chain Reaction) analysis of kiwifruit cane samples incubated in the laboratory for 13 weeks. Four canes were sampled each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

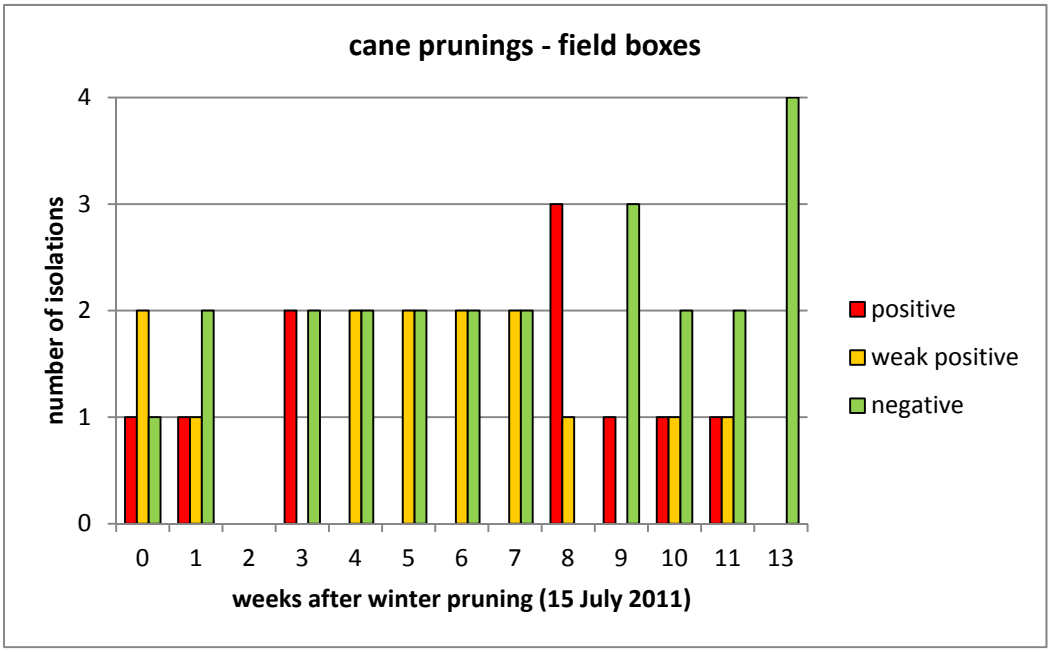


Figure 5.5 Results of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolations and detection in qPCR (Polymerase Chain Reaction) analysis of cane samples incubated on a kiwifruit orchard floor for 13 weeks. Four canes were sampled each week. Positives had CT values <30, weak positives had CT values from 30 to 35, and negatives had CT values >35.

5.4 Discussion

This work was designed to determine whether Psa survives in fallen leaves and cane prunings over winter, and if it does, for how long.

Psa survives in the leaf litter on the orchard floor for up to three months, at which time the leaves are largely decomposed. It also appears to survive in the cane prunings for at least eleven weeks. The more intermittent detection of Psa from the cane prunings mirrors the erratic distribution of Psa within live canes.

The orchard frames were exposed to the elements over the sampling period, and were therefore potentially exposed to further inoculum from the vines within the block, or to inoculum from further afield. The agreement between the orchard and laboratory isolations, where no addition of external inoculum was possible, gives confidence that bacteria derived from the initial inoculum were being detected at each weekly sampling date.

The enrichment step in this work, when the bacterium is isolated and grown from the plant material, shows that we are detecting live, viable bacterial cells, not merely DNA from dead populations.

In this project we used the bacterial growth medium KB during the enrichment step. Since starting this work, we have found that a semi-selective media (SNA++) allows greater recovery of Psa (Tyson & Curtis, unpub.). This is a semi-selective agar medium for *Pseudomonas syringae*, and is a modification of KBC medium (Mohan & Schaad 1987). It is thought that the work presented here under-estimates the amount of Psa surviving in the litter and prunings, and that if the semi-selective media had been used, more Psa would have been recovered.

The role of fallen leaves and cane prunings on the orchard floor in the life-cycle of Psa is not known; however, this work has shown that the plant debris from leaf-fall and winter pruning does harbour live Psa for at least three months and as such is a potential source of inoculum within the orchard the following spring.

5.5 Recommendations for future work

Questions that require further research include:

- Can ‘digester’ products be used to promote faster decomposition of debris and thus reduce Psa survival times?
- Does Psa survival differ in differently sized mulched pieces?
- Does Psa survive in summer pruning debris for a similar length of time?

Answering these questions will allow the development of management strategies to minimise the inoculum potential of leaf litter and pruning debris over winter and spring.

6 Psa survival on shelter and weeds

Joel Vanneste, Bridgette Moffat, Jenny Oldham, Janet Yu, Deirdre Cornish

6.1 Background

Pseudomonas syringae are good epiphytes (Hirano & Upper 2000). *P. syringae* pv. *actinidiae* has been isolated from symptomless leaves by washing (Vanneste et al. 2011), suggesting that Psa may also be able to survive epiphytically (see also section 4 of this report). The epiphytic growth of Psa on kiwifruit plants or other plants could provide the inoculum for further spread of the disease. In this study, the ability of Psa to survive on non-host plants (i.e. plants other than kiwifruit) has been determined in the laboratory under controlled conditions. The targeted plants were species commonly used as shelter belts, and weed species growing on the orchard floor. In the second part of the study, samples were collected in infected orchards and analysed in the laboratory for presence of Psa.

6.2 Methods and results

The experiments were conducted in the laboratory on potted plants using either a streptomycin-resistant derivative of Psa, or a streptomycin and rifampicin-resistant derivative. These two antibiotic derivatives grew overnight in liquid medium at 28°C at a similar rate as that of the wild type strain, indicating a similar level of fitness. Isolated colonies of those derivatives took longer to develop on agar plates with antibiotics than on plates without antibiotics. However, the final populations of the strains were not affected by the addition of antibiotics in the medium. The plants analysed were species used as shelter belts (*Cryptomeria japonica*, *Pinus radiata*, *Casuarina cunninghamiana*, *Salix* sp. and *Populus* sp.) and plants found on the orchard floor (*Crepis* sp. and *Carex* sp.).

Plants were sourced from a nursery outside the zone affected by Psa. The *Cryptomeria* were about 90 cm tall, the pine and the *Casuarina* were about 60 cm tall. For poplar and willow, detached branches were used, as the unrooted sticks bought in the nursery and rooted in the laboratory did not give enough leaf material for the experiments. All plants were inoculated with Psa by spraying with a hand-held sprayer bacterial suspensions ranging from 7.3×10^8 to 4.6×10^6 cfu/ml, and were monitored regularly for presence of the pathogen. The concentration of the inoculum was different for each experiment, as was the time at which the presence of Psa was determined. This information is given for each experiment below. Presence of the pathogen was monitored by washing 1 g of plant material (Figure 6.1) and $1/10^{th}$ dilutions made in 10 mM $MgSO_4$ of the washings were plated on King's B medium (King et al. 1954) supplemented with streptomycin (100 ppm) or streptomycin (100 ppm) and rifampicin (40 ppm). Some strains were selected for confirmation of their identity by PCR using the primers PsaF1/R2 (Rees-George et al. 2010). Control plants were treated with water instead of Psa.

Samples from shelter belts and weeds in the field were taken at two different occasions. On the first occasion, samples were taken from three different infected orchards on No. 1 Road or No. 2 Road in Te Puke. For the shelter belts, three different samples of *Cryptomeria*, two samples of pine, two samples of poplar, one sample each of willow, rimu and *Casuarina* were analysed in the laboratory for presence of Psa. For the weeds, three samples of *Crepis* sp., two samples of buttercup and two samples of *Carex* sp. plants were taken to the laboratory for analysis. Each sample was analysed three times. In the second occasion, samples of a pine tree from the

orchard on No. 2 Road were taken back to the laboratory. The washing from 1 g of tissues from each sample was plated on King's B medium without antibiotics and incubated for 72 hours.

Psa-like colonies were not recovered from the water-treated plants in any of the experiments. The results from the different experiments are presented in Figure 6.2 to Figure 6.12.

Figure 6.1. Samples of *Pinus radiata*, *Casuarina cunninghamiana* and *Cryptomeria japonica* used at each time point to determine populations of *Pseudomonas syringae* pv. *actinidiae* (Psa).

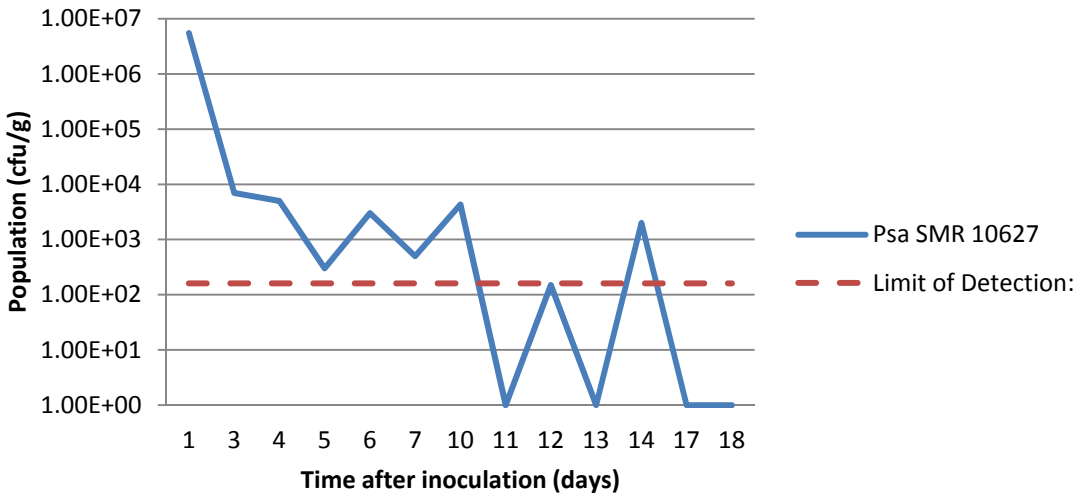


Figure 6.2. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Cryptomeria japonica* inoculated with the streptomycin-resistant derivative strain.

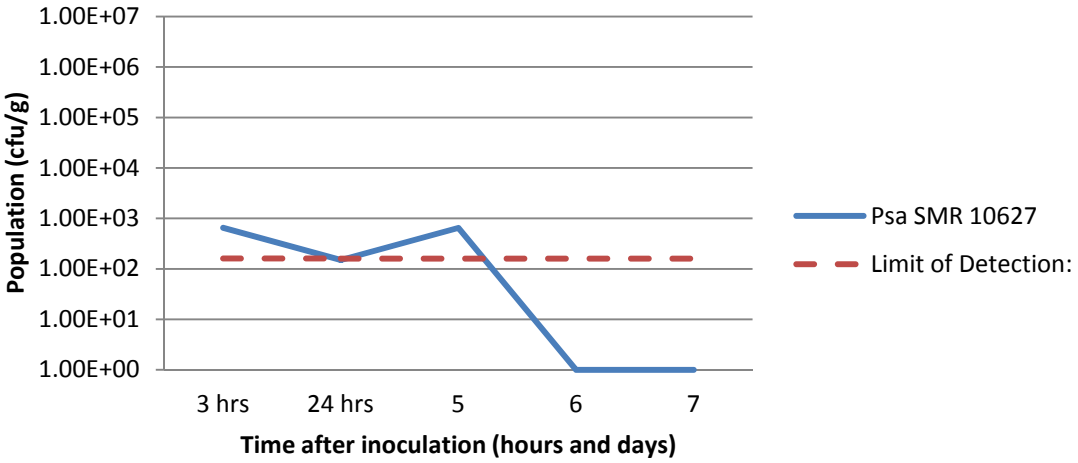


Figure 6.3. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Casuarina cunninghamiana* inoculated with the streptomycin resistant derivative strain of Psa 10627. The plant was treated with a bacterial suspension containing 9.0 x 10^7 cfu/ml.

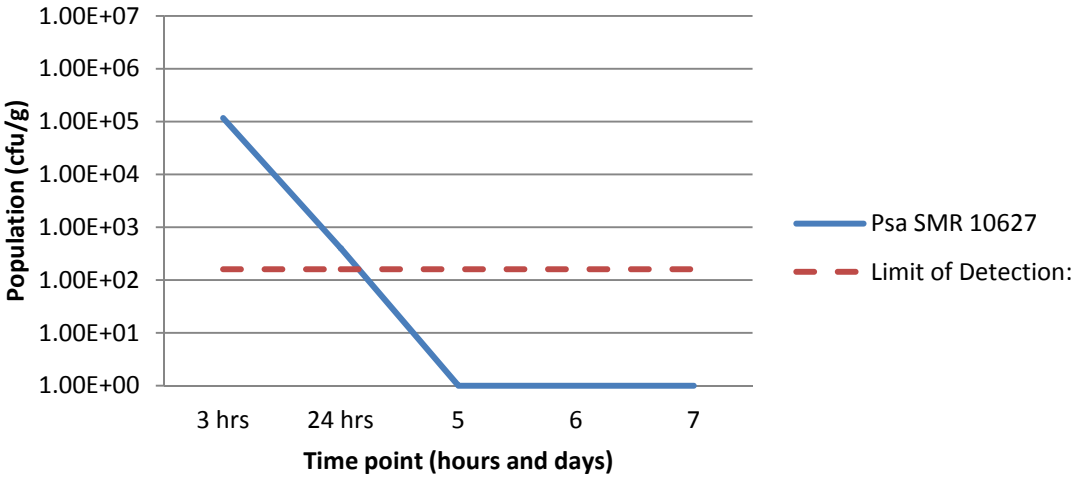


Figure 6.4. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Pinus radiata* inoculated with the streptomycin-resistant derivative strain of Psa 10627. The plant was treated with a bacterial suspension containing 9.0 x 10^7 cfu/ml.

Those initial three 'pilot' experiments have been reported in the *New Zealand Kiwifruit Journal* (Vanneste et al. 2011). From those three experiments, it was clear that Psa is able to survive epiphytically on the surface of non-host plants for a duration that varies from a few hours to several days. The variation in the population from one time point to the next, as seen in Figure 6.2 between day 10 and day 18, might not indicate a multiplication of the pathogen, but simply reflect the variability linked with the sampling method (1 g of green tissues per plant per time point).

To determine the variability between plants, independent experiments involving plants inoculated with different concentrations of inoculum were conducted in parallel. In the first series of experiments (Figure 6.5 to Figure 6.7), the streptomycin rifampicin derivative of Psa strain 10627 was used at 1.7 x 10^8 cfu/ml, 3.6 x 10^8 cfu/ml and 1.8 x 10^8 cfu/ml for experiment 1, 2 and 3 respectively. A total of six *Casuarina*, six pine and three *Cryptomeria* plants were

inoculated, such as to provide enough material to assess the Psa population at all the time points.

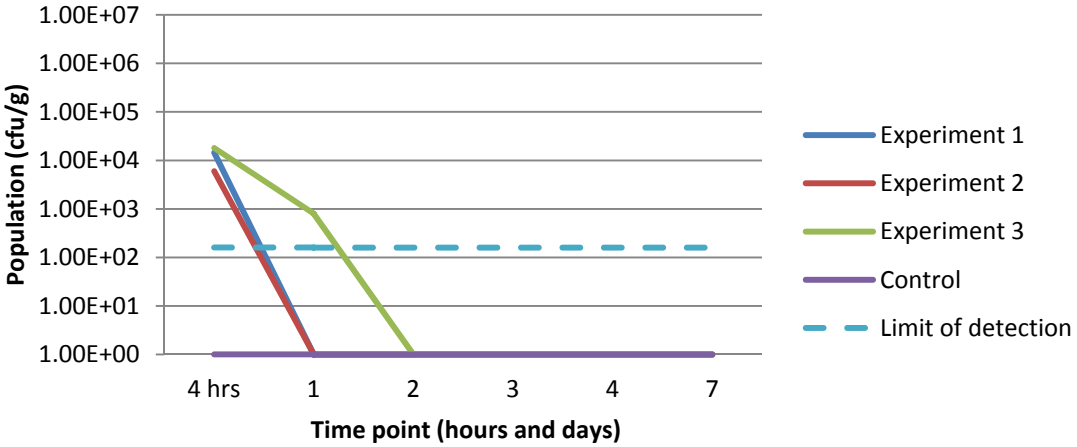


Figure 6.5. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Cryptomeria japonica* inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

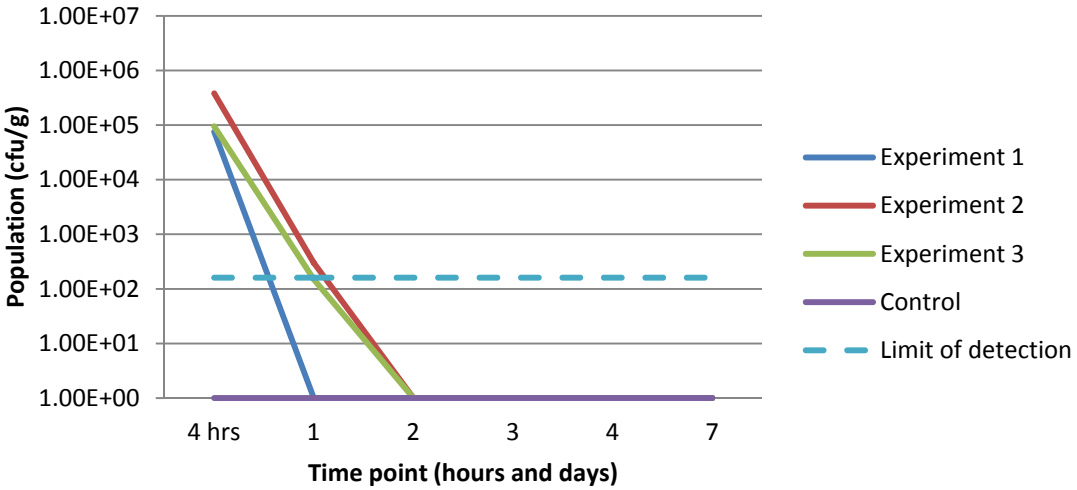


Figure 6.6. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Pinus radiata* inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

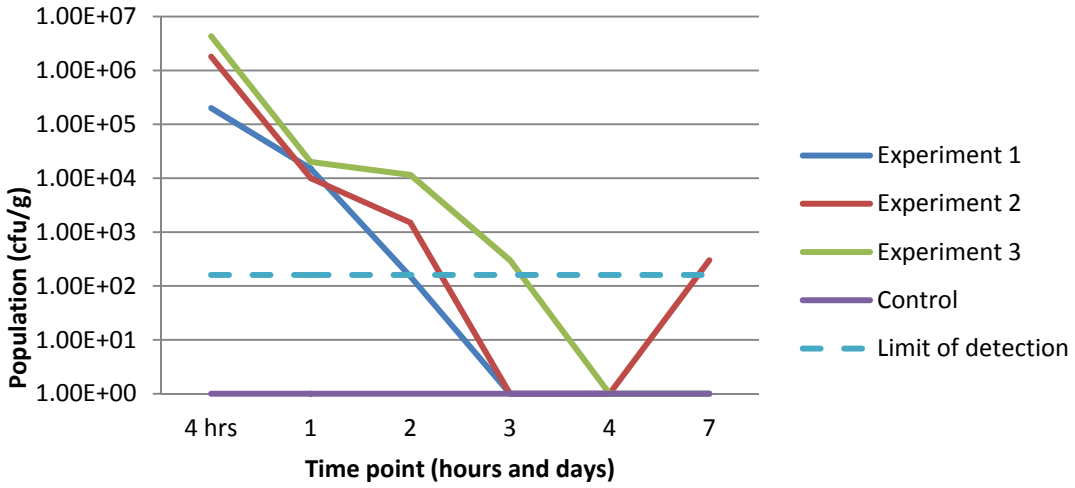


Figure 6.7. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on Casuarina *cunninghamiana* inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

Again, Psa survived epiphytically from few hours to few days on the different plants tested. The survival on *Cryptomeria* was much reduced in those experiments, probably because of the lower inoculum present on the plants at the beginning of the experiment (a thousand times less Psa per gram of tissue between the *Cryptomeria* in the first experiment (Figure 6.2) and those last three experiments (Figure 6.5)). In support of this hypothesis, the initial concentration of Psa on the *Casuarina* was much higher in those experiments (Figure 6.7) than in the pilot (Figure 6.4) and it survived for longer on *Casuarina* in those experiments.

It also seems that there is no direct relationship between concentration of inoculum and initial population on the plant. This might be because of different volumes of inoculum being sprayed or because of the state of the plants being sprayed.

This experiment was repeated on *Casuarina* and *Cryptomeria* using as inoculums the streptomycin rifampicin derivative of strain 10627 at 8.3 x 10^7 cfu/ml, 2.0 x 10^8 cfu/ml and 7.3 x 10^8 cfu/ml for experiment 1, 2 and 3 respectively. A total of six *Casuarina*, and three *Cryptomeria* plants were inoculated, to provide enough material to assess the Psa population at all the time points.

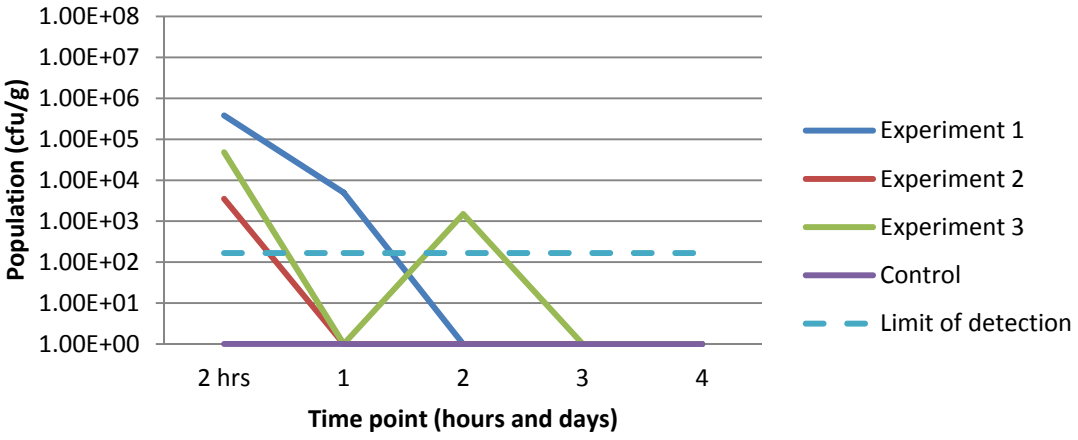


Figure 6.8. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Cryptomeria japonica* inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

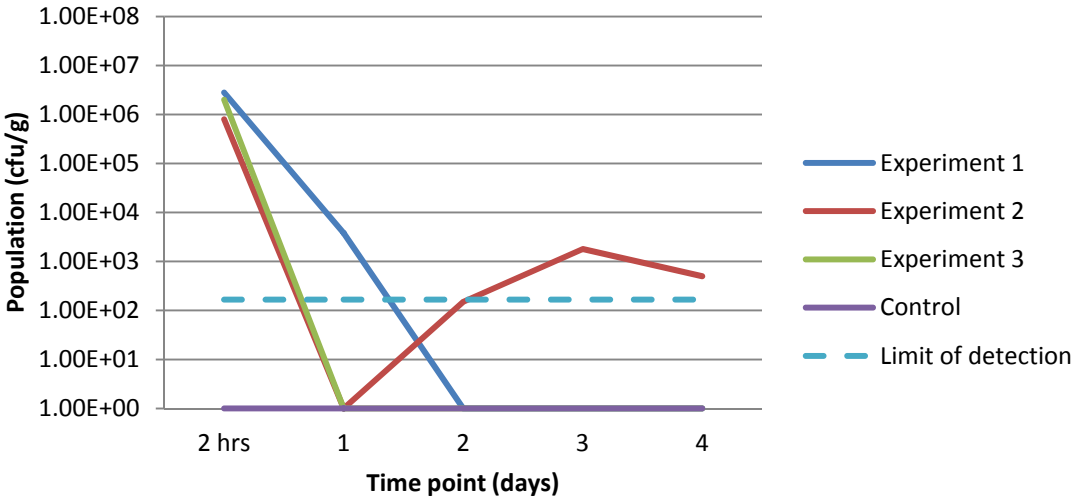


Figure 6.9. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on Casuarina *cunninghamiana* inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

These experiments confirm that the survival of Psa is dependent on the initial population of the bacteria present on the plant. It seems that Psa populations always decline on these species, suggesting that Psa is most probably surviving and not multiplying.

The survival of Psa on deciduous plants used as shelter belts was also determined. However, the amount of leaf material from plants growing in the laboratory was not sufficient to carry out an experiment. Instead of potted plants, branches collected from a Psa-free zone (Hamilton) were kept in a vase in the laboratory. The inoculum used was the streptomycin-resistant derivative of Psa 10627 at 4.6 x 10^6 cfu/ml.

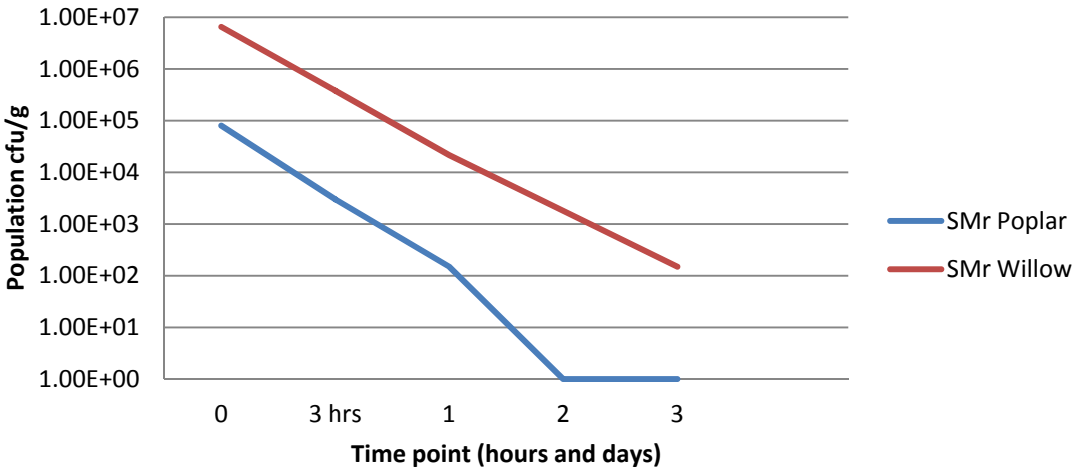


Figure 6.10. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Populus* sp. and *Salix* sp. inoculated with the streptomycin-resistant derivative strain of Psa 10627.

These results suggest that Psa survives on those plants but does not multiply, and the comparison between the survival on poplar and on willow suggests that the time Psa survives might be dependent on the initial population.

The next experiment was carried out on common Bay of Plenty orchard floor weeds, *Carex* sp. and *Crepis* sp. (Hawke's beard). The inoculum was the streptomycin- and rifampicin-resistant derivative of Psa 10627 at 2.5 x 10^8 cfu/ml and 7.3 x 10^8 cfu/ml for the experiment on 25–30 cm tall *Carex* sp. and c. 20 cm tall *Crepis* sp. respectively.

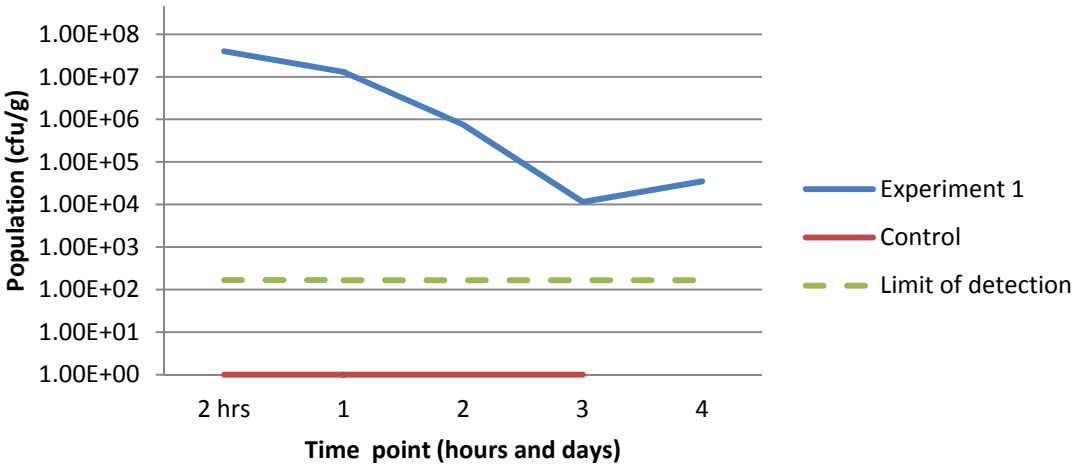


Figure 6.11. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Crepis* sp. inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

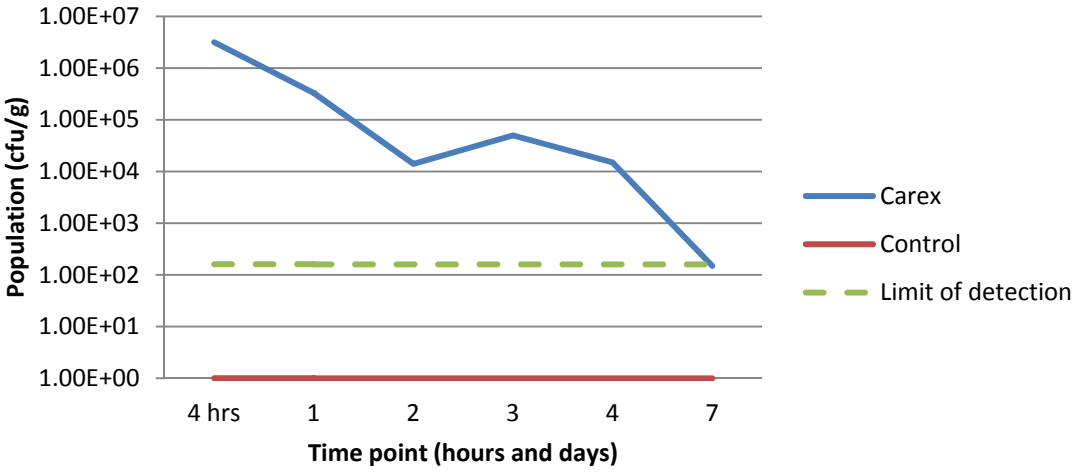


Figure 6.12. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on *Carex* sp. inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

6.3 Results from the orchard survey

With the exception of the pine sample from No. 2 Road, no Psa-like colonies were found in any of the shelterbelt or weed samples collected from Psa-infected orchards in Te Puke. PCR analyses were conducted on some of the bacteria isolated, and all were negative for Psa.

On one plate made from the washings of the pine sample from No. 2 Road, a colony with morphology similar to that of Psa has been found. This strain was positive by PCR using the Psa F1/R2 primers. This colony is being purified for confirmation of identity by biochemical means. The pine sample was taken adjacent to an infected kiwifruit vine growing among the shelter belt. A new sample from this same pine tree is being analysed in the laboratory. If those symptoms are confirmed, it would indicate that Psa can move from the canopy of infected kiwifruit vines.

6.4 Conclusions

The conclusions on survival of Psa on non-host plants are similar for all the evergreen shelterbelts, deciduous shelterbelts or weed species tested in this study. Psa might survive for a few days, but does not seem to multiply and the length of time it does survive is related to the initial concentration on the plant. Therefore, it is not surprising that we could not find Psa during the orchard inspection, except if the non-host plant was in direct contact with an infected kiwifruit vine. We might have to analyse a much larger sample to find Psa under natural conditions and/or the same area at different times, since the presence of Psa on non-host plants might only be temporary.

7 Psa survival in soil and compost

Joel Vanneste, Bridgette Moffat, Jenny Oldham, Janet Yu, Deirdre Cornish

7.1 Aim

To understand whether Psa can survive in soil, and hence whether it poses a risk as an inoculum source or has potential for spreading infection in soil moved between orchards. Potting mix has been added to the list of samples analysed, because plants are brought to the orchard in potting mix.

7.2 Method

The different soils used in this study were free-draining loamy bare soil, clayey soil from under grass (both soils from the Waikato) and potting mix (Yates Black Magic®). Samples of potting mix and outdoor soil from two separate sites (under grass and bare soil) were autoclaved. Four 10-g aliquots from each sample were weighed out and three were inoculated with 1 ml of a streptomycin-resistant derivative of Psa. A single negative control for each sample was inoculated with 1 ml sterile water. All samples were well mixed and 1 g taken for time zero plates. Two ml of sterile water was added to the 1 g soil, shaken and allowed to settle. 100 µL was then taken for dilutions and plated on plates supplemented with streptomycin. Further samples were taken after 24 h, then after 1, 2, 3, 4, 5, 8 weeks and 3 months. At three months, the residual sample was weighed and 1 ml of water was added to containers. The weights of the samples ranged from 0.28 to 0.58 g.

To test for the presence of Psa in orchard soil, four samples of soil from three different infected orchards from Te Puke were analysed for presence of Psa. Those samples were analysed as described above, with 1 g of soil washed with 2 ml of water.

7.3 Results

The starting population in the inoculum suspension was 9 x 10^8 cfu/ml. The 'Bare outdoor soil' samples were contaminated with fungal and bacterial organisms after one week and therefore discarded from the analysis.

Psa was able to survive for at least 3 months in autoclaved soil or potting mix (Figure 7.1). No Psa-like colonies could be found in samples from Psa-infected orchards and all the colonies tested by PCR were negative. It could be that the large number of non-Psa organisms in the soil masked the presence of the pathogen. Alternatively it could be that those better adapted organisms either prevented Psa from multiplying and surviving by taking away all the nutrients, or that they produced some compounds which killed Psa.

All attempts to retrieve Psa from those same soil samples non-autoclaved failed because of the high numbers of soil organisms. Even when plated on streptomycin or streptomycin and rifampicin, the natural microflora was too large to recover Psa. This does not mean that Psa did not survive; it simply means we could not find it among all the other organisms.

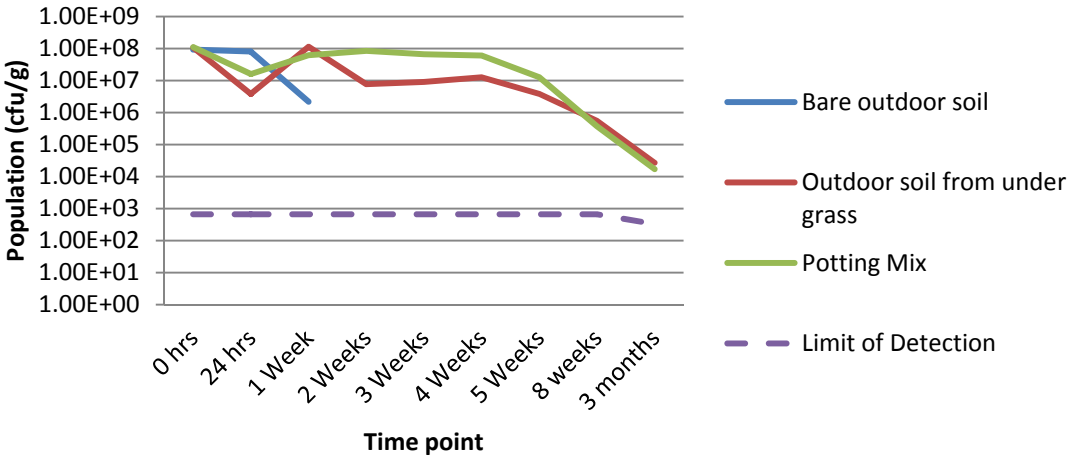


Figure 7.1. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in sterilised soil inoculated with the streptomycin-resistant derivative strain of Psa 10627.

7.4 Experiment on compost

Fresh compost samples from a company in the Bay of Plenty that delivers compost to kiwifruit growers were inoculated with the streptomycin-resistant derivative of Psa 10627. One gram of compost was mixed with 3 ml sterile water. One hundred ul of this suspension was streaked on King's B medium supplemented or not with streptomycin. Numerous bacterial colonies grew on all plates, including those supplemented with streptomycin. Any colonies resembling Psa were checked by PCR. All reactions were negative; either the Psa was killed by other microorganisms or was masked by them.

In a second experiment, the streptomycin-resistant derivative of Psa 10627 at a concentration of 6.3×10^9 cfu/ml was used to inoculate compost. At time zero, the population of Psa was 6.0×10^8 cfu/g of compost (the inoculum was recovered), but one week later the bacterial population dropped to between 4.3×10^6 to 1.3×10^6 cfu/ g of compost and no Psa could be found among those bacteria (all PCR negative). Since the medium supplemented with streptomycin was not selective enough, all further experiments were conducted with the streptomycin rifampicin-resistant derivative of Psa 10627. Using a starting inoculum of 2.0×10^9 cfu/ml, the results of two sets of compost are presented in Table 7.1. Results showed in this instance that Psa could still be detected in compost after 8 days, although populations were substantially lower than they were on the day of inoculation.

Table 7.1. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) on compost inoculated with a streptomycin rifampicin-resistant derivative of Psa 10627.

Time after inoculation in days	Lot of compost	Population of Psa on plates in cfu/g	PCR reaction	Comments
Day of inoculation	C1	8.3×10^8	+	
	C2	7.7×10^8	+	
Day 1	C1	2.3×10^8	+	
	C2	6.3×10^8	+	
Day 4	C2	1.3×10^4	+	No samples taken for C1
Day 6	C1	Overgrown cannot count the colonies	+	PCR was done on a swipe of total colonies
	C2		+	
Day 8	C1	6.3×10^3	+	
	C2	1.0×10^4	-	Needs to be redone
Day 14				Too many background bacteria to detect Psa

7.5 Conclusions

When using sterilised soil, Psa is able to survive for several months. However, it could not be found in non-sterilised soil inoculated with the pathogen. This was because of the high concentration of organisms present in the soil. Not finding Psa does not mean that the organism was not present; its presence could have been hidden by those other microorganisms. A different strategy, not relying on antibiotic-resistant derivatives, will have to be designed to determine the survival of Psa in soil.

Psa may be able to survive better on compost. Psa could be found on fresh unsterilized compost following inoculation. This might reflect that the composting process eliminates some of the microorganisms or the secondary metabolites, which in the soil kill might Psa. In aged compost, presence of Psa could not be detected because of the numbers of other microorganisms. This is the same situation as for soil samples. Again, we cannot determine whether Psa is killed or simply masked by the other organisms. This experiment is being repeated.

8 Psa survival in water

Joel Vanneste, Bridgette Moffat, Jenny Oldham, Janet Yu, Deirdre Cornish

8.1 Background

Pseudomonas syringae has been detected in waterways in New Zealand and around the world (Vanneste et al. 2008; Morris et al. 2010). The ability of this bacterial complex to survive and/or multiply in water has been credited for the wide distribution of this bacterium in the environment. This study firstly aimed at determining whether Psa could survive in water, and secondly if it could be detected in water from infected orchards.

8.2 Methods

Three types of water: tap water, reverse osmosis water and rainwater, were used in this experiment. Rainwater was collected in sterile containers outside the Hamilton laboratory. It was subsequently either sterilized by boiling or filtration through a 0.22 micron filter, or used unsterilised. The different samples of water were inoculated with Psa 10627 or a streptomycin- or a streptomycin and rifampicin-resistant derivative. At different times, aliquots were diluted and plated on King's B medium supplemented or not with antibiotics. The effect of temperature on the survival of Psa in water was also determined by keeping inoculated water at 4°C or 28°C.

8.3 Results

The first experiment demonstrated that in Hamilton tap water Psa could not survive for very long (Figure 8.1). However, in sterile water (tap water that has been boiled for at least 20 min) or reverse osmosis water, Psa was able to survive at relatively high concentrations.

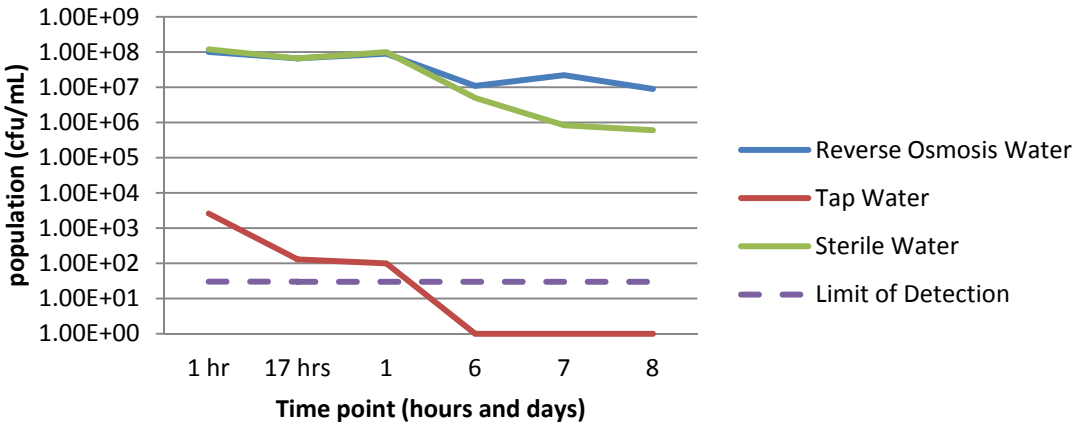


Figure 8.1. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in different types of water inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627 (1.3 x 10^8 cfu/ml).

To determine whether the streptomycin-resistant derivative of Psa 10627 could grow or survive in water as well as the wild type, the survival of Psa 10627 and its streptomycin-resistant derivative were compared in rain water that was filtered, sterilized or used without any treatment (Figure 8.2). The starting inoculum was 1.3 x 10^{10} cfu/ml and 1.0 x 10^{11} cfu/ml for the wild type strain and the streptomycin-resistant derivative respectively.

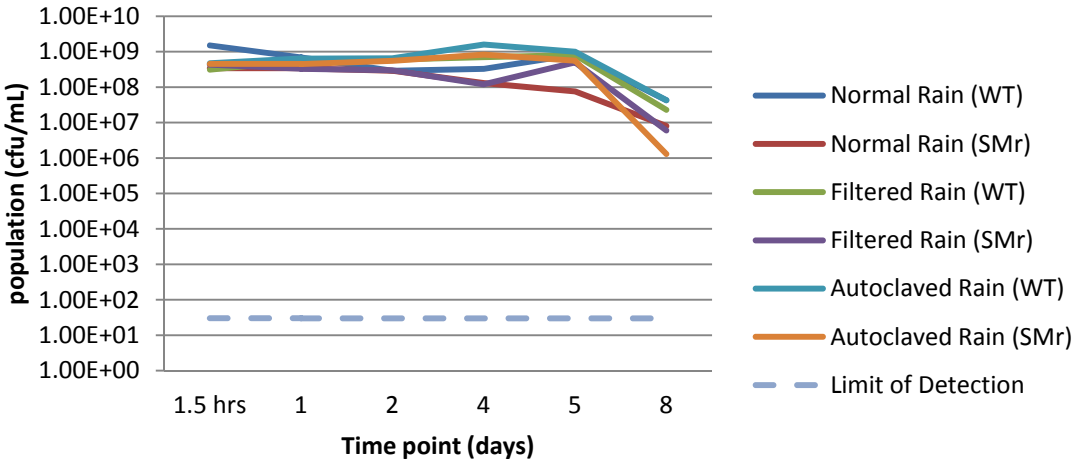


Figure 8.2. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in rain water inoculated with Psa 10627 or a streptomycin rifampicin-resistant derivative.

No differences could be observed between the survival of Psa or its streptomycin derivative in the different samples of rain water. Psa could survive for over a week in rain water at relatively high concentrations. It has to be noted that the initial inoculum was extremely high (c. 10^9 cfu/ml).

Two independent experiments were conducted to determine whether Psa could survive in tap water that had been autoclaved or not (Figure 8.3), in reverse osmosis water (Figure 8.4), or in rain water that has been autoclaved, filtered or not treated (Figure 8.5). The starting population for trial one was 3 x 10^9 cfu/ml and for trial two was 5.6 x 10^9 cfu/ml.

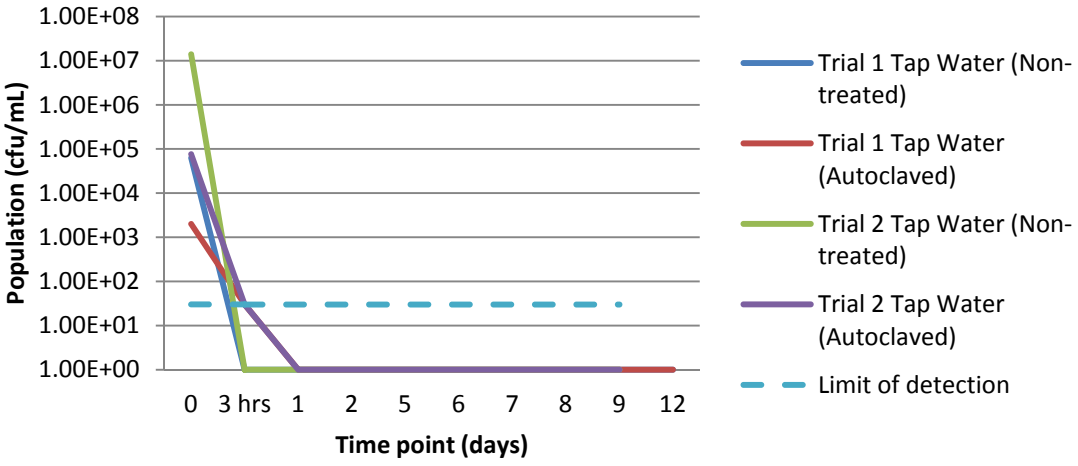


Figure 8.3. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in tap water inoculated with the streptomycin-resistant derivative strain of Psa 10627.

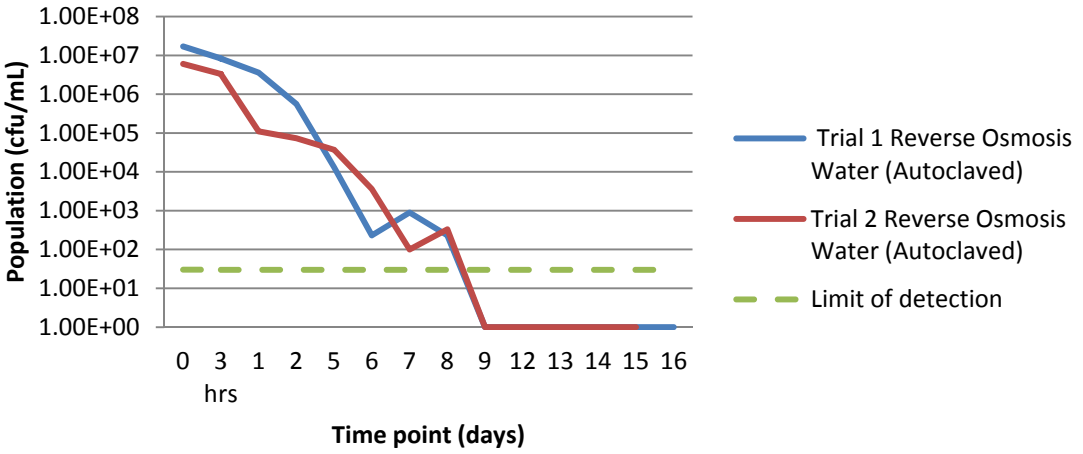


Figure 8.4. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in reverse osmosis water inoculated with the streptomycin-resistant derivative strain of Psa 10627.

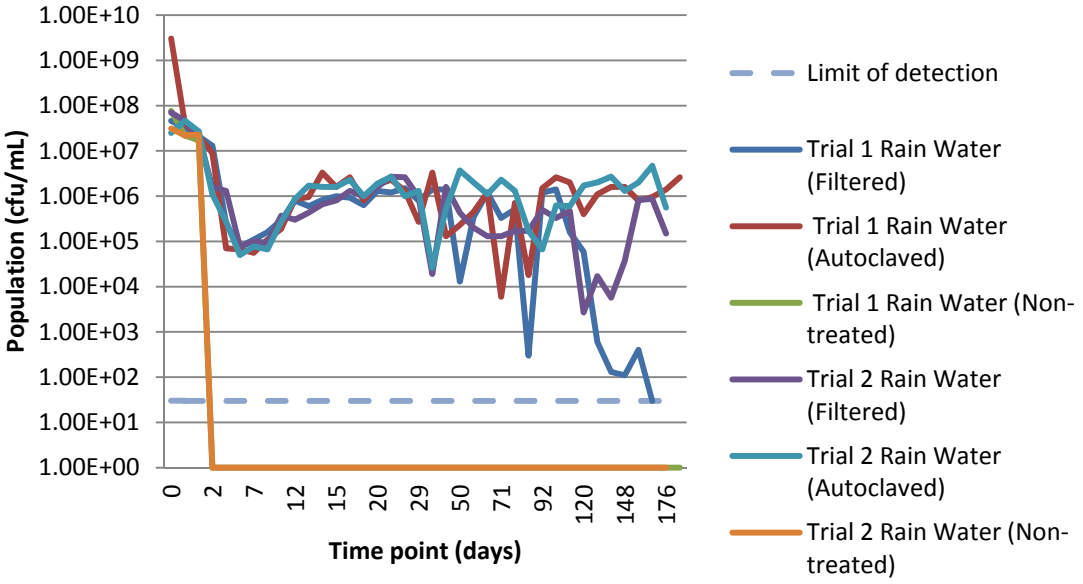


Figure 8.5. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in rain water that has been autoclaved, filtered or not treated and inoculated with the streptomycin-resistant derivative strain of Psa 10627.

In these experiments, Psa could not survive very long in tap water, presumably because of the presence of sodium hypochlorite and other chemicals added to it. Psa survived for over a week in reverse osmotic water. It might have succumbed either to unfavourable pH or to lack of nutrients. In rain water, Psa could survive for a very long time when the water had been either filter-sterilised or autoclaved. This shows that the water composition, including presence of nutrients, is adequate for survival of Psa. In the non-treated rain water in both experiments, Psa disappeared after few days. This could be because of competition with microorganisms already present in the water and better adapted at taking the nutrients present in the water, or the presence of microorganisms that produce compounds toxic to Psa. It is not clear why this effect was not seen in the previous experiment (Figure 8.2). Perhaps the microorganisms present in the water samples between the two experiments were different and affected the survival of Psa

differently. To check this hypothesis, an analysis of the microflora of the different water samples would need to be carried out.

The inability of Psa to survive in rain water that was not treated was confirmed using another strain of Psa: the streptomycin- and rifampicin-resistant derivative of Psa 10627 (Figure 8.6). The starting population was 5.9 x 10^6 cfu/ml.

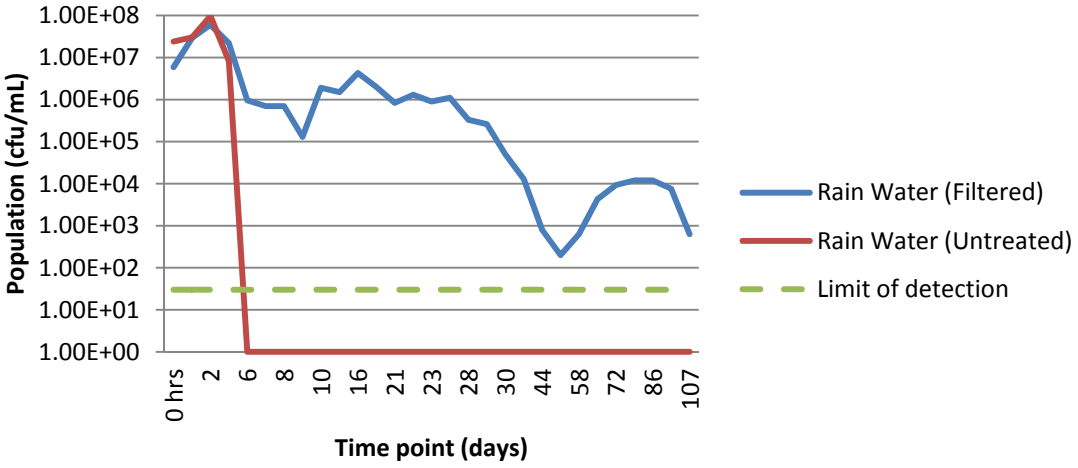


Figure 8.6. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in rain water filtered or not treated and inoculated with the streptomycin rifampicin-resistant derivative strain of Psa 10627.

To determine the influence of temperature on the survival or Psa in rainwater, a similar experiment was conducted at two different temperatures. Some filtered rain water was inoculated with the streptomycin and rifampicin-resistant derivative of Psa 10627. One sample was kept at 4°C (i.e. in the refrigerator) while the other one was kept in the 28°C incubator. The starting inoculum contained 1.2 x 10^{10} cfu/ml (Figure 8.7).

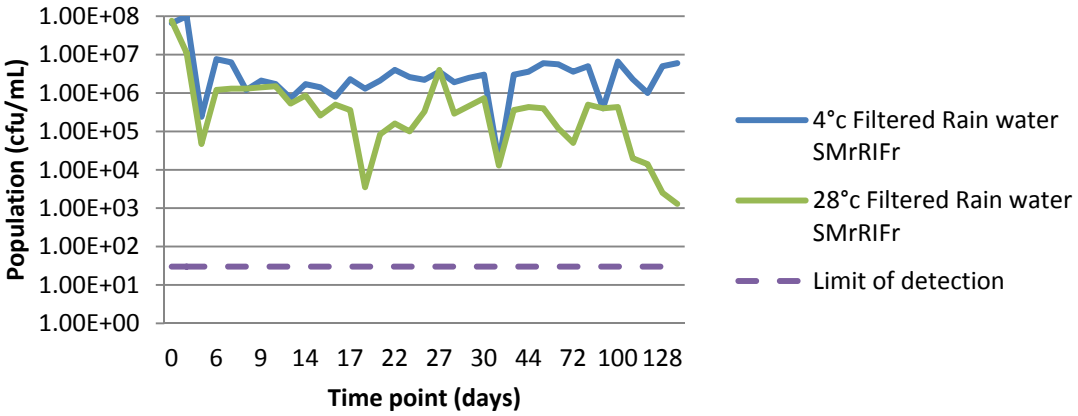


Figure 8.7. Survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) in filtered rain water inoculated with the streptomycin and rifampicin-resistant derivative strain of Psa 10627.

Psa seemed to survive well at both temperatures, but after 100 days it might be surviving better at 4°C. This could be due to a lower rate of multiplication, and therefore the nutrients available to Psa would last longer.

8.4 Orchard water sampling

Four water samples were taken from the “Bay Gold” orchard. The descriptions of the four ponds as given to us are presented below:

- Venice Pond: Earth pond that is filled from a nearby stream. The surrounding orchard land flows into this pond in rain or frost protection events; plant debris will from time to time will be swept along with the water and end up into the pond.
- Tuscany Pond: Plastic-lined pond that is filled from bores that draw water from 200 metres below the surface. The surrounding orchard land flows into this pond in rain or frost protection events; plant debris will from time to time will be swept along with the water and end up into the pond.
- Taupo pond: Earth pond that has a natural spring feeding into it, it has outflows right through the year. After frost events, the pond is recharged from bores that draw water from 200 metres below the surface. The surrounding orchard land flows into this pond in rain or frost protection events; plant debris will from time to time will be swept along with the water and end up into the pond.
- Tarawera Pond: Earth pond that is stagnant; is filled from bores that draw water from 200 metres below the surface. The surrounding orchard land flows into this pond in rain or frost protection events; plant debris will from time to time will be swept along with the water and end up into the pond. This pond only has outflows in extreme events.

When plated on King’s B medium, no Psa-like colonies could be found from any of the samples. No Psa-like organism was found by PCR either. The concentrations of other bacteria were extremely high, especially fluorescent bacteria. These high concentrations of other microorganisms might have prevented the water being contaminated by Psa. On the other hand, the presence of Psa might have been masked by the presence of those other microorganisms.

8.5 Conclusions

The laboratory experiments show the potential of Psa to survive in non-treated water. In treated water (tap water), its survival is variable and limited. The variability of the survival in tap water might be linked to the variability of the water itself. The amounts of chemicals in the tap water are not constant.

The potential of Psa to survive in sterilised rain water for considerable periods probably reflects that rain water contains enough nutrients for the initial multiplication of Psa. Later the dead cells of Psa might provide the nutrients necessary for future growth. In contrast, in non-sterilised rainwater, except in one experiment when Psa was found after 8 days, in the other experiments Psa could not be detected after five days. This might reflect that other organisms, better adapted to life in water, compete with Psa for nutrients.

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