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Influence of leaf age of *Actinidia* cultivars 'Hayward' and 'Hort16A' on infection by *Pseudomonas syringae* pv. *actinidiae*

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Executive summary

Influence of leaf age of *Actinidia* cultivars ‘Hayward’ and ‘Hort16A’ on infection by *Pseudomonas syringae* pv. *actinidiae*

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The influence of leaf age on infection by *Pseudomonas syringae* pv. *actinidiae* (Psa) was investigated using potted ‘Hayward’ and ‘Hort16A’ kiwifruit plants. Leaves were spray inoculated with an isolate of Psa (haplotype NZ-V) at a rate of 6.9×10^8 cfu/ml. Plants were maintained in a saturated environment in a plastic hot house within the confines of a containment laboratory at The New Zealand Institute for Plant & Food Research Limited, Mt Albert Research Centre, Auckland.

All leaves were assessed for symptoms eight days after inoculation and thereafter at approximately weekly intervals for four weeks. At each assessment, changes in symptom expression on the leaves were recorded.

Leaf age was found to have a significant influence on Psa infection of leaves of both kiwifruit cultivars. Flecking was evident on some of the leaves eight days after inoculation. Many of these flecks later became necrotic spots with halos similar to Psa infections that have been observed in the field. A higher percentage of leaves that were 2-3 weeks of age at inoculation had flecking and spotting than did leaves of other ages. Leaves that were seven weeks or older at inoculation had a very low incidence of Psa infection.

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1 Background

The bacterial plant pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa) is the causal agent of a damaging disease of *Actinidia* species in a number of countries around the world. It was first discovered in New Zealand in 2010 (Everett et al. 2011) and continues to be a serious threat to the New Zealand kiwifruit industry.

Tissue age, particularly leaf age, could affect infection by Psa. Anecdotal evidence from the first few months of the outbreak of Psa in Te Puke in spring 2010 suggested that leaf spots rarely appeared on the first four leaves of a given cane. This suggested that at the time of an infection event, the first few leaves on each cane (i.e. the oldest) were not infected, whereas younger leaves on the same cane had become infected. Similar observations have been made in the field in the 2011/12 season, where 'pulses' of leaf infection were observed as canes developed new leaves during the season; older leaves rarely developed new symptoms (Manning & Horner, unpub. Project VI1175).

In bacterial pathosystems, leaf age at inoculation may be positively or negatively correlated with infection. A study conducted on *Chrysanthemum morifolium* found that leaf age affected the number and size of lesions caused by *Pseudomonas cichorii*, with older leaves being less susceptible to infection than younger, immature leaves (Jones et al. 1985). Conversely, Sanchez et al. (2003) found that in coffee (*Coffea arabica*), infection by *Pseudomonas cichorii* was more frequent on older leaves.

Young fruit and leaf tissues of *Citrus* are more susceptible to *Xanthomonas axonopodis* pv. *citri* (citrus canker) than mature tissues (Verniere et al. 2003). A study on *Arabidopsis thaliana,* with *Pseudomonas syringae* pv. *maculicola,* found that defence responses to pathogens were generally higher in younger leaves and the occurrence of lesions was three times higher in young leaves than older leaves (Zeier 2005).

To date, the influence of kiwifruit leaf age on Psa infection/symptom development has not been systematically studied.

The objective of this work was to determine the relative susceptibility of leaves of different ages, ranging from newly emerged to mature, by means of a series of inoculation experiments on potted plants and *in vitro* leaf disc assays.

This work will provide growers with information on the most vulnerable stages and may provide important data for incorporation into disease risk models. This may aid in decisions on the management of vines and the optimisation of crop protection approaches.

2 Methods

2.1 Potted plant trial

2.1.1 Plants

Eight *Actinidia deliciosa* 'Hayward' plants and twelve *Actinidia chinensis* 'Hort16A' plants were obtained from the Kerikeri Plant & Food Research Station, an area that remains free of Psa. The plants were propagated from cuttings (29 July 2011) and potted into 2 L pots on 12 March 2011.

In order to track the age and growth of individual leaves, the leaves on each plant were numbered from the base to the growing tip. Leaf development was first assessed on 21 March 2012 and weekly thereafter. At each assessment, the width and length of each leaf was measured and new leaves were recorded. At time of inoculation, leaves that were classified as 'emergent' (E) at the first assessment were assumed to be 4 weeks old, new leaves nearing full expansion (N) were assumed to be 6 weeks old, and mature leaves (M) assumed to be 7+ weeks of age. Leaves younger than 4 weeks old at the time of inoculation were labelled according to the week they were first observed. For example, a leaf that appeared between 3 and 4 weeks pre-inoculation was labelled as '3 weeks'. Leaves that emerged in the week leading up to inoculation were labelled as '0 weeks'.

Plants were maintained at the Kerikeri Research Station until 16 April 2011, when they were moved to a Physical Containment Level 1+ (PC1+) laboratory at Mt Albert Research Centre (MARC), Auckland.

2.1.2 Psa strain

The isolate used for inoculations was Psa 3.2.3 (cc691), an isolate of *Pseudomonas syringae* pv. *actinidiae* taken from kiwifruit leaf spots at Te Puke Research Orchard in February 2011. This isolate is haplotype NZ-V, and is known to be pathogenic in leaf disc assays and potted plant studies (Tyson & Curtis, unpub.).

2.1.3 Inoculation

The inoculum was suspended in BS and the concentration adjusted to an optical density of 0.94 (600 nm) with an Agilent 8453 spectrophotometer. After three days, plate counts confirmed this to be a concentration of approximately 6.9×10^8 colony forming units per mL (cfu/mL).

On 18 April 2012, ten of the 'Hort16A' plants and six of the 'Hayward' were spray-inoculated with Psa. The remaining two plants of each cultivar served as non-inoculated controls and were sprayed with bacteriological saline (BS, 0.85% NaCl in sterile distilled water) only. Every leaf on each plant was spray-inoculated on both sides. After inoculation, plants were maintained in a saturated environment in a greenhouse within the confines of the laboratory.

The temperature within the plastic house was recorded using three 'temperature buttons' (Figure 1). These were situated at three locations within the plastic house (bench height at each end of the plastic house and in the centre at an elevation of 1 m). The average daily temperature in the greenhouse fluctuated between 19 and 25°C, with an average over the course of the experiment of 21.3°C (Figure 1).

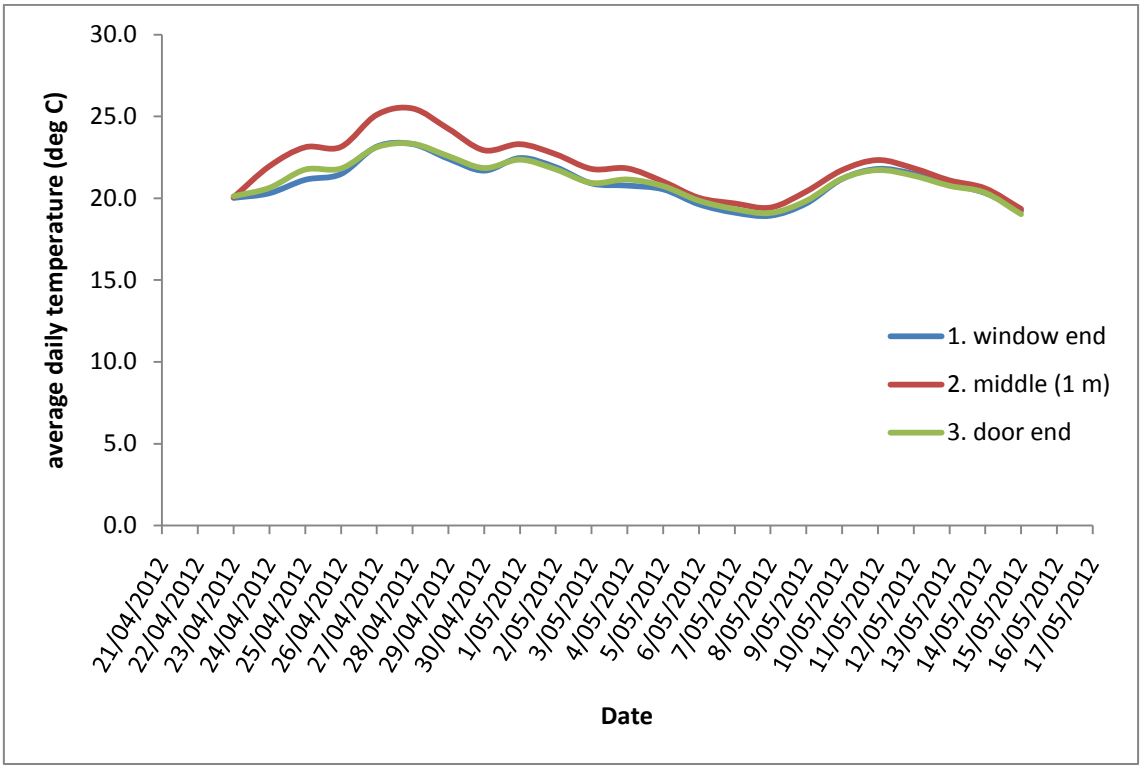

Figure 1. Average daily temperature (°C) in the greenhouse from 21 April to 17 May 2012.

2.1.4 Assessments

Assessments of plants were made 8, 12, 16, 22 and 27 days after inoculation. Any spots and flecks seen on the leaves were noted and tracked over the four-week period.

2.1.5 Isolations

Any lesions on the plants that produced exudates were swabbed and streaked across a semi-selective agar medium. Leaves with representative symptoms were surface sterilised for 20 s in 50% ethanol. Symptomatic areas were excised, macerated in 200 µL BS and left for five minutes, after which 100 µL of the resulting suspension was spread across a semi-selective agar medium. Plates were incubated at 20°C for 72 hours, and then assessed for bacterial growth. Representative colonies were confirmed as Psa by quantitative Polymerase Chain Reaction (qPCR), using the primers PsaF3/R4 of Rees-George et al. (2010).

2.2 Leaf disc trial

A range of leaves of different ages was taken from the plants used in the potted plant trial (before inoculation). Leaves from the two *Actinidia* cultivars ('Hayward' and 'Hort16A') were used in a leaf disc assay using the method of Tyson et al. (unpub.) as follows:

Leaves from each *Actinidia* cultivar were surface sterilized for 15 minutes in 0.1% NaOCl, then rinsed twice with sterile reverse osmosis (RO) water. Discs (15 mm diameter) were aseptically excised from the leaves and placed into 15 mm diameter wells made in agar plates (15 g standard agar/1 L RO water) amended with 150 ppm benzimidazole. Leaves that were too small to allow discs to be taken were cut into four. The leaf discs/pieces were placed abaxial side up to allow more potential for entry by bacteria via the stomata.

Each plate held six leaf discs or four leaf pieces; one disc on each plate was inoculated with BS only, all other discs were inoculated with droplets of Psa 3.2.3 inoculum at a rate of 6.9 x 10^8 cfu/mL. The leaf disc setup is shown in Figure 2.

After inoculation, the leaf disc assay plates were incubated at 15°C in the dark. Leaf discs were assessed for signs of infection and necrosis at day 5 and day 8.

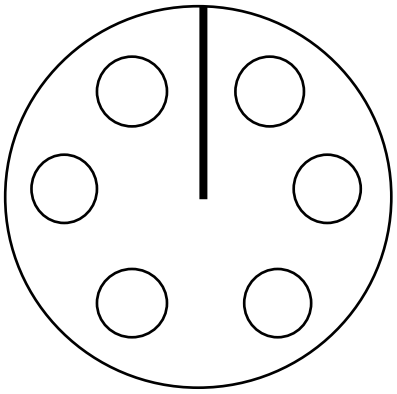

Figure 2. Kiwifruit leaf disc assay set up. Position 1, immediately to the right of the bold line, was the bacteriological saline control, all other positions were inoculated with *Pseudomonas syringae* pv. *actinidiae*.

3 Results

3.1 Potted plant trial

At the first assessment, eight days after inoculation, small, necrotic, angular spots were found on some of the leaves inoculated with Psa. Further symptoms developed over the four-week period, ranging from small brown flecks, with or without halos, to larger brown spots. Control leaves treated with only BS remained asymptomatic until the final assessment, when two leaves of one control 'Hort16A' plant were found to have developed one fleck each. Figure 3 shows typical symptoms of Psa leaf infection on 'Hort16A'.

Figure 3. Typical symptoms of *Pseudomonas syringae* pv. *actinidiae* leaf infection ('Hort16A', two-weeks-old at inoculation).

Leaf production and emergence was very variable during this trial, resulting in quite different numbers of leaves of each age group being inoculated (Table 1). Leaves were particularly sparse on the 'Hayward' vines. In addition to this variability, a substantial number of the seven-week-old leaves, in both cultivars, abscised several days after inoculation. These leaves were not included in the analysis, as it is likely that the defoliation resulted from the change of growing conditions and travel between the greenhouse in Kerikeri and the laboratory in Mt Albert, Auckland.

Figure 4 and Table 1 show the percentage of leaves of each age at inoculation showing symptoms of *Pseudomonas syringae* pv. *actinidiae* infection (flecks, spots). Leaves in the first week of emergence were susceptible to Psa. In both cultivars, leaves that were one to three weeks old were the most susceptible to infection by Psa, and thereafter there was a decline in susceptibility.

Table 1. Percentage of kiwifruit leaves of each age at inoculation showing symptoms of *Pseudomonas syringae* pv. *actinidiae* infection (flecks, spots) and average leaf area for each age class at the time of inoculation.

Cultivar and leaf age at inoculation (weeks)	% leaves with Psa symptoms	No. leaves assessed	Average leaf size (cm^2)
'Hort16A'			
0	33.3%	90	0.09
1	94.4%	18	6.38
2	94.1%	17	20.4
3	100.0%	8	33.7
4	50.0%	54	113.6
6	14.5%	62	89.1
7+	6.3%	32	90.4
'Hayward'			
0	0.0%	17	0.07
1	75.0%	4	8.96
2	100.0%	12	21.0
3	100.0%	5	84.0
4	100.0%	1	204.9
6	42.9%	14	124.7
7+	0.0%	16	49.0

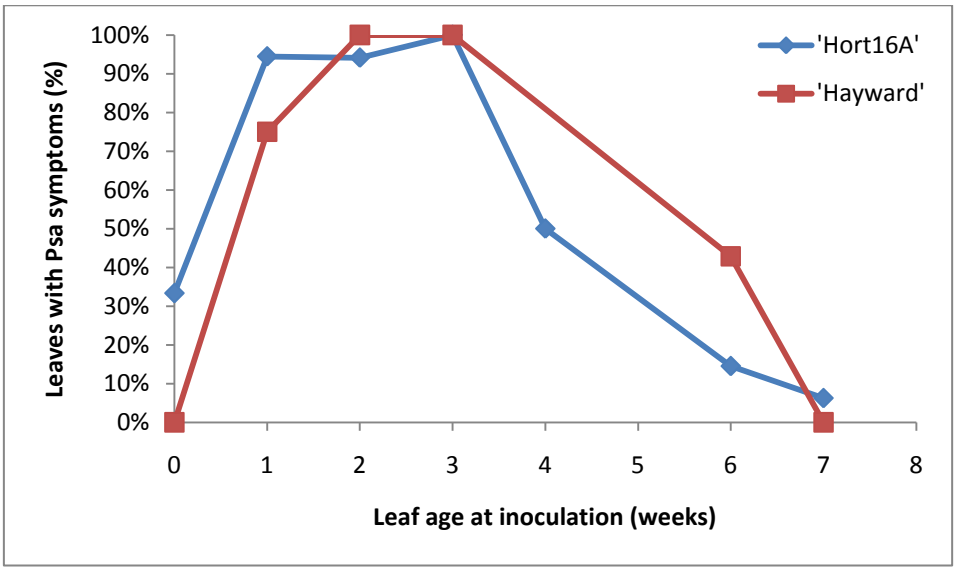

Figure 4. Percentage of kiwifruit leaves of each age at inoculation showing symptoms of *Pseudomonas syringae* pv. *actinidiae* infection (flecks, spots).

3.2 Leaf disc trial

Leaf disc infections progressed from small dark flecks or water soaked areas, to larger necrotic areas. In some cases, the entire leaf disc became infected. Figure 5 shows typical symptoms of Psa leaf disc infection after 11 days incubation ('Hayward' leaves, four-weeks-old at inoculation).

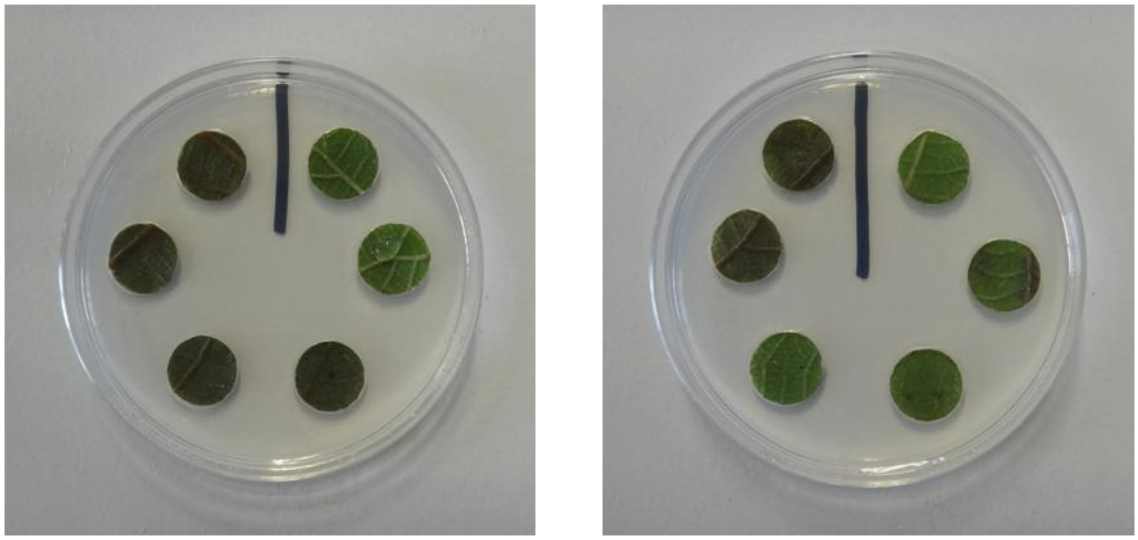

Figure 5. Typical symptoms of *Pseudomonas syringae* pv. *actinidiae* leaf disc infection ('Hayward', four-weeks-old at inoculation).

As noted for the potted plant trial, leaf production and emergence was very variable, resulting in quite different numbers of leaves of each age group being available. The 'Hayward' vines had very few leaves, with the result that no leaves in the 0-week and 3-week age groups were available for the leaf disc assays.

Figure 6 shows the average percentage of leaf disc necrosis in leaves of each age group ('Hort16A' and 'Hayward') in the leaf disc assays.

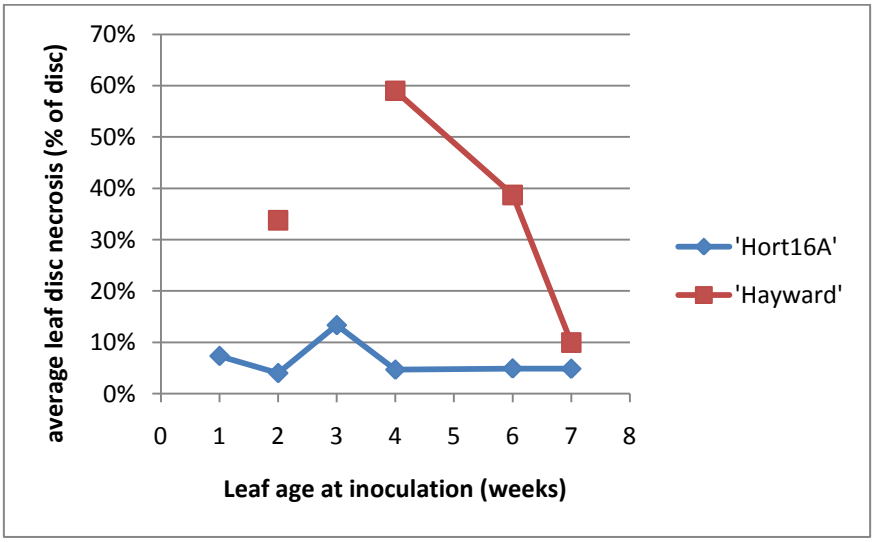

Figure 6. Average percent leaf disc necrosis in *Pseudomonas syringae* pv. *actinidiae*-inoculated kiwifruit leaves of various ages.

4 Discussion

In this study, we have found that leaf age has a significant effect on infection by Psa. Leaves from one to three weeks of age are particularly susceptible to Psa infection. This roughly corresponds to the period when leaves are still expanding. There is a considerable decline in susceptibility or symptom development in leaves older than four weeks. Orchard observations (Manning & Horner unpub.) have shown that the younger leaves are more susceptible to Psa; this has now been confirmed by this experiment.

There appears to be very little difference between 'Hort16A' and 'Hayward' in the susceptibility of leaves of different ages to infection. Although it may appear from Figure 4 that 'Hort16A' was more susceptible in the 0-week age group, there were significantly fewer leaves of this age on the 'Hayward' plants (90 'Hort16A' and 17 'Hayward' leaves: see Table 1), so the results are not directly comparable.

The lower infection rates recorded in leaves in the '0 week' class may be the result of leaf size. Most of those leaves were very small at the time of inoculation, on average only about 8 mm^2. Leaf area was approximately 100-fold greater in the '1 week' class and more than 1000-fold greater in the '4 week' age class, making the target for infection much greater in the older leaves. It is also possible that the hairiness of the newly emergent leaves could have prevented the sprayed Psa inoculum from reaching the leaf surface, or that few stomata had formed, reducing the possible entry points.

This information on the relationship between leaf age and susceptibility to Psa could be incorporated into disease prediction models and should be considered in canopy and disease management systems.

5 References

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