Systemic movement of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit vines in New Zealand

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Abstract Systemic movement of *Pseudomonas syringae* pv. *actinidiae* (Psa) was investigated in inoculated potted kiwifruit vines in a greenhouse and in naturally infected mature vines in the orchard. In the potted vine experiment, trunks of 2-year-old *Actinidia chinensis* 'Hort16A' on the rootstock *A. deliciosa* 'Bruno' were wound-inoculated with Psa. After 151 days Psa was detected up to 95 cm from the point of inoculation. Psa moved upwards and downwards within the trunks, and through the scion and the rootstock, at similar rates. In the orchard experiments, samples were taken from mature kiwifruit vines at intervals along the vines during autumn, winter and spring. Psa moved systemically throughout mature 'Hort16A' and *A. deliciosa* 'Hayward' kiwifruit vines and was detected in symptomless tissues. In both greenhouse and field situations, Psa was not inhibited by the graft union and was able to move into the rootstock and down to the base of the vine.

Keywords *Pseudomonas syringae* pv. *actinidiae*, Psa, bacterial canker, systemic migration, enrichment, qPCR.

INTRODUCTION

Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) is a relatively new disease to New Zealand and was first detected in Te Puke in November 2010 (Everett et al. 2011). Since 2010, Psa has had a devastating effect on the New Zealand kiwifruit industry, and particularly on the *Actinidia chinensis* 'Hort16A' (gold) cultivar. The disease is characterised by leaf spots, cane dieback, cankers and the production of orange-red or milky-white exudates.

Some *Pseudomonas* species have been shown to migrate systemically within their host plants. The causal agent of bacterial canker of

hazelnut, *Pseudomonas syringae* pv. *avellanae*, infects the trees through leaf scars in autumn. After overwintering in a twig, it is able to move systemically during the following spring from the infected twig to other branches and down as far as the roots (Scortichini 2010).

When bacterial canker of kiwifruit was first found in New Zealand, there was little knowledge about the spread of Psa within the vine. At that time it was thought that it may be possible to eradicate Psa from an individual vine by identifying the extent of an infection and removing the affected tissue. Understanding how the bacterium progresses once inside the

plant is essential for developing effective orchard practices and pruning methodologies, and to better understand the epidemiology of the disease in New Zealand.

In this study, systemic movement of the bacterium was examined using potted kiwifruit vines kept in controlled conditions in a polythene greenhouse and in naturally-infected mature vines in the orchard.

MATERIALS AND METHODS

Inoculated vines

Two-year-old potted *Actinidia chinensis* 'Hort16A' grafted onto the rootstock *Actinidia deliciosa* 'Bruno' were sourced from regions without Psa and kept in controlled conditions in a polythene greenhouse. Trunks of fifteen vines were wounded with a cane-grafting implement ~5 cm above the graft and inoculated with Psa at a concentration of 4×10^9 cfu/ml. Vines inoculated with bacteriological saline (0.85% NaCl in sterile distilled water) served as controls. Vines were ~2 m in height and trunks were 1.0 – 1.5 cm in diameter at the point of inoculation.

At three times after inoculation (38, 80 and 151 days), five vines of each treatment were destructively sampled by aseptically excising stem segments at intervals along the vine. At 38 days, segments were taken up to 7 cm from the inoculation site; at 80 days, segments were taken up to 30 cm from the inoculation site; and at 151 days, segments were taken from the soil line to the tip of the main shoot. At the first two sampling times, segments consisted of v-shaped excisions on one side of the stem; at the final sampling time, ~2 mm thick cross-sections of the entire stem diameter were taken. The stem sections were surface sterilised in 95% ethanol to remove external contaminants, then assayed for live Psa using a bacterial enrichment step, followed by quantitative Polymerase Chain Reaction (qPCR) identification.

Bacterial isolation and enrichment was carried out as follows. Sections were ground in 2 ml bacteriological saline (BS) and left for at least 5 min. A 200 µl aliquot of the resulting suspension was streaked onto an agar medium semi-selective for *Pseudomonas* species, a slight modification of KBC (Mohan & Schaad 1987), using sucrose-

nutrient medium as the base instead of King's B medium. Plates were incubated at ca 20°C for 48 h.

DNA extraction, and qPCR conditions and analysis, were done as described by Tyson et al. (2012), using the primers PsaF3 and PsaR4 developed by Rees-George et al. (2010). In addition, bacterial 23S primers (Anthony et al. 2000) were used in qPCR as an internal control to check that the DNA was PCR-competent.

In this study, a Cp (crossing point or threshold value) value below 30 was interpreted as a Psa-positive result, 30 – 35 as a weak positive, and a Cp value above 35 as a negative result.

Naturally-infected mature vines

Movement of Psa was examined in mature kiwifruit vines in the Te Puke district over autumn, winter and spring of 2011, the period over which the disease was first spreading throughout the district.

Five female vines in each of two *A. chinensis* 'Hort16A' (gold) orchards were sampled in late autumn (leaf fall, June), midwinter (dormancy, early August) and early spring (budburst, mid October). Five male and five female vines in one *A. deliciosa* 'Hayward' (green) orchard were sampled in mid-winter and early spring.

Samples of internal woody tissue were taken at up to 31 positions on each vine, from just above the roots to, where possible, within a cane showing dieback symptoms. The sampling positions were either 10 or 20 cm apart, depending upon the size of the vines. Samples were taken by removing the top layer of bark with a sterilised knife, ethanol-spraying the underlying wood, and then using an electric drill and sterilised drill-bit to drill into the woody tissue to a depth of 1.5 cm. The shavings adhering to the drill bit were then placed into Eppendorf tubes containing BS.

At each position on trunks and leaders, four evenly spaced sub-samples were taken from around the girth of the vine. The sub-samples were then bulked. At each position on canes, only a single sample was taken.

Bacterial isolations were made from each bulked sample onto King's B medium (King et al. 1954). DNA extractions and qPCR analyses were done as described for the inoculated vines.

RESULTS

Inoculated vines

Thirty-eight days after inoculation, Psa was detected 2 cm above and below the inoculation site. It was not detected 7 cm from the inoculation site. After 80 days, Psa was detected 30 cm above and below the inoculation site, and by 151 days Psa was found up to 95 cm from the point of inoculation (Figure 1). In all inoculated plants, Psa moved well away from the inoculation site. Psa moved upwards and downwards within the trunks, and through the scion and the graft union into the rootstock, reaching the base of the vine within 151 days in three of the five vines sampled.

Psa was never recovered from the control plants. No external symptoms typical of kiwifruit canker, such as bacterial ooze or leaf spots, were observed on any plants (inoculated or not) during the timeframe of the experiment.

On dissection, some of the inoculated vines had red staining in the tissues immediately above the inoculation site. No staining was observed below the inoculation sites, although Psa was found to be present in the symptomless tissues as far below the inoculation site as it was above the inoculation site.

Figure 1 Movement of *Pseudomonas syringae* pv. *actinidiae* within the tissues of greenhouse-grown 'Hort16A' kiwifruit vines (38, 80 and 151 days after inoculation). 1, 2, 3, 4 and 5 are five separate plants; these are different for each sampling time.

Naturally-infected mature vines

In ‘Hort16A’ Orchard 1, during the autumn and winter sampling, Psa was detected in a high percentage of the cane samples, although little was found in the trunks at those times. Canes were being removed by the grower as dieback symptoms became apparent, therefore different canes were tested at each date. By spring all canes had been removed, along with most of the trunks. At that time, Psa was detected 10 cm above the soil line in the remains of one of the five vines (Figure 2).

‘Hort16A’ Orchard 2, including the five trial vines, appeared badly affected by Psa, with a large number of dieback canes. However, little Psa was detected in the trunk and leader woody tissue at the autumn and winter samplings. By spring, Psa was detected in a large number of samples (Table 1) and appeared to be rapidly colonising entire vines (Figure 3).

Table 1 *Actinidia chinensis* ‘Hort16A’, Orchard 2. Percentage of trunk/leader woody samples positive for *Pseudomonas syringae* pv. *actinidiae* (Psa) at each sampling time.

	Autumn	Winter	Spring
Vine 1	0.0	19.0	42.9
Vine 2	7.7	0.0	92.3
Vine 3	0.0	5.6	33.3
Vine 4	0.0	21.4	35.7
Vine 5	0.0	20.0	60.0

In ‘Hayward’ Orchard 3, there was initially little visible Psa, and during the winter sampling, Psa was only detected in one position in each of one female and two male vines. At the spring sampling time, Psa was confirmed in three of the five female vines and in all five male vines. At this time, spots were evident on the leaves and blossoms had bacterial browning. Very little Psa was detected at

Figure 2 Detection of *Pseudomonas syringae* pv. *actinidiae* within the tissues of naturally-infected mature kiwifruit vines in ‘Hort16A’ Orchard 1 over autumn (A), winter (W) and spring (S), 2011. 1, 2, 3, 4 and 5 are the same five vines at each sampling date. C = cane, T = main trunk, T (C) = trunk sampled in autumn, cane sampled in winter. Sampling sites were 10 cm apart.

Figure 3 Detection of *Pseudomonas syringae* pv. *actinidiae* within the tissues of naturally-infected mature kiwifruit vines in 'Hort16A' Orchard 2 over autumn (A), winter (W) and spring (S), 2011. 1, 2, 3, 4 and 5 are the same five vines at each sampling date. C = cane, L = leader, T = main trunk. Sampling sites were 20 cm apart.

the winter sampling from most of the vines in this orchard and there was only a patchy distribution by spring (Figures 4 & 5). Psa was detected to the base of the vine in some plants.

DISCUSSION

In the inoculated vines Psa moved upwards and downwards in the trunks from the inoculation site at a similar rate. Rates of movement through the scion ('Hort16A') and the rootstock ('Bruno') were similar.

Initially the organism moved relatively slowly from the point of inoculation but the rate of movement appeared to increase with time. Psa was detected at 2 cm, but not as far as 7 cm, from the inoculation site 38 days after inoculation; this was at least 0.5 mm per day, but may have been as much as 1.6 mm per day. It had reached 30 cm from the inoculation site by 80 days after inoculation; a rate of at least 3.8 mm per day. Samples were not taken far enough out to delineate the spread in all vines at this time.

Psa moved up to 95 cm from the inoculation site within 151 days, progressing at a rate of

approximately 6.3 mm per day. In a similar study, Scortichini & Lazzari (1996) inoculated new leaf scars of hazelnut (*Corylus avellana*) with *Pseudomonas syringae* pv. *avellanae*. They found the bacterium migrated up to 45 mm in the first 2 months after inoculation. At approximately 0.75 mm a day, this is about one-eighth of the rate that was seen with Psa in the inoculated kiwifruit vines. Conversely, Momol et al. (1998) studied downwards movement of *Erwinia amylovora*, the bacterium causing fire blight of apples, from the tips of inoculated shoots and found that *E. amylovora* moved 50+ cm in 11 days, significantly faster than Psa was seen to migrate in kiwifruit.

The movement of Psa was not inhibited by the graft union and it was able to move through it into the rootstock and down to the level of the soil (roots were not sampled). The presence and location of the bacterium in the tissues could not be predicted by the symptoms.

The results from the naturally-infected mature vines indicate that Psa can move systemically throughout both 'Hort16A' and 'Hayward' kiwifruit cultivars. Psa was found in trunks as

Figure 4 Detection of *Pseudomonas syringae* pv. *actinidiae* within the tissues of naturally-infected mature male kiwifruit vines in ‘Hayward’ Orchard 3 over winter (W) and spring (S), 2011. 1, 2, 3, 4 and 5 are the same five vines at each sampling date. C = cane, L = leader, T = main trunk. Sampling sites were 20 cm apart.

Figure 5 Detection of *Pseudomonas syringae* pv. *actinidiae* within the tissues of naturally-infected mature female kiwifruit vines in ‘Hayward’ Orchard 3 over winter (W) and spring (S), 2011. 1, 2, 3, 4 and 5 are the same five vines at each sampling date. L = leader, T = main trunk. Sampling sites were 20 cm apart.

low as 10 cm above the ground, indicating that in mature vines, as with the inoculated vines in the greenhouse, the graft union does not impede the progress of the bacterium.

The pattern of detection of Psa was discontinuous throughout the trunks of mature vines and an ‘advancing tide’ of bacteria was not obvious, unlike in the ‘point-inoculated’ greenhouse vines. The distribution of the pathogen within the tissues across any cross-section of the plant was not consistent and it was

frequently isolated from some sub-samples and not others taken from the same cross-section (data not shown). From this work it could not be determined where on the plant the infections originated, or if there were single or multiple infection points.

Once established in the vine, Psa appeared to be able to spread systemically relatively rapidly. It is possible that a bacterial population below the limits of detection had become previously established in asymptomatic tissues, followed

by a rapid increase in bacterial numbers after some pre-disposing event or at a specific plant growth stage or when a critical population size was reached. Alternatively, it is possible that the bacterial titre in a vine is naturally very low over autumn and winter, and rises with the advent of spring. Under those scenarios, the results would indicate the ease of detection, not changes in distribution of *Psa* within the vines. However, given that this work was done so soon after the incursion of *Psa* into New Zealand (first detected the previous spring), neither scenario is considered likely.

From these experiments the key findings are: (1) *Psa* readily moves upwards and downwards in the plant from an inoculation site; (2) in inoculated vines, red staining in the tissue was observed only above the inoculation point, although *Psa* was detected both above and below the inoculation point; (3) *Psa* readily moved through the graft union and well down into the rootstock; and (4) internal (and external) symptoms are not an accurate guide to the distribution of the pathogen and *Psa* can be found in symptomless tissue within the plant.

The study has shown that *Psa* could be present in parts of the vine with no external symptoms. Removal of obviously diseased material therefore does not necessarily remove the bacterium as it can be present in asymptomatic tissues quite remote from symptomatic tissues. Although the extent of an infection cannot be known from the symptoms seen, the removal of visibly infected tissue will always be important, as this tissue is likely to be a major source of inoculum for further infections.

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