Understanding flower-bud rot development caused by *Pseudomonas syringae* pv. *actinidiae* in green-fleshed kiwifruit

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Abstract A greater understanding of the epidemiology of flower bud rot caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) in New Zealand green-fleshed kiwifruit cultivars is required to develop successful disease-management strategies. This study sought evidence as to whether the source of Psa bacteria that causes flower bud infection is internal or external to the flower buds as they emerge in spring. Psa was detected using qPCR in asymptomatic flower buds of two green-fleshed cultivars during spring 2016 and 2017, between bud emergence and immediately before flower opening. Bacterial isolations were made from surface sterilised and non-sterilised buds. Asymptomatic and symptomatic buds were dissected and isolations made from each of the dissected flower parts over time. Significantly more Psa was detected from non-sterilised flower buds during the early stages of bud development compared with later stages. Bud dissections showed that Psa colonisation began in the outer flower parts and moved inwards and this coincided with the development of bud rot symptoms. This study supports a hypothesis that bud rot arises when buds are externally contaminated by Psa early in their development and subsequent infection moves into the inner parts of developing flowers, destroying tissue and causing bud death. Effective control must aim to prevent initial Psa contamination.

Keywords Psa, bud rot, epidemiology, kiwifruit

INTRODUCTION

Bacterial blossom blight of kiwifruit was first recorded in New Zealand in 1973, with *Pseudomonas viridiflava* identified as the causal agent (Wilkie et al. 1973). Later, Young et al. (1997) re-examined the blossom blight bacterium and concluded that it differed from previously characterised pseudomonads so it has subsequently become known as *Pseudomonas* sp. (Hu et al. 1999). However, bacterial infection of kiwifruit flowers has increased since the incursion of *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa) into New Zealand in 2010 (Everett et al. 2011) and a new type of bud rot has been observed that is distinct from bacterial blossom blight of kiwifruit.

Bud rot of kiwifruit flowers caused by Psa can be a problem in green kiwifruit cultivars in New Zealand because of the loss of developing flowers and fruitlets it causes during spring. In an extreme case, 80% loss of potential fruit was recorded in an orchard of Green14 (*Actinidia chinensis* var. *chinensis* x *A. chinensis* var. *deliciosa* 'Zesh004'; Hunkin 2013). Symptoms of bud rot caused by Psa include discoloured sepals, flower buds that fail to open, partially opened flowers

and shrivelled flowers. Flower buds with bud rot can lead to shrivelled fruit stalks, small shrivelled fruitlets (Hunkin 2013) and abscised buds.

Recent studies have confirmed that Psa is the primary cause of recent bud rot outbreaks in the Bay of Plenty (Tyson & Manning, unpublished). Psa is known to spread in the orchard predominantly by rain splash and wind-driven rain (Tyson et al. 2014) and infected leaves and canes are likely to disseminate Psa on to flower buds during rain events. However, significant knowledge gaps still remain about the Psa bud rot disease cycle, hindering seasonal management of Psa during the pre-flowering period. This study sought evidence as to whether the Psa bacteria that causes bud rot originate internally (within the flower buds) or externally (outside the flower buds). The approach taken was to determine the presence of Psa in different flower parts within developing buds over time.

MATERIALS AND METHODS

Kiwifruit varieties, experimental sites and flower-bud sampling

Flower bud sampling was carried out over two growing seasons (2016 and 2017) on three properties in the Bay of Plenty. The two green-fleshed cultivars *Actinidia chinensis* var. *deliciosa* 'Hayward' and Green14 were each sampled at two sites. 'Hayward' site 1 was located at Omanawa (lower Kaimai Ranges) and 'Hayward' site 2 was at Paengaroa. Green14 site 1 was at the same Paengaroa site as the 'Hayward' and Green14 site 2 was near Maketu. All sampling sites were maintained following standard orchard management practices.

At each site, 10 female vines were selected randomly for flower bud sampling. Sampling began in September/October and stopped just prior to flowers opening, the timing of which varied between sites (4 to 6 weeks). Five flower buds per vine were taken weekly (50 flower buds per site) of which 4 were for Psa detection and one for Psa movement.

Psa detection in flower buds

Asymptomatic buds from all collection dates were categorised into four distinct growth stages according to the Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie (BBCH) growth stage scale (Salinero et al. 2009). These were BBCH 51 = closed buds, greenish sepals; BBCH 53 = closed buds, elongating reddish peduncles; BBCH 55 = sepals begin to separate, a white-greenish corolla is visible; BBCH 56 = sepals continue to separate, peduncles continue to elongate and thicken, corolla visible and white. After categorisation, the bud sample size was at least 40, 80, 12 and 28 buds at BBCH stage 51, 53, 55 and 56 respectively per site.

Half of the buds of each growth stage were surface sterilised (SS) in 70% ethanol for 1 min, 1% sodium hypochlorite for 3 min, 70% ethanol for 30 s, then double rinsed in sterile reverse osmosis water, before air drying in a laminar flow cabinet (based on the method of Hoyte 2001). This treatment was designed to kill bacteria on the external parts of the buds. The remaining buds were kept non-sterilised (NS) so any live bacteria on both the internal and external parts of the buds could be detected.

Psa detection was carried out from live bacteria by isolating Psa (onto a semi-selective medium), extracting DNA from isolated bacteria and then conducting qPCR on extracted DNA. For Psa isolation, both sterilised and non-sterilised whole flower buds were macerated in 1–1.5 mL bacterial saline (BS, 0.85% NaCl) and left for 5 min, after which 100 µL of the resultant suspension was spread onto King's-BC (KBC), a semi-selective medium for *Pseudomonas syringae* (Mohan & Schaad 1987). All isolation plates were incubated at room temperature for 72–120 h, to allow bacterial growth.

DNA extraction was conducted by following the method of Tyson et al. (2012). Bacterial cultures were flooded with 1 mL BS, mixed with entire cultures and an aliquot (100 µL) of each of the resultant suspension into 900 µL BS. All tubes of suspensions were then centrifuged at 8500 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 1 mL BS and centrifuged at 8500 rpm for 5 min. The supernatant was discarded and the pellet

was re-suspended in 1 mL EDTA (1 mM). An aliquot (200 µL) of the final suspension was then placed in a heat block at 100°C for 5 min, then immediately placed on ice for 10–15 min. The tubes were again centrifuged for 5 min at 13,000 rpm and the resultant DNA sample was diluted 2.5-fold, then stored at -20°C until being used for Quantitative Polymerase Chain Reaction (qPCR).

qPCR (only for presence or absence) was conducted on all flower buds for detection of Psa. qPCR was performed using a Rotor-Gene Q (Qiagen, Germantown, MD) and the Psa-specific primers PsaF3 and PsaR4, developed by Rees-George et al. (2010). Each 10 µL reaction consisted of 2.5 µL of DNA (extracted from KBC plate), 5 µL Rotor-Gene® SYBR® Green 2x, 1.5 µL RNase-free water and 0.5 µL of 5 µM forward and reverse primers. The qPCR ran under the following conditions: 95°C for 10 min 40 cycles of 95°C for 5 s, 65° for 7 s, 72°C for 7 s, followed by melting-curve analysis, with a temperature profile slope from 65°C to 97°C. In this study a crossing point value below 30 was interpreted as Psa-positive.

Incidence of Psa in flower tissues

The collected buds for all collections were individually categorised based on severity of bud browning (BB): 0 = no browning of sepals (asymptomatic), 1–5 = number/area of sepals out of five per flower showing browning (Elizabeth Popowski, personal communication). Sample size of buds were 31, 15, 29 and 8 from, 'Hayward' site 1, 'Hayward' site 2, Green14 site 1 and Green14 site 2 respectively. All buds were surface sterilised to exclude externally located bacteria and then dissected into sepal, petal, anthers and ovary to determine where bacteria were located within each bud. Dissected flower parts were then macerated in 0.3-0.8 mL BS for Psa isolation. Psa isolation, DNA extraction and qPCR were each carried out as described previously.

Statistical analysis

Statistical analyses of the qPCR detection results were done using the Two-Sample Percent Defective or Chi-Square Percent Defective test at a 95% confidence level within the Minitab18 software package (State College, PA, USA). The data were examined for each year separately and showed similar trends so the entire dataset was combined.

RESULTS

Psa detection in flower buds

Pseudomonas syringae pv. *actinidiae* biovar 3 was detected in significantly higher percentage of non-sterilised flower buds than sterilised buds for three out of the four sites at the early flower development stages, BBCH 51 or 53 (Fig. 1). However, no significant differences in detection of Psa between surface sterilised (SS) and non-sterilised (NS) flower buds occurred at the later stages of bud development at any site. For 'Hayward' site 1 there was no significant difference between the SS and NS buds at any growth stage and this site had a lower rate of Psa detection overall.

Incidence of Psa in flower tissues

The presence of Psa was assessed on separate flower parts (sepals, petals, anthers and ovary) dissected from flower buds at the following stages of bud browning symptom development: BB 0, BB 1 and BB ≥ 2 (Fig. 2).

For 'Hayward' at sites 1 and 2, before visible browning was present (BB 0), Psa presence was significantly higher in sepals than in petals, anthers and ovaries (Fig. 2). At the BB 1 stage, Psa was detected in all flower parts, with significantly higher incidence of Psa on sepals than on either anthers or ovaries. For buds in categories BB 2+, there was no significant difference in Psa detected across all flower parts.

Green14 kiwifruit at site 2, at the BB 0 stage, showed the same pattern as both the 'Hayward' sites and for Green14 at site 1 although the difference between sepals and other flower parts was less obvious. As with 'Hayward', at the BB 1 stage, the differences in frequency of Psa detection between different flower parts had diminished and at the BB 2+ stage there were no significant differences in Psa detection across all

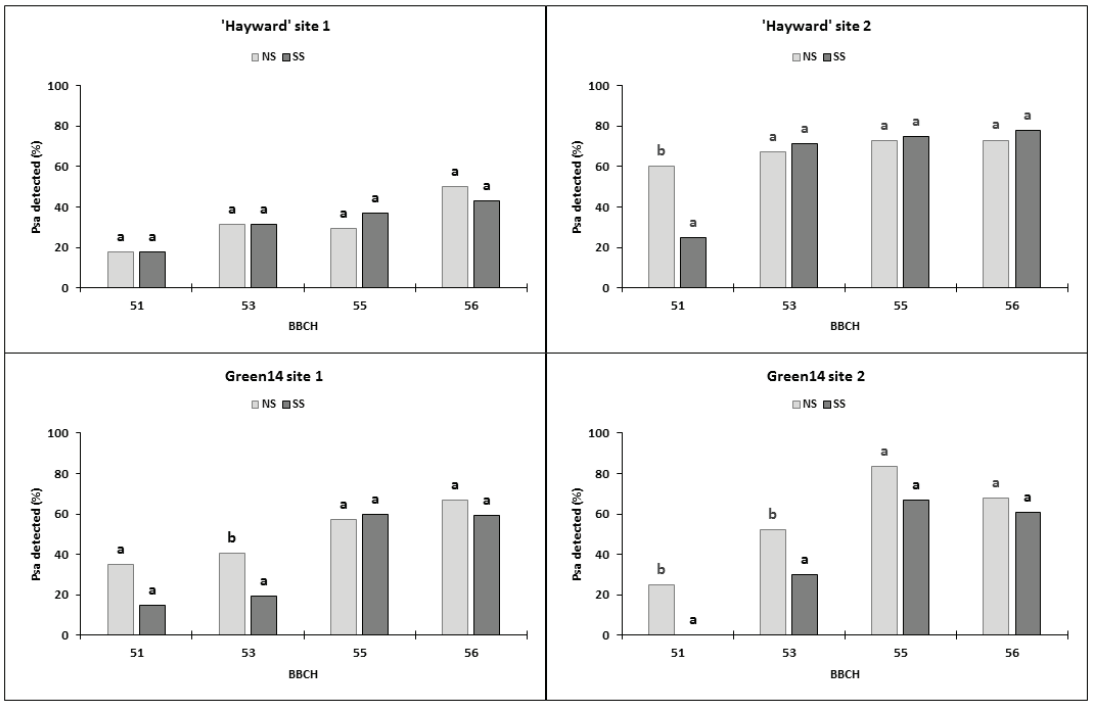

Figure 1. Detection of *Pseudomonas syringa*e pv. *actinidiae* (Psa) from non-sterilised (NS) and surface sterilised (SS) asymptomatic flower buds in *Actinidia chinensis* var. *deliciosa* 'Hayward' and Green14 (*Actinidia chinensis* var. *chinensis* x *A. chinensis* var. *deliciosa* 'Zesh004') kiwifruit using combined data from 2016 and 2017. BBCH (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) bud growth stage categories where: 51 = closed buds, greenish sepals; 53 = closed buds, elongating reddish peduncles; 55 = sepals begin to separate, a white-greenish corolla is visible; and 56 = sepals continue to separate, peduncles continue to elongate and thicken, corolla visible and white. A Two-sample Percent Defective test was used. Within each graph, pairs of bars in each BBCH category with the same letter are not significantly different (P>0.05).

flower parts.

Overall, the frequency of recovery of Psa was significantly higher from sepals on asymptomatic flower buds compared with petals, anthers or ovaries. Psa was detected more frequently across all flower parts (typically with the highest detection from sepals) on buds with more extensive visible rot symptoms.

DISCUSSION

In this epidemiology study, Psa detection was significantly higher on the non-sterile (NS) buds in early stage of bud development compared to surface-sterilised (SS) buds; and in floral dissection study, significantly higher Psa detection was noticed at outer side of the floral parts at early stages.

The more frequent detection of Psa in NS rather than SS buds at the early stages of bud development suggests that the predominant source of Psa bacteria is external, rather than internal. This is to be expected since the bacterium is spread by rain splash and wind-driven rain (Beresford et al. 2017; Tyson et al. 2014).

Dissection of asymptomatic buds (BB 0) showed that Psa was more often found in the outer flower parts (sepals) rather than further inside the buds, indicating that Psa was present

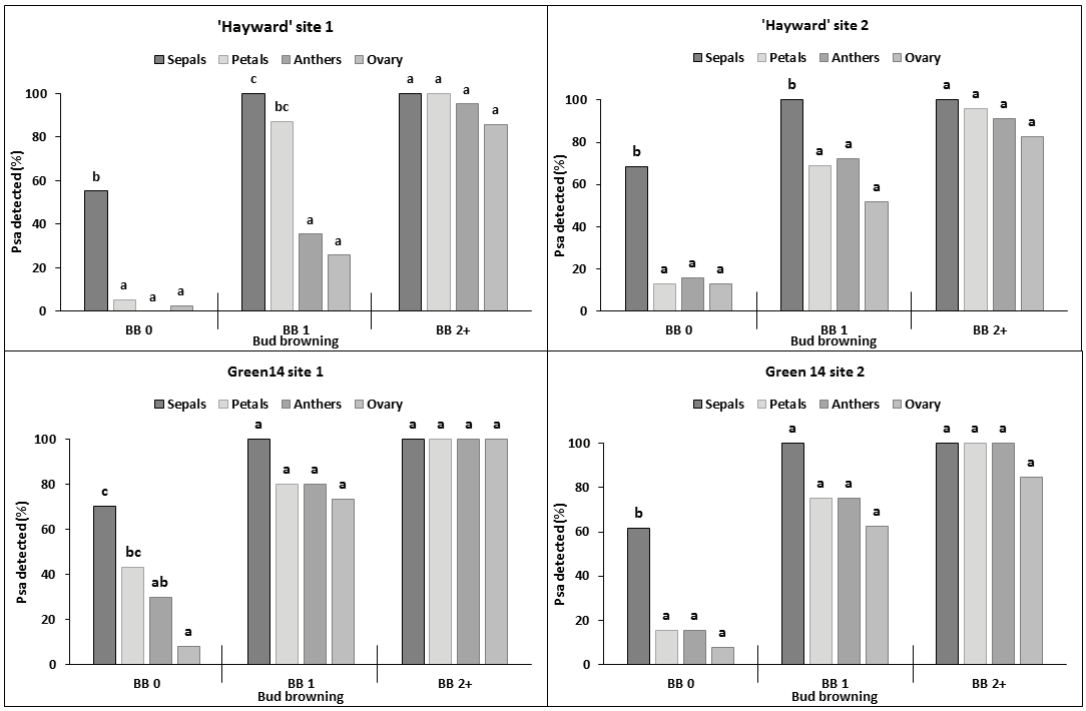

Figure 2. Frequency of recovery of *Pseudomonas syringae* pv. *actinidiae* (Psa) from the sepals, petals, anthers and ovary of flower buds displaying various degrees of bud browning symptoms (BB). BB 0= asymptomatic buds, BB 1= buds with 1 sepal brown and BB 2+= buds with 2 or more sepals brown. A Chi-Square Percent Defective test was used. Within each graph, bud browning category bars with the same letter are not significantly different (P>0.05). Data were from two sites of two commercial cultivars *Actinidia chinensis* var. *deliciosa* 'Hayward' and Green14 (*Actinidia chinensis* var. *chinensis* x *A. chinensis* var. *deliciosa* 'Zesh004') and combining seasons of 2016 and 2017.

within the outer tissues (Fig. 2). However, the possibility that some live external bacteria could have been detected cannot be ruled out even though the tissues were surface sterilised to favour detection of internal bacteria. In buds with category BB 1 bud rot symptoms (which appear when bud rot symptoms are first developing in an orchard), Psa was more frequently found further inside the buds, in petals, anthers and ovaries, although the highest frequency of detection was still in the sepals. At more advanced stages of bud rot development (category 2+), there were no differences in the frequency of Psa detection in any of the flower parts, suggesting that Psa had moved throughout the buds over time. It appears, therefore, that Psa colonisation begins in the outer flower parts (sepals) and spreads into the inner parts of the flower (petals, anthers and ovaries) as the disease progresses. This inwards movement of Psa was also suggested by Donati et al. (2018), who also detected Psa on asymptomatic buds when studying the pathway of infection in kiwifruit flowers.

These results indicate that the early stages of flower bud development may be the most important time to manage Psa in the field, by the use of contact bactericides (for example copper products).

This study found Psa was present at significantly higher rates on the exterior of young flower buds and that Psa moves from the outer to the inner flower tissues. However, it is still not known which bud stage is most susceptible to bud rot infection and which are the most favourable environmental conditions for the development of bud rot.

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