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The effect of pre-flowering girdling on the incidence of bud rot in ‘Hayward’ and Green14 kiwifruit, and an analysis of microbial populations on infected budsw (VI1509)

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CONTENTS

EXECUTIVE SUMMARY

The effect of pre-flowering girdling on the incidence of bud rot in 'Hayward' and Green14 kiwifruit, and an analysis of microbial populations on infected buds (VI1509)

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Plant & Food Research was contracted by Zespri to investigate the effect of pre-flowering girdling on the incidence of bud rot in *Actinidia chinensis* var. *deliciosa* 'Hayward' and *A. chinensis* var. *chinensis* x *A. chinensis* var. *deliciosa* 'Zesh004' (commonly known as Green14), and to define the microbial communities associated with the diseased buds.

Effect of girdling

To determine the effect of girdling, the following treatments were applied to vines of both cultivars:

1. Full girdle 30 days pre-flowering
2. Half girdle 30 days pre-flowering
3. Full girdle 20 days pre-flowering
4. Full girdle 10 days pre-flowering
5. Control (no girdling).

For 'Hayward', observations were made on buds on selected canes at regular intervals between 18 November 2014 (early bud formation) and 29 January 2015 (advanced fruitlet development). For Green14, observations were made over the period from 18 November 2014 to 30 January 2015. Because flowering commenced earlier than in 'Hayward' and the plants were in the fruitlet stage with petals still attached at the first assessment, no data were obtained for the earlier developmental stages.

On each occasion all buds on selected canes were assessed for the percentage of browning of the sepals (as a measure of bud rot). Sepal browning ranged from 0 to 100%. Numbers of buds and fruitlets that abscised during that period were also recorded.

'Hayward' vines that had received a full girdle 30 days prior to flowering had a significantly ($P<0.001$) lower proportion of sepal area affected by browning than the untreated control vines. This effect was evident on all assessment dates. None of the other treatments significantly reduced bud rot expression compared with that in the untreated control. Furthermore, the vines that had received full girdling 30 d before flowering had a significantly ($P<0.001$) lower abscission rate than vines in all other treatments.

In Green14, vines that had received full girdles 30 and 20 days before flowering had significantly ($P<0.001$) less bud rot than vines in the other treatments. Although there were no differences ($P>0.05$) between the treatments in the number of fruit that abscised, vines receiving full girdles 20 d and 30 d before flowering had fewer shrivelled fruit, a feature potentially caused by bud rot.

Microbial populations

Samples were taken from the 'Hayward' orchard only. On November 18 2014, a mixture of symptomatic and asymptomatic buds were detached from the pedicel, bulked and placed into 20 mL of phosphate buffer saline (PBS), and frozen at -20°C for processing. The same vines were re-sampled in December 2014, at early fruit development. On that occasion buds with different degrees of browning were placed individually into PBS and frozen. The December samples were unfortunately lost in transit by the courier company, during which time they thawed and remained at room temperature for several days, which compromised them.

DNA was extracted directly from 500 µL of the saline in the tubes and from a 2-3 mm section of tissue dissected from the bud using a modified CTAB-based technique (Frampton et al., unpublished).

DNA was extracted and polymerase chain reaction (PCR) amplifications were carried out for three gene targets: bacterial 16S gene, bacterial *dnaX* gene, and fungal ITS1 gene. DNA was also amplified from one mock control sample containing DNA from known isolates of bacteria (*Acidovorax* sp., *Streptomyces* sp., *Rhocococcus* sp.).

Molecular analysis of microbial populations associated with buds from the first sampling revealed *Pseudomonas syringae* pv. *actinidiae* biovar 3 (hereafter called Psa; previously called Psa-V) in all samples. The collection of a number of bud samples into a single tube apparently resulted in Psa cells being washed off the diseased buds and coming to dominate the profiles. Because the healthy and diseased buds from the first sampling were mixed in the tubes, it was not possible to compare populations on diseased and healthy buds. Fungal DNA sequences identified on the samples gave no indication that fungi were the cause of the browning of the buds.

Microbial profiles from the samples that were held up in transit were markedly different from those of the earlier samples. Several Operational Taxonomic Units (OTUs)/taxa that were not present in the first samples became dominant in these samples (for example *Pantoea* and *Rhanella* spp.). We attribute these new taxa to microbial growth during transit. Some sequences from Psa could still be detected.

Conclusions

1. Molecular analyses of microbial populations on diseased buds showed that Psa was the dominant species in the saline washes, supporting earlier findings (Tyson et al. 2015) that Psa is the most likely cause of bud rot.
2. Girdling vines 30 days before flowering resulted in significantly less bud rot than in non-girdled vines. The effect was observed in both 'Hayward' and Green14, although it was more marked in 'Hayward'.
3. The results suggest that girdling 30 days before budbreak triggered a physiological response in the vine that limited the severity of bud rot.

This is a significant finding in terms of the management both of vines and of Psa. The results suggest that girdling well before flowering can have positive effects in minimising bud rot and flower and early fruit drop caused by Psa. Those benefits, however, need to be offset against the potential risk of exposing the vines to systemic Psa infection via the girdling wound.

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1 INTRODUCTION

Bacterial blight symptoms in kiwifruit, notable for rot of floral buds and flowers, and spots on leaves, have been recorded as caused by a bacterium formerly assigned to *Pseudomonas viridiflava* (voucher specimen ICMP3272) (Young et al. 1988) and more recently referred to as *Pseudomonas* sp. New Multi Locus Sequence Typing analyses now indicate that it falls into *P. syringae* genomospecies 2/phylogroup 3, along with pathogens of woody plants (*P. s.* pv. *aesculi*) and beans (*P. s.* pv. *phaseolicola*) (Visnovsky et al. submitted). Given this, it is slightly more complicated to differentiate between *Pseudomonas syringae* pv. *actinidiae* biovar 3 (hereafter called Psa; previously called Psa-V) and the earlier described bud rot strain on leaves than earlier believed.

An extensive five-year survey established the association of the disease with rainfall during the blossom period (Pennycook & Triggs 1992). The epidemiology and ecology of the kiwifruit blossom blight was also studied in the 1990s (Everett & Henshall 1994). This was before the 2010 incursion of Psa (Everett et al. 2001). More recent studies by Tyson et al. (2015) have demonstrated a constant association of Psa with browning of buds, although *Pseudomonas* sp. was also found in approximately 30% of rotted buds. Nevertheless they concluded that the majority of bud rot symptoms seen in their study were the result of infection by Psa.

The objective of this research was to determine the effect of pre-flowering girdling on the expression of bud rot and to ascertain the microbial communities associated with bud rot symptoms.

2 MATERIALS AND METHODS

The trial was undertaken on two commercial kiwifruit orchards in the Bay of Plenty:

1. A 'Hayward' orchard at McLaren Falls
2. A Green14 orchard located at Maketu.

Girdling was undertaken with a chain on the scion of each cultivar. A full girdle, where the cambial layer was "cut", was performed on the entire perimeter of the scion. In a half girdle, the cut was made to only half the perimeter of the scion. The following treatments were undertaken, where the scion was girdled the specified number of days before flowering:

1. Full girdle 30 days pre-flowering
2. Half girdle 30 days pre-flowering
3. Full girdle 20 days pre-flowering
4. Full girdle 10 days pre-flowering
5. Control (no girdling).

2.1 Site 1. 'Hayward'

At the 'Hayward' site there were 10 replicate vines per treatment, with a total of 50 vines. The layout was a randomised block design. On each vine, all flowers on four entire canes (two on either side of the scion) were counted. The same canes were counted at each assessment. The vines began to flower in November 2014 and were assessed five times. The first three assessments on 18 November 2014, 04 December 2014 and 12 December 2014 were made over the period covering the development stages from "popcorn" to flowers fully open. On each occasion each bud on the cane was assessed for the percentage of browning of the sepals (as a measure of bud rot). Sepal browning ranged from 0 to 100% (Figures 1-4).

The final two assessments (9 January 2015 and 29 January 2015) were made at the fruitlet and advanced fruitlet stages respectively. Assessments were made by counting the number of healthy fruit remaining on the cane, the number of shrivelled fruitlets remained on the cane, and the number that had dropped off (indicated by bare pedicels still attached to the cane). Fruitlets that had dropped off were assumed to have been infected by bud rot, as the pedicel exhibited a brown discolouration, similar to that observed on the sepals of the bud rot-infected flowers.

In addition, 10 male vines in the orchard were assessed, with observations made on four canes per vine. Every bud on each cane was assessed in the same manner as above.

2.2 Site 2. Green14

At the Green14 orchard, five replicate vines were used per treatment, giving a total of 25 vines. The layout was a randomised block design. On each vine, six canes (three on each side of the scion) were used and every flower on each of the canes was observed at assessment time.

Assessments were made on 18 November 2014, 10 December 2014, 9 January 2015 and 30 January 2015. Flowering in the Green14 vines began in September 2014, much earlier than in the 'Hayward' orchard. As a result, the Green14 vines were at the fruitlet stage with petals still attached at the first assessment on 18 November 2014. Thus no data were obtained for the developmental stages from popcorn to fully open.

Figure 1. Kiwifruit sepals showing no symptoms.

Figure 2. Bud with 70% browning.

Figure 3. Bud with 100% browning.

Figure 4. Buds with different degrees of browning.

2.3 Microbial communities associated with bud rot symptoms

Samples were taken from the 'Hayward' orchard only. In the first November collection, five or more buds per vine were detached from the pedicel and placed into 20 mL of phosphate buffer saline (PBS). Each sample comprised a mixture of symptomatic and asymptomatic buds. The same vines were re-sampled in December 2014, at early fruit development. On this occasion, samples with no symptoms ranging through to those with nearly 100% 'bud' discolouration, were placed individually in tubes of PBS, returned to the laboratory, and stored at -20^{0}C. The November samples were hand couriered to PFR Lincoln, and remained frozen throughout the journey. The December samples were sent via a courier company and unfortunately delayed in transit, during which time they thawed and remained at room temperature for several days. A number of secondary fungi were observed to have grown on those samples during that time.

2.4 DNA extractions and PCR

DNA was extracted using a modified CTAB-based technique (Frampton et al., unpublished).

DNA was extracted from two sample types:

- Directly from 500 µL of phosphate saline in which the kiwifruit buds were stored. It was anticipated that the saline would carry a selection of bacteria and fungi washed off the buds, an approach used in other phyllosphere studies
- A 2- to 3-mm section of tissue dissected from a kiwifruit bud.

DNA extractions and PCRs were carried out on the first 15 samples taken from the experiment that arrived without any delay in Lincoln. Four further saline samples from the later consignment that was delayed in transit were also extracted.

PCR amplifications were carried out for three gene targets that were chosen to allow profiling of broad bacterial and fungal populations:

1. Bacterial 16S gene (targeting all bacteria)
2. Bacterial *dnaX* gene (targeting pseudomonads)
3. Fungal ITS1 gene (targeting all fungi).

DNA was amplified from one mock control sample containing DNA from known isolates of bacteria (*Acidovorax* sp., *Streptomyces* sp., *Rhocococcus* sp.).

PCR reactions were carried out in duplicate, then purified using Agencourt® AMPure® XP beads. PCR products were equalized in concentration then indexed and sequenced on a single Illumina MiSeq plate by New Zealand Genomics Ltd (NZGL).

2.5 Processing of DNA sequences

DNA sequences were processed to filter out chimeras and cluster the sequences into Operational Taxonomic Units (OTUs) based on 97% similarity (Frampton et al., unpublished). The most closely related sequence to each OTU was then identified by BLAST against GenBank databases.

2.6 Analysis of data

Field data were analysed using Genstat ANOVA with a treatment*date random effect, and the block effect being the single vine. Proportional data were angular transformed [arcsin(sqrt(x))] to ensure the residuals were evenly distributed.

3 OBSERVATIONS AND RESULTS

3.1 Site 1. 'Hayward'

When assessments commenced the flowers were at tight popcorn stage and symptoms were beginning to develop. Bud rot was estimated as the percentage of the sepals covered by browning. The numbers of shrivelled fruit and pedicels that remained after flowers or fruit had dropped were also recorded.

3.1.1 Percentage of sepal area with browning

Percentages of buds covered by browning for the different treatments on each assessment date are shown in Table 1. Overall, there was a significant (P>0.05) treatment effect of girdling. Vines that had received a full girdle 30 days prior to flowering had a significantly (P<0.001) lower proportion of sepal area affected by browning than the untreated control vines. This effect was evident on all assessment dates. None of the other treatments significantly reduced but rot expression compared with that in the untreated control. There was, however, a high degree of variability. On two assessment dates (18 November 2014 and 12 December 2014), although there was a large difference in the values, there were no significant (P>0.05) differences in symptom severity between the vines in the 10 d, the 20 d full girdle and the 30 d half girdle treatments. There was no significant treatment by date of assessment interaction.

Table 1. Percentage of sepal browning on 'Hayward' kiwifruit vines at the McLaren Falls orchard.

Treatment	18 November 2014[*]		4 December 2014		12 December 2014	
10 d full girdle	28.93	b	32.55	b	19.96	b
20 d full girdle	17.46	ab	23.93	b	18.98	ab
30 d full girdle	3.53	a	3.4	a	3.25	a
30 d half girdle	14.31	ab	16.45	ab	17.1	ab
Control	25.42	b	30.65	b	23.85	b

[8]Within the same column, values with the same letter do not differ significantly (P>0.05).

3.1.2 Flower and fruit drop

The cumulative proportion of flowers and fruitlets falling from the tagged canes increased steadily from November 2014 to January 2015 (Figure 5). Vines that had received full girdling 30 d before flowering had a significantly (P<0.001) lower abscission rate than vines in all other treatments. There was no difference in the number of fruit that dropped because of bud rot in the 20 d before flowering full girdled vines and the 30 d before flowering half-girdled vines (P=0.373). Statistically there was a significant (P<0.001) difference between the control and all treatments except the 10 d before flowering full girdle, which did not differ significantly (P=0.231) from the control.

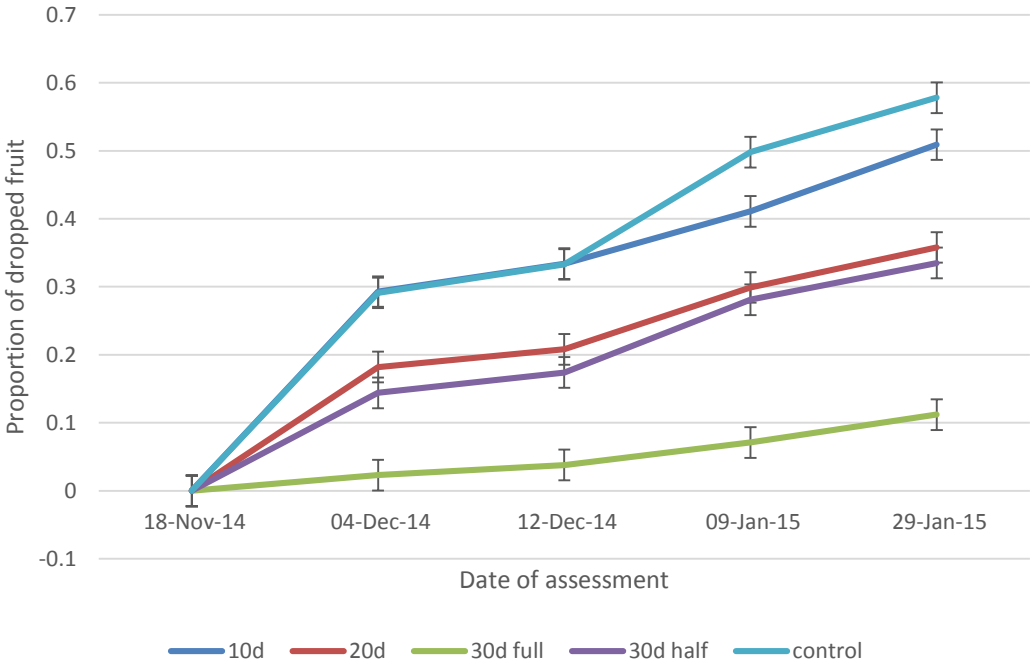

Figure 5. Back-transformed proportion of flowers and dropped fruit attributable to bud rot on 'Hayward' kiwifruit vines at McLarens Fall orchard. From 18 November to 12 December 2014, flowers were in the popcorn to fully flowering stage, with fruitlet development having occurred after this date. full = full girdle; half = half girdle, applied at 10, 20 or 30 d before flowering.

3.1.3 Bud rot on male buds

Bud rot expression on male flowers was very low, with an average of 3%, 4% and 3% on 18 November 2014, 4 December 2014 and 12 December 2014, respectively. There were no significantly ($P>0.05$) differences in bud rot severity between any of the assessment dates.

3.2 Site 2. Green14

When initial assessments of Green14 began in November 2014, the flower development was quite advanced and by December vines were at fruitlet stage. The initial assessment on 19 November indicated there were significant differences in the percentage expression of bud rot between the treatments (Figure 6). Vines in the 30 day before flowering full girdle and the 20 day before flowering girdle treatments had significantly ($P<0.001$) less bud rot than vines in the other treatments. However, once fruitlets developed there were no difference ($P>0.05$) between the treatments in the number of fruit that abscised (Figure 7). There was, however, a significant ($P<0.001$) difference between the treatments for the number of shrivelled fruit remaining on the vine, a feature potentially caused by bud rot. Vines receiving full girdles 20 d and 30 d before flowering had fewer shrivelled fruit (data not shown).

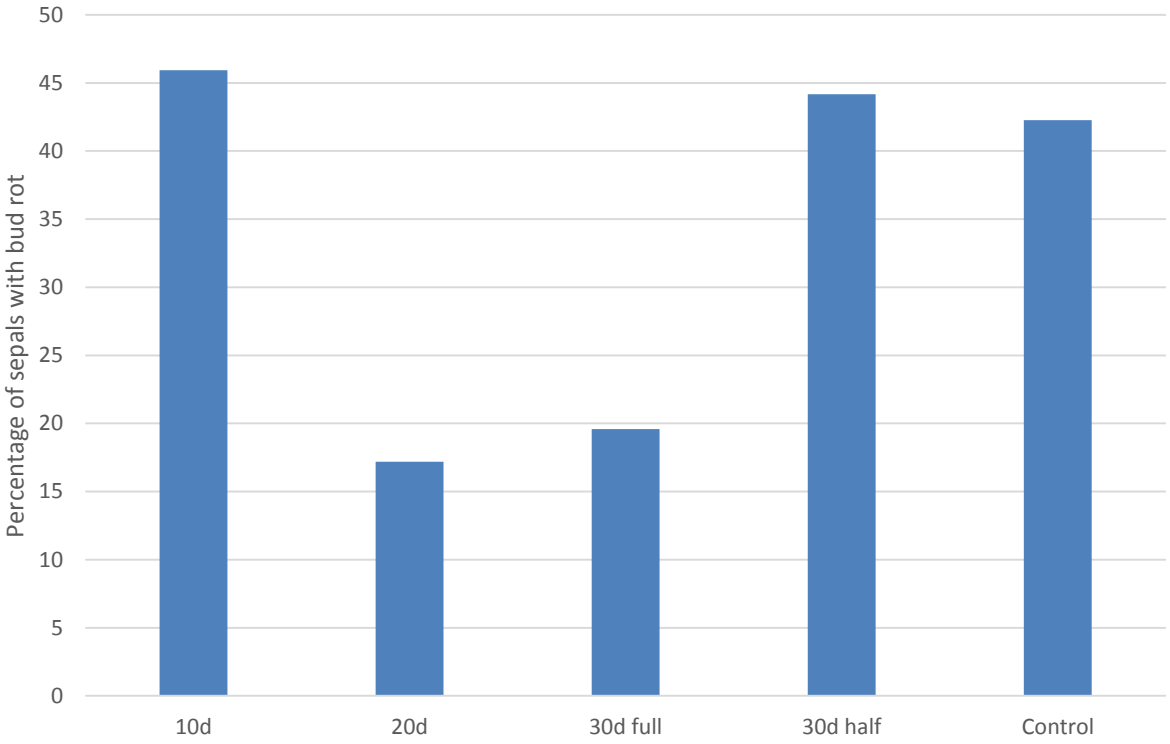

Figure 6. Back-transformed mean bud rot percentage on 19 November 2014 on 'Yesh004' (Green14) kiwifruit vines that had undergone different girdling treatments at 10, 20 and 30 days (half and full girdle) before flowering. Control vines had no girdling.

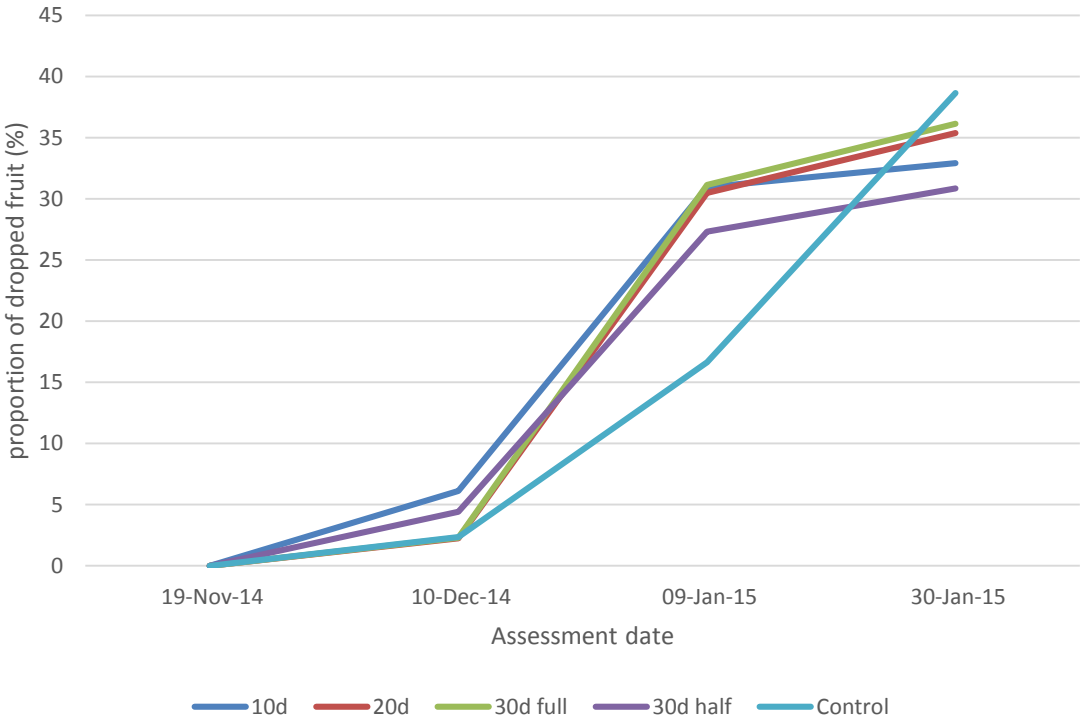

Figure 7. Back-transformed proportion of 'Yesh004' (Green14) kiwifruit dropped at the four assessment dates from vines that had undergone girdling treatments at 10, 20 and 30 days (half and full girdle) before flowering. Control vines had no girdling.

3.3 Microbial communities associated with bud rot symptoms

The results are summarized below.

3.3.1 Bacterial 16S gene

Saline extractions

- The distribution of sequence reads in our mock sample was, as expected, based on input DNAs and prior NGS runs i.e. the process appears to have functioned correctly.
- The predominant sequence from the saline/popcorn stage samples corresponded to *Pseudomonas syringae* pv. *actinidiae.* It must be noted, however, that a small number of other *Pseudomonas* spp., such as *P. cannabina,* have the same degree of identity across this DNA fragment. Samples 2, 15 and 17 had less Psa than other samples.
- The second and third most prominent sequences were from plant chloroplast and mitochondrial sequences, indicating that plant cells had sloughed off the buds and co-extracted with microbial cells.
- Other bacteria with moderate abundance were from *Sphingomonas*, *Acinetobacter* and *Pseudomonas* (near *fluorescens*). *Sphingomonas* bacteria are consistently detected from the phyllosphere of plants.
- Overall, the profiles from saline samples were consistent with our expectations of the microflora that should be obtained from bud washes.

Tissue extractions

- Microbial profiles from bud tissue DNA were essentially the same as those from the saline except that sequence reads from the plant host were much more dominant and the underlying profile of bacteria was not as easily observed (i.e. the saline was the best approach from these samples).

Later samples

- Microbial profiles from the four samples that were held up in transit were markedly different from those of the other fresh (popcorn) samples. Several OTU/taxa that were not present in the fresh samples became dominant in these samples (for example *Pantoea* and *Rhanella* spp.). We attribute these new taxa to microbial growth during transit. Some sequences from Psa could still be detected.

3.3.2 dnaX

- PCRs for the *dnaX* gene were carried out with newly designed PCR primers. These primers produced the most abundant sequences with highest (99%) similarity to *P. s.* pv. *actinidiae*. Two other less abundant *P. syringae* lineages were evident in some samples.

3.3.3 Fungal ITS1

- A large number of high quality fungal sequence reads were obtained from the saline samples.
- The profiles observed were relatively consistent with those observed in our prior studies of kiwifruit leaf microflora. The profiles were dominated by basidiomycete yeasts (e.g. *Sporobolomyces*, *Cryptococcus*, Ustilaginaceae), and ascomycetes (e.g. *Cladosporium*, *Epicoccum*).

4 DISCUSSION

Molecular analysis of microbial populations associated with buds revealed Psa in all samples. The collection of a number of bud samples into a single tube apparently resulted in Psa cells being washed off the diseased buds and coming to dominate the profiles. Because the healthy and diseased buds from the first sampling were mixed in the tubes, it was not possible to compare populations on diseased and healthy buds.

Amplification of *dnaX* gene has previously given improved discrimination of Psa and *Pseudomonas syringae* pv. *actinidifoliorum* when using 454 sequencing. However, further optimization of our primers is required for MiSeq amplification of *dnaX*. We are currently experimenting with other primer combinations for better differentiation of *P. syringae* strains in environmental samples.

Fungal DNA sequences identified on the samples gave no indication that fungi were the cause of the browning of the buds. To correlate abundance of each fungal taxon with disease severity, buds of different degrees of infection would need to be analysed separately, although it is worth noting that Psa appears to be the dominant component of these samples.

The results from this study suggest that Psa was the primary cause of the observed browning symptoms. This is consistent with the earlier findings of Tyson et al. (2015). However, we cannot exclude the possibility that other closely related strains of *P. syringae* were also present.

This work has shown that girdling vines 30 days before flowering resulted in significantly less bud rot than in non-girdled vines. The effect was observed in both 'Hayward' and Green14, although this was more marked in 'Hayward'. The results suggest that girdling 30 days before budbreak triggered a physiological response in the vine that limited the severity of bud rot.

This is a significant finding in terms of the management both of vines and of Psa. The results suggest that girdling well before flowering can have positive effects in minimising bud rot, and flower and early fruit drop caused by Psa. Those benefits, however, need to be offset against the potential risk of exposing the vines to systemic Psa infection via the girdling wound.

5 REFERENCES

Everett KR, Henshall WR 1994. Epidemiology and population ecology of kiwifruit blossom blight. *Plant Pathology* 43: 824-830.

Everett KR, Taylor RK, Romberg MK, Rees-George J, Fullerton RA, Vanneste JL, Manning MA 2011. First report of *Pseudomonas syringae* pv. *actinidiae* causing kiwifruit bacterial canker in New Zealand. *Australasian Plant Disease Notes* 6: 67-71.

Frampton RA, Anderson CR, Park D, Weir BS, Bulman SR (unpublished) Detection of *Pseudomonas* spp. by microbial profiling of kiwifruit lesions.

Pennycook SR, Triggs CM 1992. Bacterial blossom blight of kiwifruit - a 5-year survey. *Acta Horticulturae* 2(297): 559-565.

Tyson JL, Curtis CL, Manning MA. February 2015. Budrot in 'green' kiwifruit (*Actinidia* sp.) varieties - Spring 2014. A Plant & Food Research report prepared for: Zespri Group Limited. Milestone No.58315. Contract No. 30965 Job code: P/345130/01. SPTS No. 11140.

Young JM, Cheesmur GJ, Welham FV, Henshall WR 1988. Bacterial blight of kiwifruit. *Annals of Applied Biology* 1121: 91-105.

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