Using qPCR to monitor populations of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit vines after spray application of Bacstar™

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Abstract Populations of *Pseudomonas syringae* pv. *actinidiae* (Psa) on vines sprayed with a biological control agent (Bacstar™; *Bacillus amyloliquefaciens* D747; Ba) and on untreated control vines were monitored using qPCR. Leaf discs were taken from vines in February 2014, 3 months after the final of three spray applications of Bacstar™ in October and November 2013. An aliquot of leaf washings in bacterial saline (BS) was spread on to potato dextrose agar in Petri plates and the numbers of colonies of Ba were counted. DNA was extracted from the remaining BS and the quantity of Psa DNA was determined using qPCR. Ba was recovered from leaf discs that had been sprayed with Bacstar™ in greater numbers than from unsprayed leaf discs ($P < 0.05$). Psa was recovered in greater numbers from the unsprayed control leaf discs than from the Bacstar™ sprayed discs ($P < 0.05$).

Keywords field trials, Psa, *Bacillus amyloliquefaciens* strain D747.

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa) causes kiwifruit canker, a destructive disease first described in 1989 in Japan (Takikawa et al. 1989). The disease was then described from Korea in 1992 (Koh & Lee 1992), and Italy in 1994 (Scortichini 1994). More recently, there have been reports from Italy and New Zealand of a more severe strain on yellow kiwifruit (Ferrante & Scortichini 2009; Everett et al. 2011). Examination of whole genome DNA sequence of strains of Psa from around the world identified several distinct clades, referred to

as Korean (K), Japanese (J), Global outbreak (V) and New Zealand Low virulent (LV) (McCann et al. 2013). The bacterial colonies from the different clades are morphologically similar, but can be reliably separated on DNA sequence (Chapman et al. 2012). The strains from New Zealand, Italy and China isolated during the recent global outbreak were shown to be a single clone and showed no difference in core sequence (McCann et al. 2013). The origin of this clone was hypothesised to be no more than 10 years prior to 2012. Those Psa-V

strains isolated from New Zealand immediately after the outbreak in November 2010 were shown to be genetically identical following analysis of single nucleotide polymorphisms (SNPs) (McCann et al. 2013).

A biological control agent, Bacstar™ (Highland et al. 2012), has performed well in field trials in Italy against a virulent strain of *Pseudomonas syringae* pv. *actinidiae* (Psa-V) in kiwifruit orchards (Biondi et al. 2012). The active ingredient of Bacstar™ is an isolate of *Bacillus amyloliquefaciens* strain D747 (Ba). The results in Italy were from experiments on kiwifruit vines that had been inoculated in the field with Psa-V, which is not allowed on producing plants in New Zealand. In New Zealand, the only orchards available for testing products are located in kiwifruit growing zones that are already infected with Psa-V. Proving that Bacstar™ is an effective control measure against Psa-V in the field in New Zealand is a difficult technical challenge because of the possible uneven distribution of natural infections, and because of the difficulties of quantifying Psa-V using conventional PCR technology. If inoculations are conducted, more of the variation in the data is likely to be explained by the treatment effects. Quantification is required to demonstrate that application of Bacstar™ reduces Psa populations and thus shows potential for controlling the kiwifruit canker disease.

The adaptation of conventional Psa primers to use in a quantitative polymerase chain reaction (qPCR) (Rees-George et al. 2010; Everett et al. 2012a) and the design of primers that are specific to Psa-V (Rikkerink et al. 2011) provide a tool that could be used to monitor populations of Psa-V in the orchard. This study was conducted to investigate whether qPCR could be used to test the effectiveness of Bacstar™ to protect kiwifruit against natural infections of Psa-V.

MATERIALS AND METHODS

Application to kiwifruit vines

A kiwifruit orchard within the zone of Psa-V infections in Bay of Plenty was selected for this trial. There were two treatments: vines were either untreated or had Bacstar™ applied in combination with Bond® Xtra. Bacstar™

was applied on three occasions (10 October, 21 October and 4 November 2013) using a Solo motorised knapsack sprayer. The treatment was applied at 1500 kPa pressure with hollow cone nozzles at the rate of ca 1000 litres/ha. Bacstar™ (Certis USA) (25% ai) was applied at a rate of 1.5 kg/ha and Bond Xtra (Loveland Industries Inc., USA) at a rate of 700 ml/ha.

The trial layout consisted of 12 plots, with each plot being a single bay within a 'Hayward' pergola canopy. Six rows were selected as replicates, with each treatment being randomly assigned to one of the two bays in each row, giving a randomised block design.

Leaf collection

Leaves were sampled on 3 February 2014. Two 1 cm diameter leaf discs were sampled with a leaf punch from each of 25 leaves per plot selected randomly at ca 1.6 m above the ground. The leaf punch was sprayed with 70% ethanol between each plot. Leaves were folded down the middle and two discs cut at once. Leaf discs from each plot were collected in a single plastic vial. Lids on plastic vials were tightly sealed, and immediately transported by overnight courier to the Plant & Food Research Mt Albert Research Centre under a Ministry of Primary Industries permit for the movement of Psa-infected material.

Determination of *Bacillus* populations by dilution plate methodology

Twenty-five leaf discs from each plot were washed in 2 ml 0.5% Bacto™-peptone solution and ten-fold dilutions were made. A 100 µl aliquot of each dilution was placed onto potato dextrose agar (PDA; Difco™) and spread using a sterile bent glass rod. *Bacillus* colonies were identified by morphology with the aid of the dissecting microscope and counted. The morphology of these colonies was compared with colonies isolated from Bacstar™.

Quantification of Psa-V using qPCR

The remaining 25 leaf discs from each plot were washed with 2 ml 0.85% NaCl. A 1.5 ml aliquot of the washing was removed and centrifuged (16,200 × g for 10 min). The pellet was resuspended in 180 µl Buffer ATL of a Qiagen® DNeasy kit, and,

after extraction and washing, was eluted in 200 µl. A 20 µl aliquot of this elution was diluted in 50 µl of 0.1 mM EDTA pH 8 before conducting qPCR.

A 10 µl aliquot of a bacterial suspension of an overnight culture of two strains of *Psa*-J (ICMP 9617 and ICMP 9855) grown in Oxoid® nutrient broth at 28°C with shaking (180 rpm) was taken from a ten-fold dilution series. The concentration was determined by counting colonies (Barbosa et al. 1995) on King's medium B (King et al. 1954) in Petri plates and measuring the optical density (OD 534 nm) using a Unicam SP1800 dual beam Ultraviolet spectrophotometer. The concentration of a subsequent overnight culture of *Psa*-V (strain BF isolated immediately after the November 2010 outbreak in New Zealand) was determined with the aid of a spectrophotometer (A_{535}) and adjusted to 10^9 cfu/ml. The relationship between the concentration determined by the spectrophotometer and number of colonies of *Psa*-V isolate BF has been repeatedly validated elsewhere (Everett et al. 2012b). DNA was extracted from a dilution series (10-fold to 10^6 cfu/ml) and amplified using the *Psa*F3/R4 primers of Rees-George et al. (2010) and the HopZ2b primers of Rikkerink et al. (2011).

Quantitative polymerase chain reaction (qPCR)

Psa-V was quantified using the HopZ2b primers of Rikkerink et al. (2011) and the *Psa*F3/R4 primers of Rees-George et al. (2010). The 10 µl/well reaction consisted of 1 µl of DNA, 0.5 µl SYBR Green I Master, 3 µl GIBCO™ water and 5 µM of each forward and reverse primers, and was conducted in the LightCycler® 480 Real-Time PCR System under the following conditions: 95°C for 10 min, 45 cycles of 95°C

for 5 s, 60°C for 7 s, 72°C for 7 s, followed by melting-curve analysis with a temperature profile slope from 65°C to 97°C with continuous fluorescence measurement.

Data analysis

Minitab®16.1.1 was used for data analysis and for calculations. Graphs were generated using Microcal® Origin 8.5. Crossing threshold (Ct) values generated by the qPCR were subjected to a logarithmic transformation prior to analysis. An analysis of variance was conducted to take into account the structure of the experiment (randomised block design) followed by a one-tailed post hoc Dunnett's test to determine if *Psa* populations on treated vines were less than those on untreated vines, and if *Ba* populations were more on treated vines than untreated.

RESULTS

Determination of *Bacillus* populations on leaf discs

Bacteria identified as similar in morphology to *Bacillus amyloliquefaciens* strain D747 were recovered from leaf discs taken from kiwifruit vines in February 2014 that had either been sprayed with Bacstar™ in October/November 2013 or left untreated. The mean number of *Ba* colony-forming units isolated from leaves from Bacstar™ treated vines (1.81×10^3 cfu/cm²) was significantly higher ($P=0.005$) than from leaves of the untreated control vines (1.07×10^2 cfu/cm²) (Table 1).

Quantification of *Psa*-V on leaf discs by qPCR

The relationship between the mean of the colony-forming units (cfu) for two strains of *Psa*-J

Table 1 The number of *Bacillus* or *Psa* bacterial cells (colony-forming units/leaf disc) from 1 cm diameter leaf discs from an orchard naturally infected with *Pseudomonas syringae* pv. *actinidiae*. Values are mean $\pm$ standard errors.

	Ba ¹ – dilution plating	<i>Psa</i> – F3/R4 primers	<i>Psa</i> -V ² – HopZ2b primers
Unsprayed	$1.07 \times 10^2 \pm 6.42 \times 10^1$	$1.25 \times 10^8 \pm 4.78 \times 10^7$	$1.63 \times 10^8 \pm 8.38 \times 10^7$
Bacstar™	$1.81 \times 10^3 \pm 5.61 \times 10^2$	$3.01 \times 10^7 \pm 2.28 \times 10^7$	$1.49 \times 10^7 \pm 5.75 \times 10^6$
P (ANOVA)	0.005	0.09	<0.01
P (Dunnett's) ³	<0.05	<0.05	<0.05

¹Ba= *Bacillus amyloliquefaciens* strain D747 (the active ingredient of Bacstar™).

²*Psa*-V = *Pseudomonas syringae* pv. *actinidiae* virulent strain.

³Dunnett's one-tailed test.

and the OD 535 nm reading was determined following regression analysis (Figure 1).

For both primer sets a 1:100 v/v dilution of the extracted DNA was required to enable the highest concentration of Psa-V to be quantified accurately. Using the results for this dilution, a log-linear relationship was produced with 99% of the variation in the data explained by a highly significant ($P < 0.0001$) linear regression (Figure 2).

The calculated mean number of Psa-V recovered from leaf discs sprayed with Bacstar™ was lower than the number recovered from the unsprayed controls (Table 1). Results of the analysis of variance on $\log_{10}$ transformed Ct values derived by using the F3/R4 primers showed that the means were not significantly different ($P = 0.09$). However, a one tailed Dunnett's test showed a significant reduction in recovery of Psa DNA following application of Bacstar™ ($\alpha = 0.05$) (Table 1).

When the HopZ2b primers were used in the qPCR assay there was a significant difference ($P < 0.01$) in the Ct values between the untreated and Ba treated leaf washings. Similarly, a one-tailed Dunnett's test showed that less ($\alpha = 0.05$) Psa-V DNA was recovered from Bacstar™ treated leaves than from the untreated controls (Table 1). When the Ct values were converted to bacterial numbers, it was apparent that Bacstar™ applications had reduced bacterial numbers of

Psa-V from ca 10^8 cfu/cm² to ca 10^7 cfu/cm², a one log-fold reduction (Table 1).

DISCUSSION

Differences between numbers of Psa-V bacterial cells on untreated vines and those treated with Bacstar™ were able to be detected using qPCR. The results showed a significant difference between treated and untreated vines when the HopZ2b primers were used for qPCR. When the F3/R4 primers were used, the differences were less significant. In a similar experiment conducted using a rifampicin-resistant strain applied to kiwifruit flowers in Italy, a reduction of one order of magnitude was also reported following application of Bacstar™ (Biondi et al. 2012).

The significance of the statistical analysis was reliant on the removal of an outlier value. This vine was treated with Bacstar™ and was immediately adjacent to shelter on the orchard. More Psa-V DNA was extracted from this one plot than from any of the other treated plots, suggesting that there may have been a confounding environmental factor, such as a gap in the shelter (Anonymous 2013). The leaf samples were collected from the same height in the canopy, and replication was increased compared to previous unpublished work (data not shown), but variability in the distribution of natural infections still affected the statistical analysis.

Figure 1 Relationship between the mean number of colony-forming units (cfu) of *Pseudomonas syringae* pv. *actinidiae* (ICMP 9617 and 9855) and the corresponding optical density (OD 535 nm) values.

Figure 2 Relationship between number of colony-forming units of the virulent strain of *Pseudomonas syringae* pv. *actinidiae* (isolate BF) and the corresponding Crossing threshold (Ct) values generated using qPCR.

These results show that the HopZ2b primers and qPCR could be used to determine the effect of bactericides applied in the field on populations of *Psa-V*. It would be useful to compare populations immediately before and immediately after spray applications. If this type of sampling was conducted, differences in the population of *Psa-V* on individual vines or leaves would have less effect on the statistical analysis.

In the absence of symptoms early in an epidemic or on tolerant varieties of kiwifruit (Anonymous 2012), qPCR could be used to monitor the effectiveness of treatments on bacterial populations. It would also be useful to record any symptoms such as leaf spotting, bud rot and development of secondary symptoms such as cane dieback and canker formation (Everett et al. 2011) once they appear.

Bacterial colonies that were morphologically similar to *Bacillus amyloliquefaciens* strain D747 were detected on leaves in the kiwifruit orchard on which Bacstar™ was applied 3 months after application. There were significantly more *Bacillus* colonies isolated from treated leaves, showing that it had established in the orchard. Because colonies were also found on untreated leaves, it is also possible that Ba had been spread by wind and rain to adjacent vines, and/or there may be naturally occurring strains of *Bacillus* that are morphologically similar to Ba. A similar study in Italy showed that Ba established on kiwifruit leaves 96 h after application, and that it survived at population levels of 10^6 cfu/ml for a further 10 days (Biondi et al. 2012). Other studies monitoring bacterial biocontrol populations in the orchard using qPCR (Cabrefiga et al. 2011; Rosello et al. 2013) showed colonisation of flowers by *Lactobacillus plantarum* and *Pseudomonas fluorescens* was higher (10^5 – 10^8 cfu/flower) than by Ba on the leaves of the present study. Survival of *Pseudomonas fluorescens* on leaves of glasshouse plants declined from 10^7 cfu/g to 10^4 cfu/g by 28 days after application (Pujol et al. 2005), more similar to the numbers of Ba found on kiwifruit leaves 3 months after application. However, a direct comparison is difficult due to differences in the sampling units and the time after application. Although plate counting effectively demonstrated survival of

Ba on treated leaves in this study, development of a specific Ba qPCR assay would be useful to determine population dynamics more accurately.

There was a concomitant one log-fold decrease in numbers of *Psa-V* on kiwifruit leaves that had been colonised by Bacstar™, from ca 10^8 to 10^7 cfu/cm². These results provide evidence that Bacstar™ applications reduce numbers of *Psa-V* bacterial cells in the orchard 3 months after application, but the question that remains is whether this reduction is sufficient to limit the destructive disease caused by this organism. Similar studies on leaf infections by citrus canker (*Xanthomonas citri* subsp. *citri*) showed that a one log-fold reduction from ca 10^8 to 10^7 cfu/cm² following application of several compounds resulted in a noticeable reduction in leaf spot symptoms in the greenhouse (Li & Wang 2014). However, further study is advised to investigate the effect this reduction of bacterial numbers has on kiwifruit canker symptoms in the field in New Zealand.

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