Heat treatments to kill *Pseudomonas syringae* pv. *actinidiae* on contaminated pollen

K.R. Everett, D. Cohen, I.P.S. Pushparajah, M.J. Vergara, C.L. Curtis, N.J. Larsen and Y. Jia

The New Zealand Institute for Plant & Food Research Limited, Private Bag 92169, Mt Albert, Auckland 1142, New Zealand
Corresponding author: Kerry.Everett@plantandfood.co.nz

Abstract Various combinations of heat, time and relative humidity (RH) were tested for their ability to kill *Pseudomonas syringae* pv. *actinidiae* (Psa), the cause of kiwifruit canker, on freshly harvested anthers artificially contaminated with this bacterium. Pollen viability and Psa survival were tested after treatment. Although short durations at high temperatures and high RH did kill Psa, these treatments were also lethal to pollen. When RH was reduced, the time before pollen viability was lost increased, but Psa survival was also enhanced. The most promising treatment was 35°C at a RH of 50% or less, at which pollen viability was not affected even after 20 h, but Psa did not survive when applied at 10^6 cfu/ml. Modelling suggests that extending the time of treatment would kill higher concentrations of Psa. The mechanism for Psa death at 35°C is probably by a combination of heat and desiccation.

Keywords bacteria, desiccation, biofilms, aggregates, thermal death point.

INTRODUCTION

An epidemic of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) began in New Zealand as the result of an incursion that was first detected in the Bay of Plenty in November 2010 (Everett et al. 2011). Since then, this bacterial disease has devastated plantings of *Actinidia chinensis* 'Hort16A' (ZESPRI® GOLD Kiwifruit) in the Bay of Plenty, with an estimated 50% loss of vines in 18 months (Greer & Saunders 2012). This cultivar of kiwifruit is particularly susceptible to this disease and, since 2007, has resulted in almost complete removal of 'Hort16A' plantings in Italy (Balestra et al. 2009). Recently it was shown that the severe epidemic in Italy was caused by a highly virulent strain (Ferrante & Scortichini 2010), and that the strain causing the epidemic in New Zealand is similar (Chapman et al. 2011).

Despite the statement made in a recent review that bacteria are not transmitted on pollen (Card et al. 2007), there is evidence that pollination of flowers by naturally infected pollen can transmit the plant pathogenic bacterium *Xanthomonas juglandis* (walnut bacterial blight) to cause blossom end blight disease (Ark 1944b; Bradbury 1967; Phatak 1980). This bacterium was isolated from pollen dissected from catkins of infected walnut trees on several occasions in California (Ark 1944b), but was not able to be isolated from pollen collected in Oregon by other workers (Miller & Bollen 1946). Observational evidence in Oregon suggested that pollen was not implicated in the

disease epidemic in this cooler climate. Further observational evidence of pollen transmission of *X. juglandis* in California was based on spread of the disease from infected trees that had no cankers or lesions, to previously healthy trees (Ark 1944a). In Italy, introduction of infected catkins into closed nylon bags placed around flower buds was able to transmit the disease to the developing fruit, but healthy catkins and infected twigs were not able to transmit the disease to fruit (Ercolani 1962). Infected twigs in this experiment transmitted the disease to leaves. Although transmission was not proven, *Erwinia amylovora* (fire blight) was isolated from pollen from infected apple trees (Johnson & Stockwell 1998). It has long been known that a bacterial disease of honeybees (American foulbrood, *Paenibacillus larvae*) can be transmitted from colony to colony on contaminated pollen (Gochnauer & Corner 1974; Morse 1978) and some human pathogens, such as *Pseudomonas aeruginosa*, have also been isolated from pollen (Al-Dabbas et al. 2012). It is possible that bacteria on pollen could be a source of infections of human lungs by this bacterium (Lanotte et al. 2004). In a review article on seed and pollen transmission of plant pathogens, Phatak (1980) states "There is a real danger of host pollen contamination of practically any bacterial pathogen which may have infected the host before or at the time of pollen formation/maturation".

Kiwifruit pollen is applied artificially as part of normal agronomic practice in New Zealand and elsewhere to enhance fruit size and consistency of pollination (Hopping 1982). Testing by the Ministry of Primary Industries (MPI) using real-time polymerase chain reaction (PCR) and the primers of Rees-George et al. (2010) suggested that New Zealand pollen was contaminated with Psa (MAF 2010). Psa was not able to be isolated at that time, but has since been isolated from kiwifruit pollen from Italy (Vanneste et al. 2011) and New Zealand (MAF 2011). More importantly, kiwifruit flowers pollinated with contaminated pollen were shown to be epiphytically colonised by Psa in glasshouse experiments in Italy (Gallelli et al. 2011; Stefani & Giovanardi 2011). It is important to develop treatments to eliminate viable Psa from pollen to prevent potential spread and to reduce the inoculum load introduced into infected orchards, so that the yield of tolerant cultivars of kiwifruit can be maximised in the presence of this bacterium.

Treatments reported to remediate American foulbrood include antibiotics and irradiation, and the most effective method for control in hives is incineration (Liu & McRory 1994; Melathopoulos et al. 2004; Bogdanov 2006). Irradiation of kiwifruit pollen resulted in commercially unacceptable damage through a reduction in fruit size and set when the pollen was subsequently used (Musial & Przywara 1998, 1999). It is well known that the use of antibiotics cannot guarantee complete removal of bacteria from the treated substrate (Tuomanen et al. 1986; Tuomanen & Tomasz 1990; Charpentier & Tuomanen 2000), and antibiotic resistance can quickly develop in a bacterial population as a result. In contrast, heat treatments can completely remove spoilage bacteria from food products (Bremer et al. 1998), and heat is used to remove pathogenic bacteria (*Agrobacterium tumefaciens*) from grape cuttings utilising a 'window' of temperature and time in which the cuttings survive, but bacteria do not (Burr et al. 1989). Although several experiments have been conducted on the effect of humidity and temperature on pollen, the aim of these experiments was to investigate the effect of environmental factors on pollination and fruit set (Aronne 1999; Aronne et al. 2006; Nepi et al. 2010). The time and temperature combinations that are lethal to Psa in an aqueous suspension have been determined (Pushparajah & Everett 2011). This paper describes a series of experiments on fresh pollen in an attempt to find a 'window' combination of temperature, RH and time that kills Psa but does not kill pollen. This is the first report of using heat treatments for this purpose.

MATERIALS AND METHODS

Contamination of pollen

A rifampicin-resistant strain of Psa was grown on King's medium B in Petri plates (King et al. 1954) amended with 50 µg/ml rifampicin at 28°C for 48 h. Cells were harvested by washing with sterile

distilled water and concentration was adjusted following determination of the optical density with the aid of a spectrophotometer (measured at an absorbance of 535 nm). An aliquot of 10 µl of water as a control and 10 µl suspensions of 10^6, 10^7 and 10^8 cfu/ml of this rifampicin-resistant strain was added to each of four scintillation vials containing 50-90 mg (mean of 18 replicates = 65 mg ± 1.4 mg) of freshly harvested anthers from *Actinidia deliciosa* males. These levels of contamination were chosen to replicate natural inoculum levels. Addition of 10 µl of 10^6 cfu/ml to 0.05 g anthers equates to 10000 bacteria or 2×10^5 cfu/g. Naturally contaminated anthers contained 1.8×10^7 cfu/g (Braggins & Saunders 2012), which is similar to the 10^8 cfu/ml used in the present experiments.

Treatments by combinations of relative humidity (RH) and temperature

Open vials containing anthers were then placed on a shelf in an incubator with or without a fan, or in a chamber with controlled RH, airflow and temperature (Surefruit® machine, Plant & Food Research) for various times. The optimum airflow for use in the Surefruit machine was experimentally determined to be 0.5 m/s (data not shown).

The two separate investigations during the course of this study were short periods at high temperatures (Table 1) and extended times at lower temperatures (Table 2). The Surefruit machine was used to investigate the effect of different RH regimes for an extended time at 35°C, and incubators were used to investigate several lower temperatures at ca 30%, 50% and 100% RH. The ca 30% RH was achieved by incubating at 35°C at ambient RH in a fan incubator; 50% RH was achieved by placing an open container with 500 ml of water into an incubator without a fan; and 100% RH was achieved by closing the vial. The 100% RH was calculated from the volume of the vial and the amount of water that was added. RH and temperature in the incubators were logged using an Easylog® USB-2 datalogger. The Surefruit machine automatically recorded airflow, temperature and RH.

Table 1 Combinations of temperature and relative humidity (RH) (indicated by X) used to test *Pseudomonas syringae* pv. *actinidiae* bacterial survival in the Surefruit® machine at an airspeed of 0.5 m/s for up to 60 min.

Temperature	Relative Humidity (%)					
(°C)	20	24	25	28	30	50
35						X
40						X
45						X
50				X		X
53			X			X
55		X				X
60	X				X	X

Table 2 Combinations of temperature and relative humidity (RH) (indicated by X) used to test *Pseudomonas syringae* pv. *actinidiae* (Psa) bacterial survival at temperatures of 28°C and 35°C.

Temperature	Relative Humidity (%)			
(°C)	30	40	50	60
28	X			
35	X	X	X	X

Determination of pollen viability and bacterial survival

After treatment, 500 µl of pollen germination medium containing 10% sucrose and 0.01% boric acid was placed in each vial. Pollen grains were released by natural dehiscence of anthers that occurred during treatments. After 1 h, 100 µl of the pollen germination medium was removed and 10 µl of this placed on Kings' medium B amended with 50 µg/ml rifampicin in Petri plates, and dilutions of 1:10, 1:100, 1:1000 and 1:10000 v/v with sterile deionised water using the droplet plate method (Barbosa et al. 1995). After incubation for 48 h at 28°C, colonies were counted and results expressed as cfu/ml. After 2 h, pollen germination was observed with the aid of the compound microscope and number of pollen grains germinating out of 25 pollen grains was counted. There were four 25 pollen grain replicates.

Decimal reduction time (D-values)

The time required at a certain temperature to kill 90% of the organisms being studied, the *decimal reduction time* (D-values), was calculated from graphs of percentage survival of bacteria versus time, and was derived from the value on the X axis (time) at which percentage survival was reduced to 10% (Bremer et al. 1998). This can also be defined as the time required for the number of surviving microorganisms to decrease one logarithmic unit. D-values were plotted against temperature using Origin® version 8.5 and the linear regression function of Origin was used to derive formulae to predict the time after which 90% of the bacterial cells were killed at various temperatures. Pollen viability was calculated using the same method, except that these values were derived from the value on the X axis at which viability was reduced to 50%. A linear regression was fitted to each line. The best fit for pollen viability required a $\log_{10}$ transformation of Y, and the best fit for bacterial survival at a concentration of 10^6 and 10^7 cfu/ml was without transformation.

Solving simultaneous equations

The simultaneous linear equations systems were set up based on the observations of percentages of survival of Psa bacteria and the time taken to achieve such percentages, while the percentage of survival of Psa bacteria was logit transformed and the time was $\log_e$ transformed. Two observations were the percentages of survival of Psa bacteria at the 15th and 20th hours. A formula explaining the relationship between the percentage survival of Psa bacteria and time was formed by solving simultaneous linear equations. The formula is as follows:

$$\text{Logit}(P_{survival}) = a + b[\log_e(\text{Time})]$$

where a is the intercept and b is the slope.

RESULTS

The effect of relative humidity on bacterial survival in an incubator

The time required to reduce viable bacterial numbers to zero at 60°C decreased with increasing RH (Figure 1). At 100% RH 8 min was required to kill the bacterial cells, at 50% RH the time increased to 60 min, and at 30% RH 2×10^7 cfu/ml bacterial cells survived after treatment at 60°C for 60 min.

Pollen in an incubator at 60°C at 100% RH did not survive any of the times tested. In an incubator at 65°C and 50% RH, pollen viability remained above 50% for at least 5 min, but declined to below 50% after 60 min (data not shown).

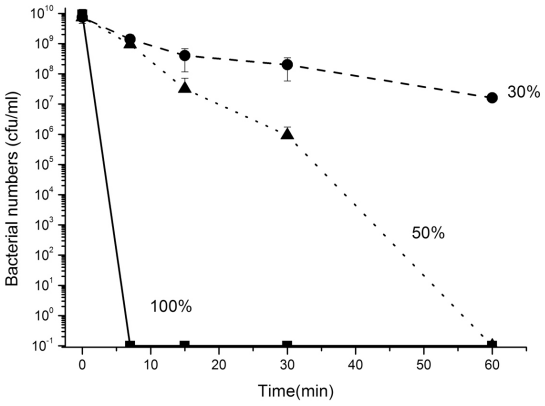

Figure 1 Reduction in *Pseudomonas syringae* pv. *actinidiae* cell numbers on kiwifruit anthers (cfu/ml) over time at 60°C and three RH values (30, 50 and 100%). A small constant value (0.01) was added to bacterial numbers to allow zero values to be plotted.

Short periods at high temperatures and 50% relative humidity

At 50% RH, pollen viability was adversely affected by increasing time at temperatures of 40°C or higher (Figure 2). When applied at a concentration of 10^8 cfu/ml, less than 90% of the bacteria were killed at temperatures lower than 50°C at all the times tested, and increasing temperature above 50°C to 55°C did not significantly increase mortality of bacteria. At temperatures up to and including 50°C, more time was required to reduce the numbers of bacteria by one logarithmic unit at all three concentrations (10^6, 10^7 and 10^8 cfu/ml) than was required to reduce pollen viability to 50%. That is, pollen viability declined to below commercially acceptable levels more rapidly than the bacteria were killed. At lower bacterial concentrations of 10^6 and 10^7 cfu/ml, 90% of

bacterial cells were killed at 53°C with exposure times of 4.8 and 10.4 min respectively, and pollen retained a viability of 50% at an exposure of 7.1 min. At 55°C, the time required for 90% mortality of bacterial cells when applied at a concentration of 10^7cfu/ml was 3.1 min. The calculation was not possible for a concentration of 10^6 cfu/ml. At 55°C, pollen viability of 50% was retained after an exposure of 5.6 min. At 60°C, pollen retained 50% viability for 3.2 min, but the predicted fit could not calculate bacterial survival for this part of the curve. Observed data at these temperatures showed that there was no survival of bacteria after 1 min at 55°C at concentrations of both 10^6 and 10^7 cfu/ml, nor after 2 min at 53°C for 10^6 cfu/ml.

Short periods at high temperatures at low relative humidity

When RH was set to 20% on the Surefruit machine, the set RH was affected by ambient conditions (Table 1). This made the results difficult to interpret, and all these results are not reported.

When the response for the pollen and bacteria was plotted for 50, 53 and 55°C (24, 25 and 28% RH, respectively), pollen viability of 50% was retained for a longer treatment time than at 50% RH, but bacterial cells were also able to survive (Figure 3). Bacteria, when applied at the highest concentration (10^8 cfu/ml), were able to survive for ca 9 min at 55°C, but pollen viability was lost after 5 min. There was no 'window' where pollen survived for longer than bacterial cells when applied at the highest concentration. These relationships were only significant for the linear regression with pollen, and for a bacterial concentration of 10^7 cfu/ml.

Long treatment periods at low temperatures

When bacterial cells were applied to pollen at a concentration of 10^6 cfu/ml, no bacteria could be

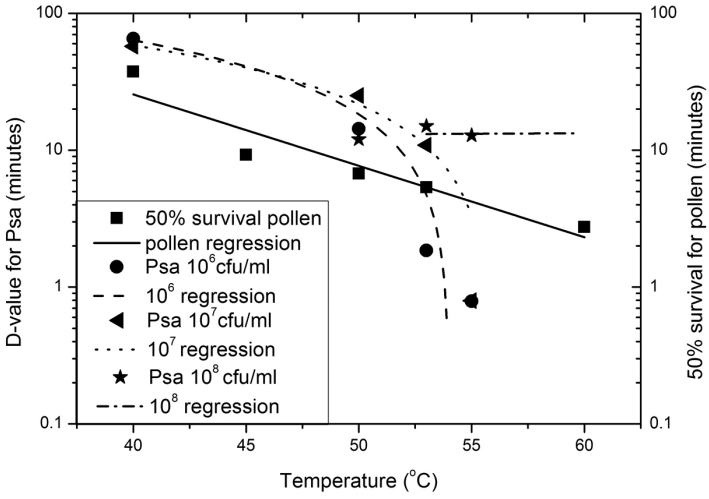

Figure 2 Time (min) to reach 50% germination of kiwifruit (*Actinidia deliciosa*) pollen and 90% kill (D-values) of a rifampicin-resistant strain of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied to pollen at three different concentrations (10^6, 10^7 and 10^8 cfu/ml). The experiment was carried out at five different temperatures for up to 60 min under conditions of 0.5 m/s airspeed and 50% RH. Less than 90% of the bacteria were killed at temperatures lower than 50°C at a concentration of 10^8 cfu/ml after the times that were tested. Linear regression fit of $\log_{10}$ transformed time for 50% viability loss of kiwifruit pollen at various temperatures was $Y = 3.5 - 0.05X$ ($R^2 = 0.87$, $P = 0.01$) and linear regression fit of D-values to temperature for three concentrations of Psa were $Y = 244.9 - 4.53$ X, ($R^2 = 0.97$, $P=0.01$) for 10^6 cfu/ml; $Y = 206 - 3.69$ X ($R^2 = 0.98$, $P = 0.005$) for 10^7 cfu/ml and not significant for 10^8 cfu/ml.

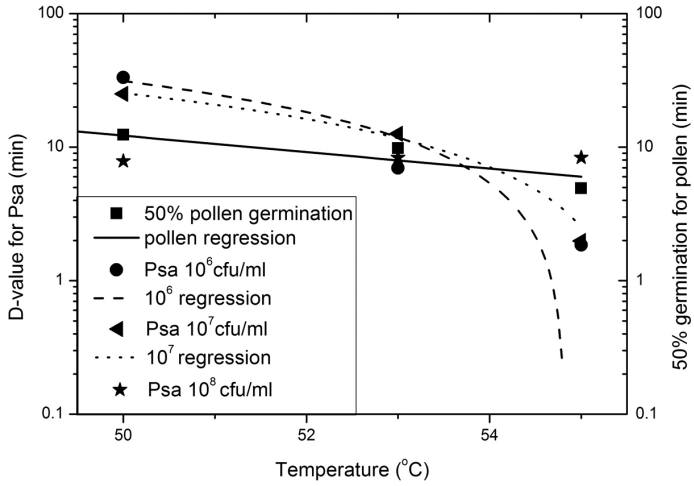

Figure 3 Time (min) to reach 50% germination of kiwifruit (*Actinidia deliciosa*) pollen and 90% kill (D-values) of a rifampicin-resistant strain of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied to pollen at three different concentrations (10^6, 10^7 and 10^8 cfu/ml). The experiment was carried out at three different temperature:RH combinations (50°C and 24% RH, 53°C and 25% RH, and 55°C and 28% RH) under conditions of 0.5 m/s airspeed for up to 32 min. Linear regression fit of $\log_{10}$ transformed time for 50% viability loss of kiwifruit pollen at various temperatures was Y = 4.2 - 0.06X (R^2 = 0.97, P = 0.01) and linear regression fit of D-values to temperature for three concentrations of Psa were Y = 355.2 – 6.5 X, (R^2 = 0.87, ns) for 10^6 cfu/ml; Y = 254.2 - 4.6 X (R^2 = 0.99, P = 0.04) for 10^7 cfu/ml and not significant for 10^8 cfu/ml.

detected after 20 h at 35°C and 30% RH in any of the three repeats of this experiment. However, bacteria applied to pollen at a higher concentration (10^7 and 10^8 cfu/ml) did survive after 20 h. If these data are used to predict the time it would take bacterial numbers applied at those concentrations to fall to 0.01% of the original concentration (a four-fold log reduction), the time required is calculated as between 34 and 54 h (Table 3).

When the temperature was lowered to 28°C and at 30% RH, bacterial numbers after 20 h did not fall to zero for any of the applied concentrations (data not shown).

When the effect of increasing RH at 35°C was tested on survival of pollen and bacteria treated for 20 h, bacteria applied to anthers at a concentration of 10^6 cfu/ml were completely killed at 30% RH, but not at 40, 50 and 60% RH (Figure 4). The reduction of bacterial numbers at higher rates of application (10^7 and 10^8 cfu/ml) was greater at 30% RH than at the higher RH values. There was a greater reduction in bacterial

Table 3 Predicted time at 35°C and 30% RH after which *Pseudomonas syringae* pv. *actinidiae* (Psa) bacterial numbers declined by a four-fold log reduction (0.01% survival). Bacterial numbers were calculated from three repeats of the experiment. The value of 0.01% represents total kill.

Concentration of Psa applied to pollen	Coefficients		Predicted hours for 0.01% survival
	Intercept	Slope	
10^6 cfu/ml	76.13	-31.47	15.05
10^7 cfu/ml	3.95	-3.3	53.63
10^8 cfu/ml	3.33	-3.57	33.67

numbers at 60% RH than at 40 and 50% RH. Pollen germination was high (over 60%) at 30, 40 and 50% RH, but declined to less than 10% at 60% RH. At 30% RH, two- and three-fold log reductions of bacterial numbers were achieved, and at an applied concentration of 10^6 cfu/ml, no viable bacteria could be detected.

DISCUSSION

The most promising treatment for the removal of Psa from fresh pollen was an extended time at 35°C at low RH. Pollen viability was unaffected by this temperature for up to 20 h at RH values up to, but not including, 60%. In contrast, Psa cells were killed after 20 h when present at low concentrations (10^6 cfu/ml). Modelling the rate of mortality of bacteria at higher application concentrations suggests that extending the time to 60 h may result in complete kill of bacterial cells, but this would need to be confirmed experimentally before any recommendations could be made. The results suggest that pollen will not be detrimentally affected by extending treatment time at this temperature, but this would also need to be confirmed experimentally.

The present results suggest that the conditions for loss of viability of Psa at 35°C were a combination of heat and desiccation, because bacterial numbers were most effectively reduced at the lowest RH tested. The survival response of bacteria to increasing RH was an inverted U shape, and following a lower rate of mortality at intermediate RH values, there was an increase again at the highest RH, demonstrating that heat alone was also having an effect. The results showed that at high temperatures, an increase in RH resulted in greater mortality of bacteria. In contrast, Psa was able to withstand dehydration at 28°C even when present at low concentrations. This suggests that the mechanism that enables Psa to survive desiccation at 28°C was ineffective at 35°C; that the rate of desiccation was greater at the higher temperature; that 28°C was not lethal after 20 h; or that the mechanism involves a combination of these factors. The combination of heat at 35°C and desiccation at the lowest RH (30%) had the greatest detrimental effect on Psa survival. For these reasons, procedures to increase the rate of desiccation at 35°C, such as increasing the airspeed and lowering the RH, may shorten the time required to kill the bacterium.

When the temperature was raised to 40°C, pollen viability was adversely affected after a relatively short time (32 min) at 50% RH.

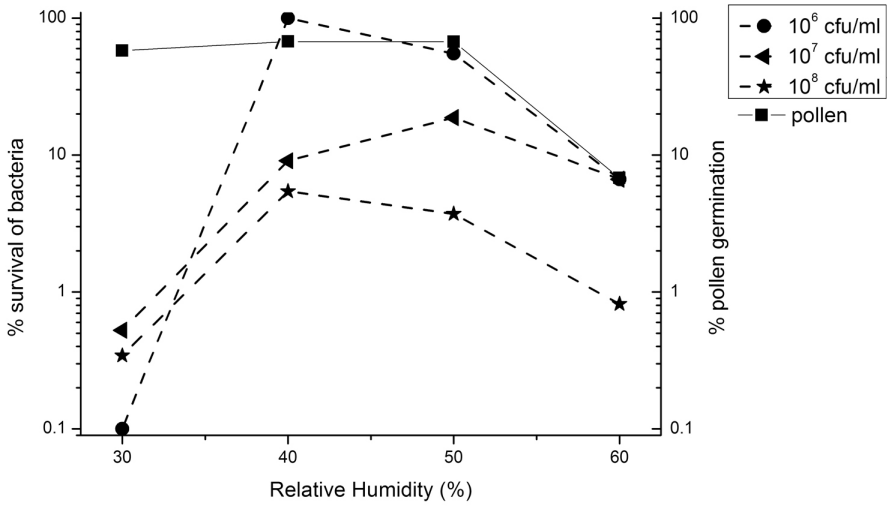

Figure 4 Survival of *Pseudomonas syringae* pv. *actinidiae* bacteria (%) and kiwifruit pollen germination (%) at different RH values at 35°C for 20 h.

Because this time is very short, pollen may not survive reducing the humidity to 30% followed by extending the duration of treatment at this temperature.

A 'window' of high temperature and RH at which all Psa cells, at all the concentrations applied, were killed, but at which pollen survived, was not found. For low concentrations of bacteria, at 50% RH, the time required to reduce viability of the pollen to 50% was between 6 and 7 min, and the time to kill 90% of the bacterial cells was between 3 and 5 min, leaving a margin of error ('window') of ca 2 min. Under commercial conditions, a small error in timing would result in economically damaging loss of pollen viability. There was no improvement of the margin of error when RH was reduced to 24-28%, and high concentrations of bacterial cells survived longer treatment times than did the pollen. It is possible that reducing airspeed would allow more margin of error, but from these results it appears that it is not likely to expand the 'window' to more than a few minutes, and thus the treatments would not be commercially viable. In contrast, the predicted 'window' of treatment for extended time at 35°C is commercially viable and should be investigated further.

The ability of Psa to survive these treatments when present at high concentrations could be explained by the formation of a biofilm or aggregates (Alhede et al. 2011; McDougald et al. 2012). Bacteria use different biochemical pathways in a biofilm than when present as single cells, which can increase resistance to environmental stressors such as antibiotics and antimicrobials (Barraud et al. 2006). Biofilms can take up to 5 days to form under artificial conditions (Barraud et al. 2009), but it is possible that the addition of high concentrations of Psa to pollen induces similar bacterial responses that enhance resistance to environmental stressors. Similar responses to environmental stressors were found for both aggregates and biofilms of *Pseudomonas aeruginosa* (Alhede et al. 2011). Exploring this hypothesis further could reveal a treatment that would reduce treatment times, for example if the biofilm or aggregate could be disrupted by another complementary method.

Nitric oxide is reported to disrupt biofilms, as well as cis-2 decenoic acid, dispersin B and deoxyribonuclease (Kaplan et al. 2003; Barraud et al. 2006). Of these compounds, nitric oxide is relatively non-toxic to humans, and is readily available in gaseous form. Some studies have been conducted on the effect of nitric oxide on pollen tube growth, and although orientation and growth rate were affected, pollen death was not reported (Prado et al. 2004). Investigation of the introduction of these compounds into the Surefruit machine at the same time as extended duration at 35°C and low RH could be worthwhile.

Once a suitable protocol has been devised and thoroughly tested on naturally contaminated pollen, artificial pollination using cleaned pollen would be preferable to using bees to pollinate kiwifruit flowers. Psa has been found to survive on bees, pollen and in hives, and there is certainly a risk that Psa on bees could be carried from an infected orchard to an uninfected orchard to cause disease (Pattemore et al. 2011). The application of pollen contaminated with Psa to flowers resulted in the establishment of epiphytic populations on leaves and fruit (Stefani & Giovanardi 2011). Ark (1933a) concluded that new leaf infections by *Xanthomonas juglandis* on previously healthy walnut trees were due to spread of the bacterium on contaminated pollen from adjacent diseased walnut trees, and was able to cause this bacterial disease on fruit by pollinating walnut flowers with contaminated pollen, as was Ercolani (1962) for the same disease by placing infected catkins adjacent to flowers. In November 2010, it was discovered that kiwifruit flowers were naturally infected and damaged by Psa (Everett et al. 2011). Live Psa-V has been isolated from kiwifruit pollen harvested from infected orchards in New Zealand (MAF 2011). During the course of this present study, it was shown that Psa-V will survive drying at 28°C, and this is the standard commercial practice used for processing pollen in New Zealand. Thus the evidence so far suggests that pollen could be a vector for Psa-V, but the definitive experiment would be to pollinate kiwifruit flowers with Psa-V contaminated pollen and by this means transmit the disease. If indeed Psa-V is vectored on

kiwifruit pollen, then even in the presence of high inoculum pressure, using Psa-free pollen would reduce the amount of inoculum in kiwifruit orchards and could improve productivity of tolerant kiwifruit cultivars in the future. Using kiwifruit pollen known to be free of Psa has the potential to reduce the spread of bacterial canker within New Zealand and elsewhere.

ACKNOWLEDGEMENTS

This study was supported by a grant from ZESPRI Ltd, Plant & Food Research and The Foundation for Research Science and Technology (Contract CO2X0501 Better Border Biosecurity). Many thanks to the pollen companies for providing pollen for testing. Thanks to the Pollen Technology Communication Team for providing feedback, advice and for facilitating communication. Also thanks to Rod McDonald and Bruce Freeth for advice on adjusting the parameters of the Surefruit machine, and to Barbara Waddell, Graham Fletcher, Mark Large and Bob Fullerton for valuable discussion.

REFERENCES

Al-Dabbas MA, Abbas MA, Al-Khafaji RM 2012. Dust storms loads analyses-Iraq. Arabian Journal of Geosciences 5(1): 121-131.

Alhede M, Kragh KN, Qvortrup K, Allesen-Holm M, van Gennip M, Christensen LD, Jensen PO, Nielsen AK, Parsek M, Wozniak D and others 2011. Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm. PLoS One 6(11): e27943.

Ark PA 1944a. Further evidence of pollen dissemination of walnut blight. Phytopathology 34: 933 (abstract).

Ark PA 1944b. Pollen as a source of walnut bacterial blight infection. Phytopathology 34: 330-334.

Aronne G 1999. Effects of relative humidity and temperature stress on pollen viability of *Cistus incanus* and *Myrtus communis*. Grana 38(6): 364-367.

Aronne G, De Micco V, Scala M 2006. Effects of relative humidity and temperature conditions on pollen fluorochromatic reaction of *Rosmarinus officinalis* L. (Lamiaceae). Protoplasma 228(1-3): 127-130.

Balestra GM, Mazzaglia A, Quattrucci A, Renzi M, Rossetti A 2009. Current status of bacterial canker spread on kiwifruit in Italy. Australasian Plant Disease Notes 4: 34-36.

Barbosa HR, Rodrigues MFA, Campos CC, Chaves ME, Nunes I, Juliano Y, Novo NF 1995. Counting of viable cluster-forming and non cluster-forming bacteria - a comparison between the drop and the spread methods. Journal of Microbiological Methods 22(1): 39-50.

Barraud N, Hassett DJ, Hwang S-H, Rice SA, Kjelleberg S, Webb JS 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. Journal of Bacteriology 188(21): 7344-7353.

Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, Kjelleberg S 2009. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. Journal of Bacteriology 191(23): 7333-7342.

Bogdanov S 2006. Contaminants of bee products. Apidologie 37(1): 1-18.

Bradbury JF 1967. *Xanthomonas juglandis*. Descriptions of Fungi and Bacteria. CAB International, Wallingford UK. 130 p.

Braggins TJ, Saunders S 2012. Mitigating the risk of *Pseudomonas syringae* pv. *actinidiae* introduction on pollen. Zespri Innovation Project V11285 Report. 28 p.

Bremer PJ, Osborne CM, Kemp RA, van Veghel P, Fletcher GC 1998. Thermal death times of *Hafnia alvei* cells in a model suspension and in artificially contaminated hot-smoked Kahawai *(Arripis trutta)*. Journal of Food Protection 61(8): 1047-1051.

Burr TJ, Ophel K, Katz BH, Kerr A 1989. Effect of hot water treatment on systemic *Agrobacterium tumefaciens* biovar-3 in dormant grape cuttings. Plant Disease 73(3): 242-245.

Card SD, Pearson MN, Clover GRG 2007. Plant pathogens transmitted by pollen. Australasian Plant Pathology 36(5): 455-461.

Chapman J, Taylor R, Alexander B 2011 Second report on characterization of *Pseudomonas syringae* pv.*actinidiae* (Psa) isolates in New Zealand. Report of the Ministry of Agriculture and Fisheries, Wellington, New Zealand. March. 10 p.

Charpentier E, Tuomanen E 2000. Mechanisms of antibiotic resistance and tolerance in *Streptococcus pneumoniae*. Microbes and Infection 2(15): 1855-1864.

Ercolani GL 1962. Individuazione di *Xanthomonas juglandis* (Pierce) Dowson in Emilia. Phytopathologia Mediterranea 2: 1-10.

Everett KR, Taylor RK, Romberg MR, Rees-George J, Fullerton RF, Vanneste JL, Manning MA 2011. First report of *Pseudomonas syringae* pv. *actinidiae* causing kiwifruit bacterial canker in New Zealand. Australasian Plant Disease Notes 6(1): 67-71.

Ferrante P, Scortichini M 2010. Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. Plant Pathology 59(5): 954-962.

Gallelli A, Talocci S, L'Aurora A, Loreti S 2011. Detection of *Pseudomonas syringae* pv. *actinidiae*, causal agent of bacterial canker of kiwifruit, from symptomless fruits and twigs, and from pollen. Phytopathologia Mediterranea 50(3): 462-472.

Gochnauer TA, Corner J 1974. Detection and identification of *Bacillus larvae* in a commercial sample of bee-collected pollen. Journal of Apicultural Research 13(4): 265-267.

Greer G, Saunders C 2012. The costs of Psa-V to the New Zealand kiwifruit industry and the wider community. Agribusiness and Economics Research Unit Report, Lincoln University. 62 p.

Hopping ME 1982. 80,000 honeybee hives not needed; spray pollination of kiwifruit possible by 1990. New Zealand Agricultural Science 16(1): 46-48.

Johnson KB, Stockwell VO 1998. Management of fire blight: a case study in microbial ecology. Annual Reviews in Phytopathology 36: 227 - 248.

King EO, Ward MK, Raney DE 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44: 301-307.

Lanotte P, Watt S, Mereghetti L, Dartiguelongue N, Rastegar-Lari A, Goudeau A, Quentin R 2004. Genetic features of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients compared with those of isolates from other origins. Journal of Medical Microbiology 53: 73 - 81.

Liu TP, McRory D 1994. The use of gamma-radiation from Co-60 in a commercial facility in Ontario to disinfect honeybee equipment. 1 American foulbrood disease. American Bee Journal 134(3): 203-206.

MAF 2010. New Zealand pollen tests positive for Psa. http://www.biosecurity.govt.nz/media/20-11-10/new-Zealand-pollen-tests-positive-for-Psa (retrieved 25/3/2012).

MAF 2011. Detection of *Pseudomonas syringae* pv. *actinidiae* from leaves and pollen collected from symptomatic and asymptomatic *Actinidia chinensis* in Te Puke, Bay of Plenty. Report to Zespri. 49 p.

McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S 2012. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nature Reviews Microbiology 10(1): 39-50.

Melathopoulos AP, Nelson D, Clark K 2004. High velocity electron-beam radiation of pollen and comb for the control of *Paenibacillus larvae* subspecies larvae and *Ascosphaera apis*. American Bee Journal 144(9): 714-720.

Miller PW, Bollen WB 1946. Walnut bacteriosis and its control. Technical Bulletin, USDA and Agricultural Experiment Station, Oregon State College 9: 107.

Morse RA 1978. Honey bee pests, predators, and diseases. Morse RA ed. Cornell University Press, Ithaca, NY, USA. 329 p.

Musial K, Przywara L 1998. Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Annals of Botany 82(6): 747-756.

Musial K, Przywara L 1999. Endosperm response to pollen irradiation in kiwifruit. Sexual Plant Reproduction 12(2): 110-117.

Nepi M, Cresti L, Guarnieri M, Pacini E 2010. Effect of relative humidity on water content, viability and carbohydrate profile of *Petunia hybrida* and *Cucurbita pepo* pollen. Plant Systematics and Evolution 284(1-2): 57-64.

Pattemore D, Hoyte S, McBrydie H, Goodwin R, Moffat B, Yu J, Parry F, Ah Chee A 2011. Survival of *Pseudomonas syringae* pv. *actinidiae* and *P. s.* pv. *syringae* on bees and in beehives. Plant & Food Research Report SPTS No. 6109.

Phatak HC 1980. The role of seed and pollen in the spread of plant pathogens particularly viruses. Tropical Pest Management 26(3): 278-285.

Prado A, Porterfield D, Feijo J 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 131(11): 2707-2714.

Pushparajah IPS, Everett KR 2011. Time and temperature combinations that are lethal to *Pseudomonas syringae* pv. *actinidiae* New Zealand Plant Protection 64: 291 (abstract only).

Rees-George J, Vanneste JL, Cornish DA, Pushparajah IPS, Yu J, Templeton MD, Everett KR 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using Polymerase Chain Reaction (PCR) primers based on the 16S-23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. Plant Pathology 59: 453-464.

Stefani E, Giovanardi D 2011. Dissemination of *Pseudomonas syringae* pv. *actinidiae* through pollen and its epiphytic life on leaves and fruits. Phytopathologia Mediterranea 50(3): 489-496.

Tuomanen E, Tomasz A 1990. Mechanism of phenotypic tolerance of nongrowing pneumococci to beta-lactam antibiotics. Scandinavian Journal of Infectious Diseases, Supplementum 74: 102-12.

Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A 1986. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. Journal of General Microbiology 132: 1297-1304.

Vanneste JL, Giovanardi D, Yu J, Cornish DA, Kay C, Spinelli F, Stefani E 2011. Detection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen samples. New Zealand Plant Protection 64: 246-251.