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Cleaning infected pollen

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A report prepared for

ZESPRI Group Limited
Ref: VI1249-30

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Plant & Food Research, Mt Albert Research Centre

SPTS No. 6536

This report has been prepared by The New Zealand Institute for Plant & Food Research Limited (Plant & Food Research), which has its Head Office at 120 Mt Albert Rd, Mt Albert, Auckland.

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Executive summary

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Physical and chemical treatments

A series of physical and chemical treatments were tested for, firstly, removal of all contaminating bacteria from pollen, then for removing Psa from pollen that was artificially cleaned and then recontaminated with Psa.

A number of the wet treatments were tested with a quick wash in 95% ethanol showing the most promise for killing Psa whilst retaining viability of pollen. The effect of this treatment on bacterial survival would need to be investigated further before it could be recommended. However, the Pollen Technology Communication Team made the decision that wet treatments did not align with current commercial practice and were to be pursued no further.

The freeze/thawing treatments were ineffective at reducing total bacterial contamination on pollen, but it is not known what effect these treatments would have on Psa survival. An investigation of the effect of freeze/thawing on Psa cells at different concentrations may be fruitful.

Heat treatments

During the research both temperature and humidity were identified as important in the killing of Psa and/or the retention of pollen viability. A window of temperature and humidity at which all Psa was killed but pollen survived was not found. Even at a temperature of 55 °C at which pollen survived longer than did Psa at a low concentration, the time required to kill the pollen was between 4 and 8 minutes. In commercial conditions a small error in timing would result in loss of the pollen. It is possible that a further reduction in airspeed would allow more margin of error at 55°C, but this would need further investigation.

The most promising treatment was extended time at 35°C at low humidity. Pollen viability was unaffected by this temperature at humidities up to, but not including, 60%. In contrast, Psa was eventually killed when present at low concentrations (10^5 - 10^6 cfu/ml) in eight trials conducted under these conditions.

These results suggest that the mechanism for loss of viability of Psa at 35°C was dessication. If this is correct then at this temperature Psa was unable to survive moisture loss unless present at high concentrations. In contrast, Psa was able to withstand moisture loss at 28°C even at low concentrations. This suggests that the mechanism that enables Psa at low concentrations to survive dessication at 28°C did not function at 35°C.

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1 Introduction

The bacterial kiwifruit pathogen, *Pseudomonas syringae* pv. *actinidiae* (Psa), was not known to be present in New Zealand until it was isolated from kiwifruit leaves and flowers in November 2010 (Everett et al. 2011). A delimiting study by MAF Biosecurity and subsequent typing showed that there were two strains of Psa in New Zealand that are now called Psa-LV and Psa-V (Chapman et al. 2011). Psa-LV was widespread and thus has probably been present in New Zealand for a significant period of time, probably less than 20 years (Park et al. 2011), but symptoms were not severe and were not noticed. Psa-V, which causes leaf spots, flower wilting and browning, cane dieback, wilting, brown staining, cankers and death in as little as 4 weeks on susceptible varieties such as 'Hort16A' (ZESPRI® GOLD), is a recent introduction to the Te Puke district of New Zealand. Since November 2010 this strain has spread from the original site where systemic symptoms were found to over 50% of ZESPRI® GOLD orchards in New Zealand.

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 MAF Biosecurity using real-time PCR and the primers of Rees-George et al. (2010) suggested that New Zealand pollen was contaminated with Psa, although live Psa was not able to be isolated at that time. As a precautionary response to this information, a project to clean artificially infected pollen was initiated by ZESPRI to reduce the risk of New Zealand pollen spreading Psa. Since this project started a recent finding by MAF has found that live Psa-V can be isolated from pollen in closed New Zealand flowers (MAF, 2011). An earlier hypothesis that Psa was not likely to be present on New Zealand pollen because it is harvested from closed flowers (Vanneste et al. 2011) has now been refuted.

A number of different chemicals and procedures were first used to treat dried stored pollen to remove Psa, but either the pollen was killed or Psa was not killed, rendering most of these treatments ineffective (Everett 2011). The most promising of these treatments was heat, because there were industry reports of the pollen being able to survive temperatures that were known to kill Psa (Pushparajah & Everett 2011). This report describes these earlier findings in more detail and describes a series of heat experiments on dessicated stored pollen and on fresh pollen to attempt to find a 'window' of temperature and time combinations that kill Psa but do not kill pollen.

Part 1. Chemical and physical treatments

1.1 Methods

Raw pollen

Pollen that had tested positive for Psa by PCR was supplied by MAF Biosecurity and by PFR Te Puke. For the first set of experiments the removal of all bacteria from this pollen was attempted.

Pollen was weighed into 1.5ml Eppendorf tubes. There were three replicate tubes containing 0.03g of pollen each from three different pollen containers for each treatment.

Freeze/thawing.

Tubes containing pollen were placed in the freezer (-18°C) and then at ambient (c. 25°C) for the times described in Table 1, 2 and 3. After the final freeze/thaw cycle, 400 µl of Oxoid nutrient broth (NB) was added to the dry pollen and vortexed once immediately after addition of nutrient broth, and again after 30 mins. After another 30 mins 10 µl was placed on King's medium B (KB) undiluted, and at a dilution of 1:10, 1:100, 1:1000 and 1: 10000 v/v with sterile deionised water. After 48 hours incubation at 25°C colonies were counted and the number of colony forming units (cfu) per ml of nutrient broth per g of pollen was calculated and presented as a logarithmic function.

Table 1: Freeze/thaw cycles for Experiment 1

Treatment	Freeze	Thaw	Freeze	Thaw	Freeze	Thaw
1.1	1 h	1 h				
1.2	1 h	1 h	1 h	1 h		
1.3	1 h	1 h	1 h	1 h	1 h	1 h
2.1	2 h	2 h				
2.2	2 h	2 h	2 h	2 h		
2.3	2 h	2 h	2 h	2 h	2 h	2 h

Table 2: Freeze/thaw cycles for Experiment 2

Treatment	F	T	F	T	F	T	F	T	F	T	F	T	F	T
1	+	+												
2	+	+	+	+										
3	+	+	+	+	+	+								
4	+	+	+	+	+	+	+	+						
5	+	+	+	+	+	+	+	+	+	+				
6	+	+	+	+	+	+	+	+	+	+	+	+		
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= 30 minutes, F = Freeze, T= Thaw

Table 3: Freeze/thaw cycles for Experiment 3

Treat-ment	12 hr	F	T	F	T	F	T	F	T	F	T	F	T	F	T	F	T
1		+	+														
2	+	+	+	+	+	+	+										
3	+	+	+	+	+	+	+	+	+								
4	+	+	+	+	+	+	+	+	+	+	+						
5	+	+	+	+	+	+	+	+	+	+	+	+	+				
6	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

+ = 1 hour except for column 1 (12 hours)

F = Freeze

T= Thaw

Ethanol and hypochlorite

1. An aliquot of 400 µl of 95% ethanol was added to 0.03g dry pollen in an Eppendorf tube and then vortexed. After 1 minute the tubes containing pollen were centrifuged for 8500 rpm for 5 mins in a Heraeus Pico 21 microfuge. Following centrifugation the supernatant was removed and the pellet resuspended in 400 µl of 1:3 v/v sodium hypochlorite, then vortexed. After 3 minutes the tubes containing pollen were centrifuged as before, the supernatant removed, and the pellet resuspended in 400 µl of sterile deionised water (SDW). Following centrifugation the pellet was resuspended in 400 µl NB. After an hour 10 µl was placed on KB and at a dilution of 1:10, 1:100, 1:1000 and 1: 10000 v/v with SDW. After 48 hours' incubation at 25°C colonies were counted and the number of colony forming units per ml of nutrient broth per g of pollen was calculated and presented as a logarithmic function.
2. An aliquot of 400 µl of 95% ethanol was added to 0.03g dry pollen in an Eppendorf tube and then vortexed. After 5, 10 and 20 minutes the pollen plus ethanol was centrifuged for 5 mins at 8500 rpm and the ethanol was removed, then 400 µl of NB was added and the suspension was immediately vortexed. After 1 hour 10 µl was placed on KB undiluted, and at a dilution of 1:10, 1:100, 1:1000 and 1: 10000 v/v with SDW. After 48 hours' incubation at 25°C colonies were counted and the number of colony forming units per ml of nutrient broth per g of pollen was calculated and presented as a logarithmic function.

Acetic acid

1. An aliquot of 400 µl of 17.5M acetic acid (glacial), 5M acetic acid, 1 M acetic acid (the concentration in vinegar) and 0.1M acetic acid was added to three replicate Eppendorf tubes of pollen and immediately vortexed. After 30 minutes tubes were vortexed again, and after another 30 minutes tubes containing pollen and acetic acid were centrifuged for 5 mins at 8500 rpm and the acetic acid was removed, then 400 µl of NB was added and the suspension was immediately vortexed, then vortexed again after 30 minutes. After 1 hour in total 10 µl was placed on KB undiluted. An aliquot of 10 µl of nutrient broth and 10 µl sterile deionised water were also placed on KB after all other tubes to check for contamination. After 48 hours' incubation at 25°C colonies were counted and the number of colony-forming units per ml of nutrient broth per g of pollen was calculated and presented as a logarithmic function.
2. An aliquot of 400 µl of 5M, 3M, 2M and 1M acetic acid was added to three replicate Eppendorf tubes of pollen and immediately vortexed. After 30 minutes tubes were vortexed again, and after another 30 minutes tubes containing pollen and acetic acid were centrifuged for 5 mins at 8500 rpm and the acetic acid was removed, then 400 µl of NB was added and the suspension was immediately vortexed, then vortexed again after 30 minutes. After 1 hour in total 10 µl was placed on KB undiluted. An aliquot of 10 µl of NB and 10 µl SDW were also placed on KB after all other tubes to check for contamination. After 48 hours incubation at 25°C colonies were counted and the number of colony-forming units per ml of nutrient broth per g of pollen was calculated and presented as a logarithmic function. The Eppendorf tubes containing 390 µl NB were centrifuged for 5 mins at 8500 rpm immediately after the 10 µl aliquots were removed. The NB was removed and the pollen was resuspended in 400 µl pollen germination media (10% sucrose and 100 mg/L boric acid). Following vortexing, an aliquot of 10 µl was placed in the centre of a microscope slide with a well and inverted on glass rods in a sealed plastic container with a damp paper towel (a hanging drop method). The plastic containers and the remaining pollen in germination media in Eppendorf tubes were then placed in the dark at 20°C for 24 hours. The glass slides were removed, a cover slip placed on the well, and examined with the compound microscope. A 100 µl aliquot of the pollen in Eppendorf tubes was placed on a glass slide, covered with a cover slip and examined with the compound microscope. Germination was recorded when the pollen tubes were the same length as the pollen grains, and 100 pollen grains per treatment per replicate were examined.

Controls

For each treatment except for freeze/thaw experiment 2 and 3 there was an untreated control where three replicate Eppendorf tubes containing 0.03 g of pollen was amended with 400 µl of NB and the suspension was immediately vortexed. After 30 minutes the tubes were vortexed again, and after 1 hour 10 µl was placed on King's medium B undiluted, and at a dilution of 1:10, 1:100, 1:1000 and 1: 10000 v/v with sterile deionised water. After 48 hours' incubation at 25°C colonies were counted and the number of colony-forming units per ml of nutrient broth per gram of pollen was calculated and presented as a logarithmic function.

Heated pollen

Due to the presence of spore-forming bacteria such as *Bacillus* spp. and the known difficulties associated with killing these bacteria, pollen was subsequently treated to remove all bacteria,

then it was contaminated with known strains of Psa. To achieve this, pollen was heated at 100ºC for 24 hours, then placed at room temperature for 24 hours, then heated again. Twice-heated pollen was then contaminated with Psa-V or Psa-LV by placing 0.03g in 400 ml of bacterial suspension for one hour. Contaminated pollen was then dried on filter paper in Petri plates at 28ºC overnight. For heat treatments in Part 2, pollen was heat treated by placing the Petri plates containing pollen at the different temperatures. For treating with various substances, the contaminated pollen was scraped off the filter paper and placed in 1.5 ml Eppendorf tubes.

Buffered hypochlorite

Hypochlorite was buffered to pH 7 with hydrochloric acid at a range of concentrations from 50-800 ppm for a treatment time of 30 sec.

After an exposure time of 30 sec, sodium sulphite solution was added to neutralise the hypochlorite. As a control, a sample of pollen was treated with a solution of 800 ppm buffered hypochlorite that had been neutralised with sulphite solution

The effect of short durations of ethanol on pollen viability

Treatments were carried out on filter paper in a Buchner flask with a vacuum. The treatments were:

1. Moisten pollen with Pollen Germination Medium (PGM), leave 15 min then wash with 80% EtOH, followed by 95% EtOH
2. Wash dry pollen with 95% EtOH
3. Wash dry pollen with 80% EtOH
4. Wash dry pollen with 80% EtOH, followed by 95% EtOH

A sample of the dry pollen from each treatment was transferred to a filter paper moistened with PGM. At the times shown below, a sample of pollen was spread onto PGM-agar coated slide.

1.2 Results

1.2.1 Removing all bacteria from PCR positive pollen

Freeze/thawing

Bacterial numbers on pollen were reduced 10-fold following three 1 hour freeze/thawing cycles (Figure 1). Decreasing the length of the cycles to 30 minutes resulted in no reduction in bacterial numbers with increasing numbers of freeze/thaw cycles (Figure 2). Repeating the first experiment and increasing the number of 1 hour duration freeze/thawing cycles did not reduce bacterial numbers (Figure 3). In all experiments bacterial numbers were not reduced to zero.

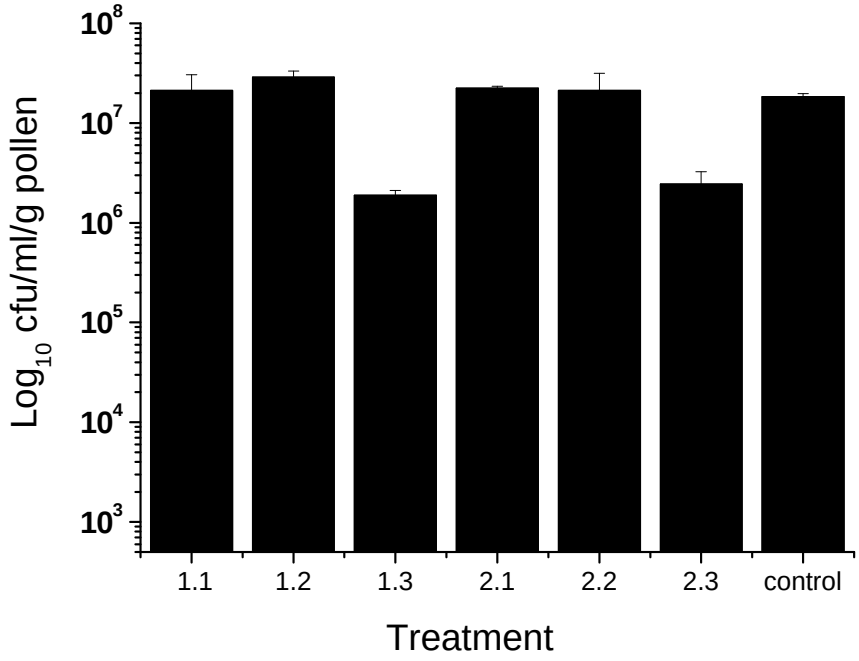


Figure 1 Experiment 1. Freeze/thaw 1 and 2 hour cycles. The first number is the duration in hours, and the second number is the number of freeze/thaw cycles (Table 1). .

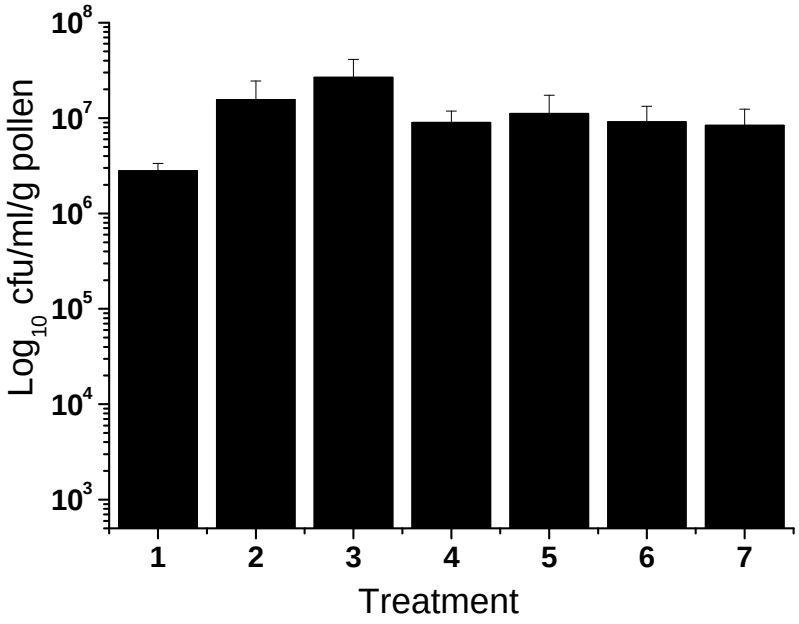


Figure 2. Experiment 2. Freeze/thaw 30 minute cycles. The treatment number represents the number of cycles. (Table 2).

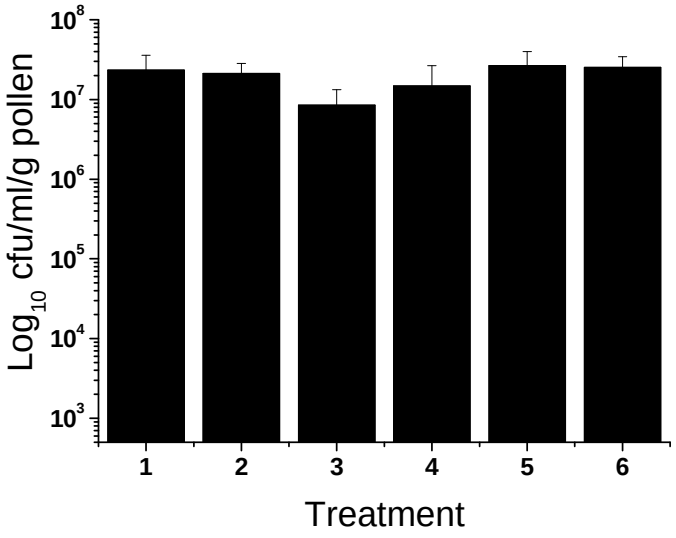


Figure 3. Experiment 3. Freeze/thaw 1 hour cycles. The treatment number represents the number of cycles, treatment 1 is one 1 hour cycle, treatment 2, 3, 4 and 5 are, respectively, three, four, five, and six 1 hour cycles followed by 12 hours in the freezer, and treatment 6 is seven 1 hour cycles interrupted by two cycles of 12 hours in the freezer (Table 3).

Ethanol and hypochlorite

The treatment of naturally contaminated pollen by 95% ethanol for increasing time showed no improvement for killing bacteria (Figure 4).

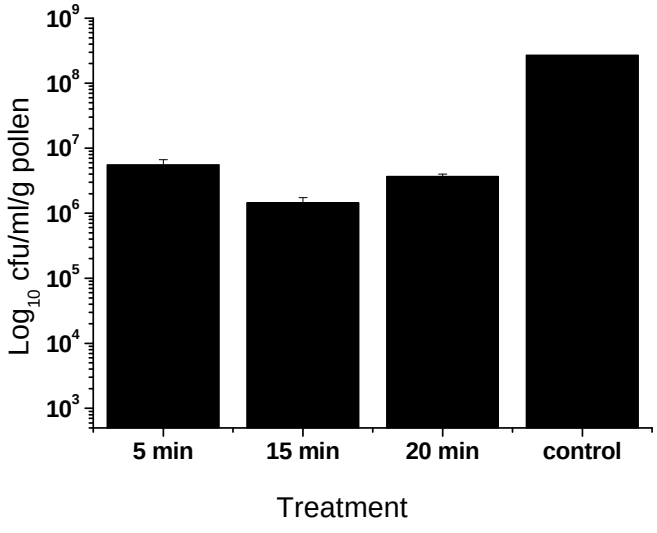


Figure 4. Number of bacteria isolated from pollen following different durations of exposure in 95% ethanol.

Hypochlorite by itself did not kill the bacteria (Table 4). In combination with ethanol the pollen was also killed (Table 5), and chlorine dioxide did not kill Psa (Table 6). Buffered hypochlorite above a concentration of 50 ppm killed the pollen (Table 7).

Table 4: The effect of 70% ethanol and hypochlorite on percent recovery of bacteria.

Time (mins)	70% ethanol + hypochlorite	hypochlorite
0	100	100
3	0	100
6	0	100
12	0	100
24	0	100

Table5: The effect of 80% ethanol, hypochlorite, hexane and hydration on pollen germination.

	treatment				pollen germination (%)	
	80% ethanol	hypochlorite	hexane	hydrated	mean	stdev
1	-	-	-	-	58.7	6.8
2	-	-	-	+	51.3	9.3
3	+	+	-	-	0	0
4	+	+	-	+	0	0
5	+	+	+	-	0	0
6	+	+	+	+	0	0

Acetic acid

Concentrations of acetic acid greater than 1M were able to kill all bacteria on naturally contaminated stored pollen (Figure 4). However, the pollen was not viable (Figure 5).

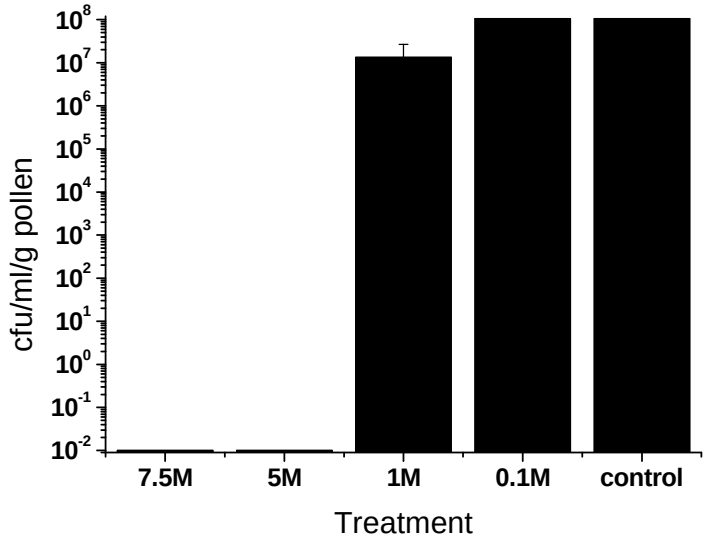


Figure 5: The effect of treating naturally contaminated pollen with 0.1, 1, 5 and 7.5M acetic acid on bacterial populations.

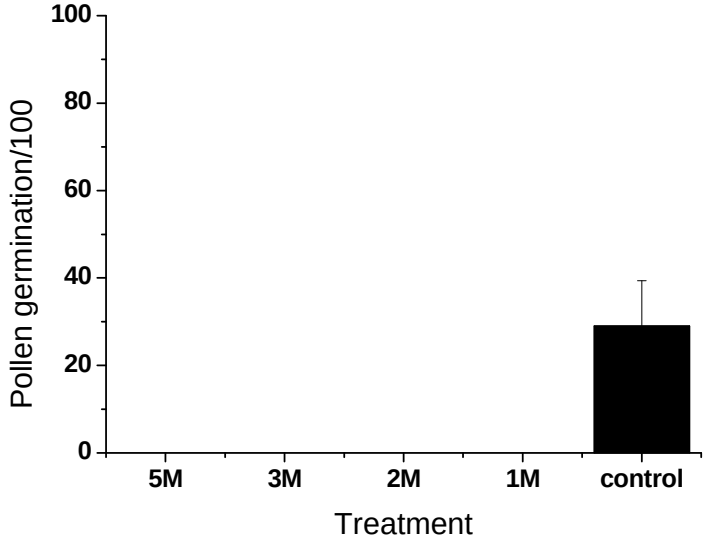


Figure 6: The effect of treating naturally contaminated stored pollen with 1, 2, 3 and 5M acetic acid on germination.

1.2.2 Removing Psa from contaminated cleaned pollen

Treatment of pollen that had been cleaned of contaminating bacteria by 'cooking' (100°C for 24 hours, room temperature for 24 hours, then 100°C for 24 hours) showed that ethanol did kill Psa-V and Psa-LV (Table 6). However, it also reduced pollen viability (Table 6).

Table 6: Cleaned pollen contaminated with Psa-LV or Psa V, then treated with different concentrations of ethanol for 3 minutes followed by washing with sterile deionised water.

	Psa LV		Psa V		pollen viability	
		washed		washed	mean	stdev
70%	0	0	0	0	0.0	0.0
80%	0	0	0	0	0.0	0.0
95%	0	0	0	0	2.3	1.5
untreated control	100	100	100	100	32.7	2.1

Table 7: The effect of chlorine dioxide on cleaned pollen contaminated with Psa-LV and Psa-V on percent recovery of bacteria.

concentration (ppm)	Psa LV	tween 20	Psa V	tween 20
0	100	100	100	100
50	100	100	66	100
100	100	100	100	66
1000	100	100	100	100

Table 8: The effect of buffered hypochlorite on pollen viability.

Treatment	5 hr	20 hr
0 ppm	++	+++
50 ppm	++	+++
100 ppm	-	+
200 ppm,	-	-
400 ppm	-	-
800 ppm	-	-
800 ppm neutralised	++	+++

+++ high pollen germination, ++ reduced pollen germination, and – no pollen germination.

Treatment with a concentration above 50 ppm of buffered hypochlorite killed the pollen (Table 8). A few grains of pollen from the 100 ppm treatment germinated by 20 hours but the pollen tubes were very short.

Table 9: The effect of a brief ethanol wash on pollen viability.

		Time on PGM in filter paper after treatment				
Ethanol concentration in washes	15 min PGM pre-treatment	0 min	15 min	30 min	60 min	60 min
80% then 95%	+	0	0	0	0	0
95%	-	22	13	41	25	38
80%	-	0	0	0	0	0
80% then 95%	-	0	0	0	0	0

Note: PGM = pollen germination medium

It appears that a 95% ethanol wash on dry pollen is not toxic to pollen (Table 9). However, 80% ethanol killed pollen.

Part 2: Heat treatments of stored pollen

1.3 Method

A series of heat treatments were conducted on dry stored pollen. Some results were not repeatable.

1.4 Results

These results are summarised from two PowerPoint presentations made to ZESPRI, KVH and pollen producers on 3rd March 2011 and 25th August 2011.

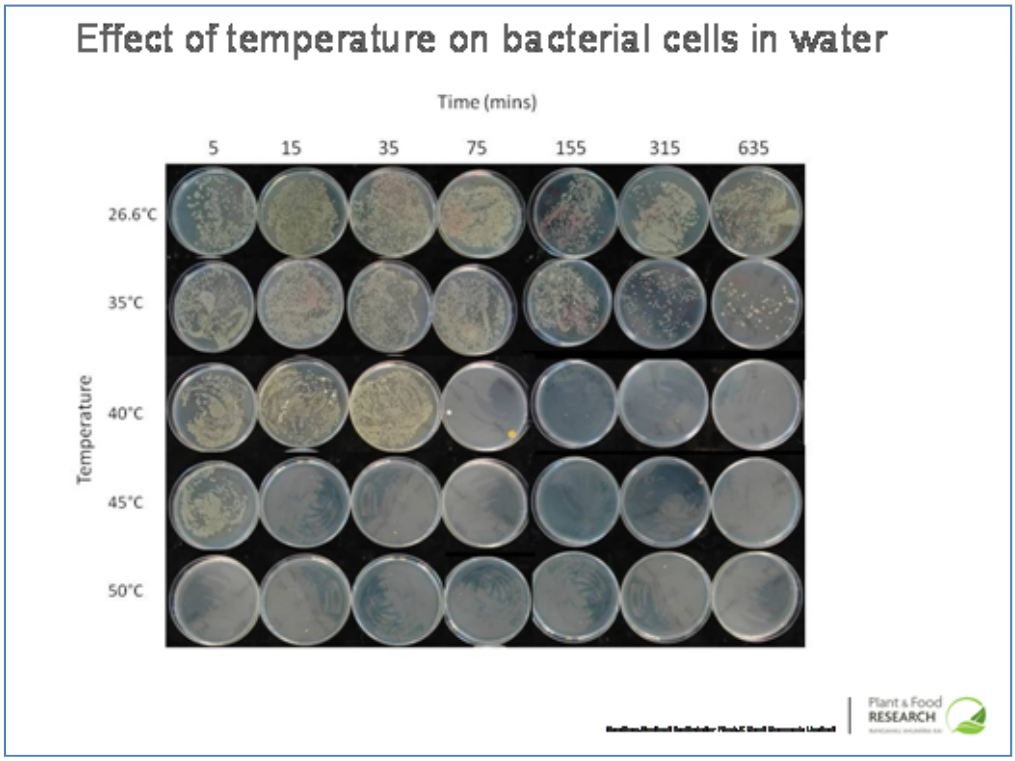
Slide 1

Slide 2. A summary of the treatments that had been tried up to this point and the conclusion that heat showed the most potential.

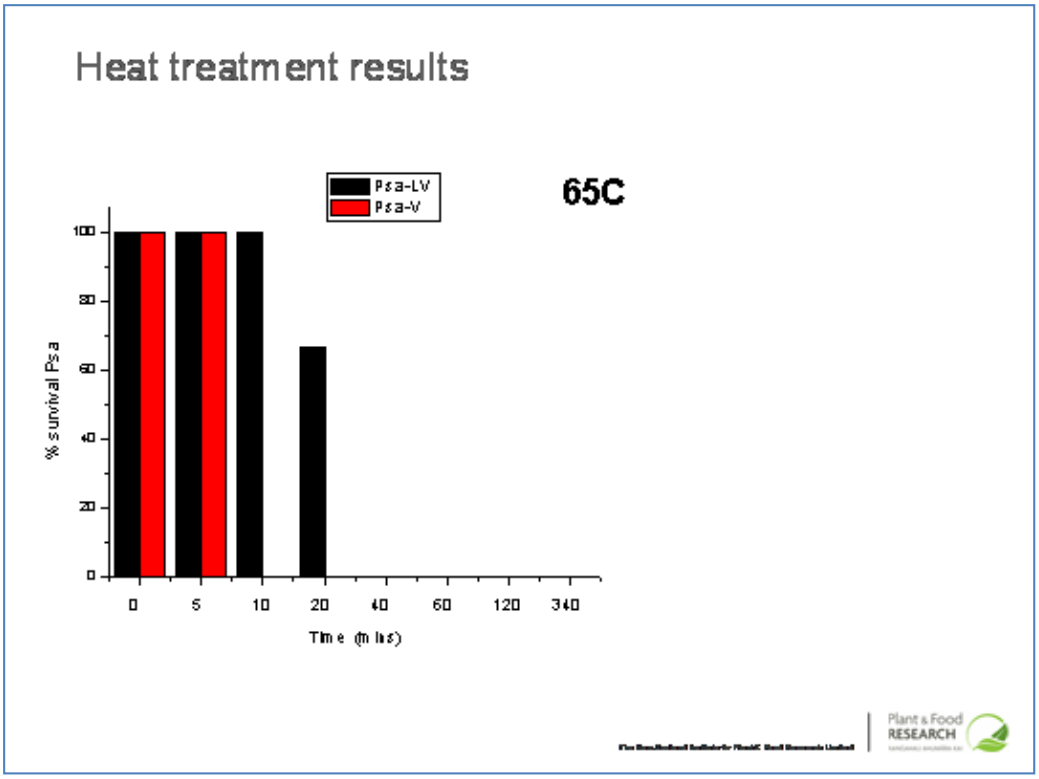
Summary

Ultraviolet C Light (UVC)	Unsuccessful	Extreme physical difficulty in ensuring all Psa exposed to UVC during treatment.
Freeze/thawing cycles	Unsuccessful	Completely ineffective.
Acetic acid	Unsuccessful	Bacteria still able to grow post-treatment.
Hexane	Unsuccessful	Bacterial levels not reduced.
Hypochlorite	Unsuccessful	Kills pollen- one test pollen survived- but never been able to repeat it.
Ethanol	Unsuccessful	Psa killed- but so is pollen- one test pollen survived, but never been able to repeat it
Hypochlorous acid	Unsuccessful	Killed pollen
Chlorine dioxide	Unsuccessful	Pollen survives- but Psa not killed
Combinations	Unsuccessful	As above
Heat	Good potential	Pollen survives, and Psa killed. Lots of margin for error. Pollination in Italy looks promising. Psa test needs repeating

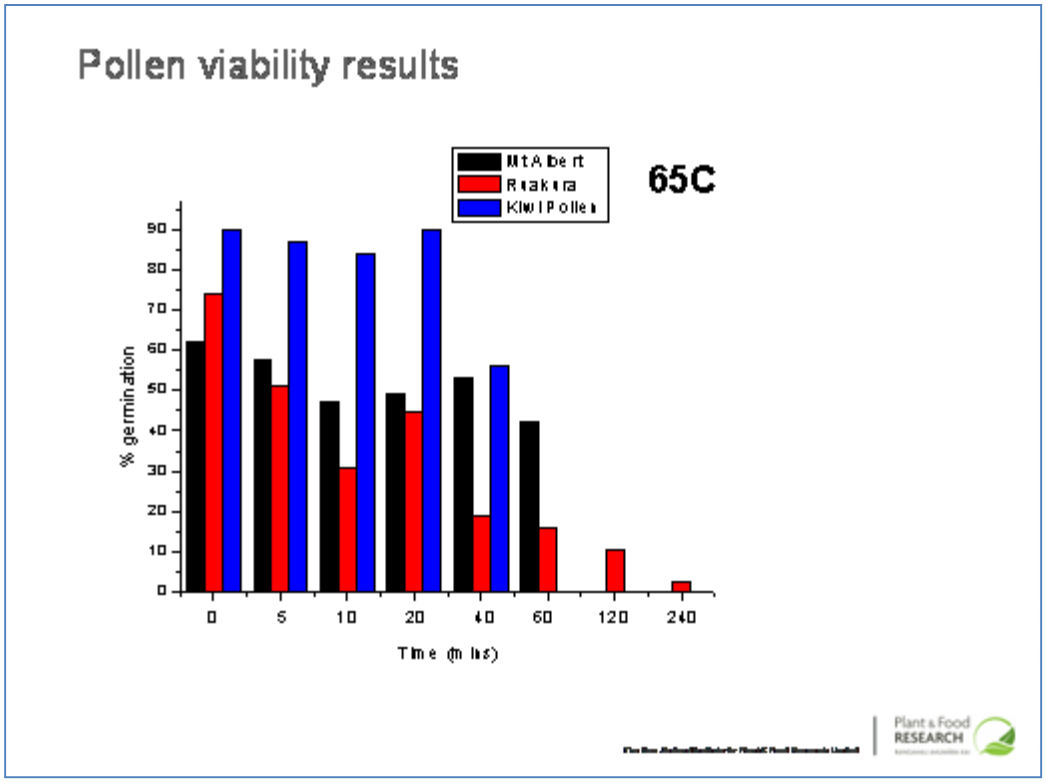
Slide 3. Results of heat treating *Pseudomonas syringae* pv. *actinidiae* cells in a suspension in sterile deionised water showed that short durations of temperatures of 45°C and above were lethal to Psa. Reports from industry that pollen would survive treatments of 65°C for an hour provided good potential for a wide “window” between pollen survival and killing Psa.



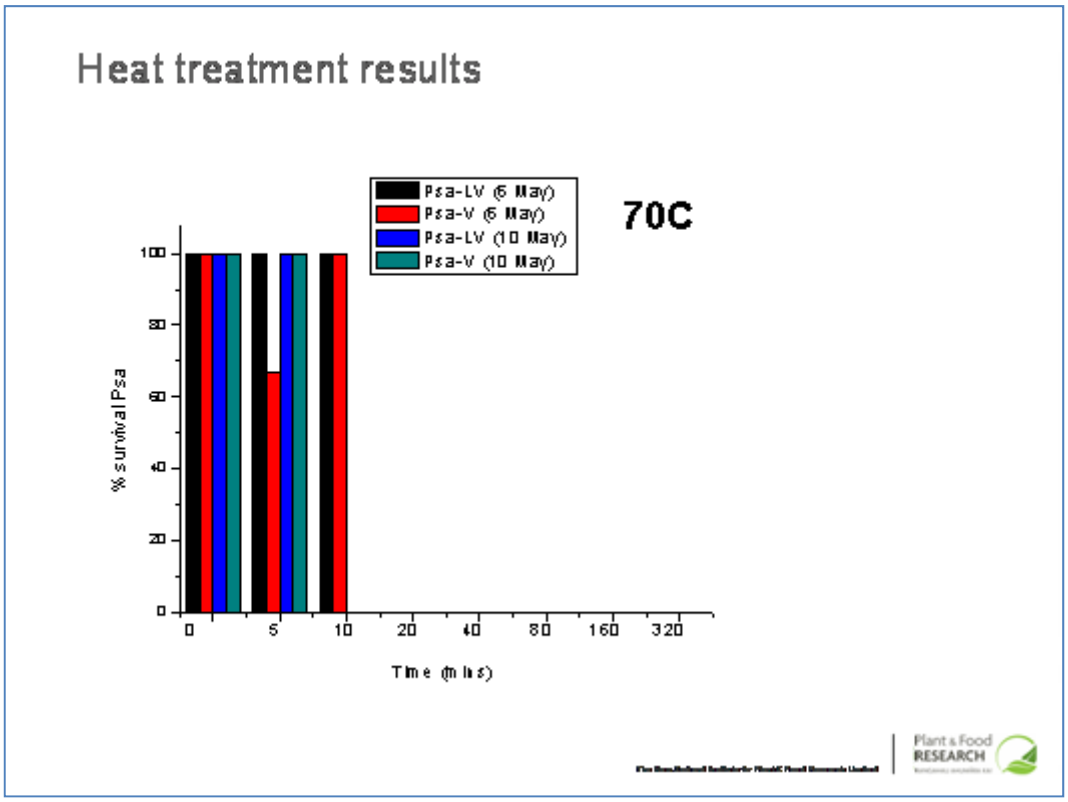
Slide 4. A result on bacterial survival of heat treating dried stored cleaned pollen that had been contaminated with Psa-V and Psa LV. This result could not be repeated.



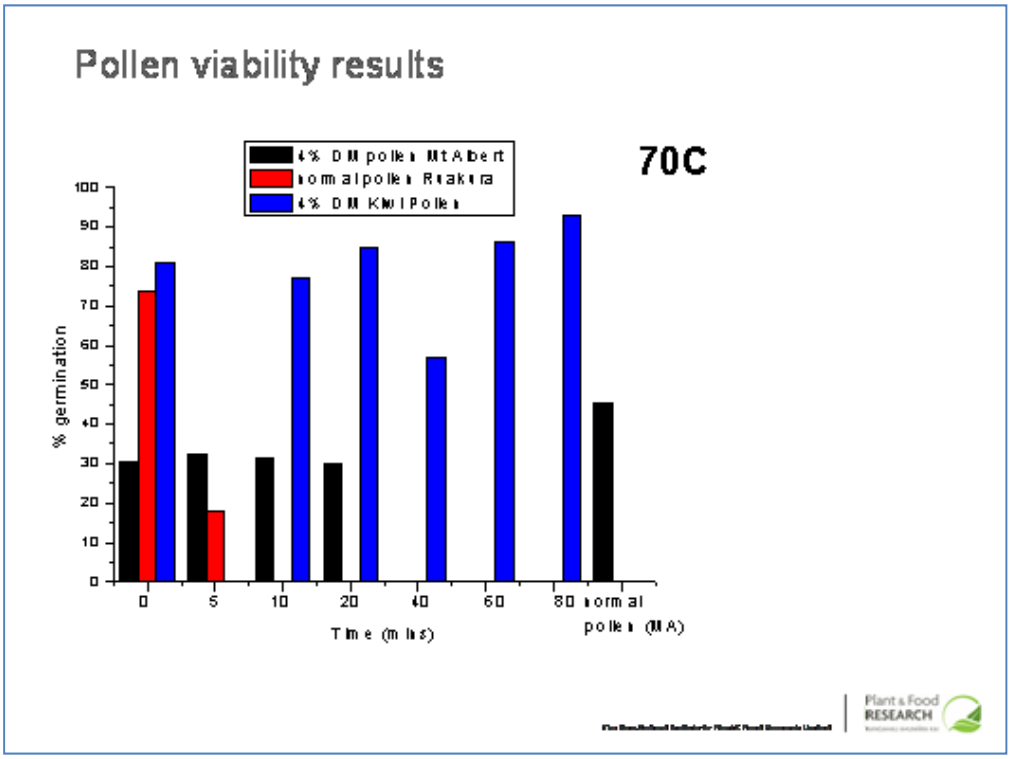
Slide 5. Results of the effect of 65°C on pollen viability from three different laboratories. Dried, stored pollen did survive this temperature for up to 60 minutes with reasonable germination. Pollen in the experiments at Ruakura was hydrated prior to heat treatments, and did not survive as well as dehydrated pollen used at Mt Albert and by KiwiPollen. Pollen germination for hydrated pollen was 48% following 20 minutes at 65C compared with 74% for untreated pollen. This treatment was lethal for Psa in the previous experiment but could not be repeated.



Slide 6. Results of heat treating pollen at 70°C on survival of Psa. This result was achieved twice.

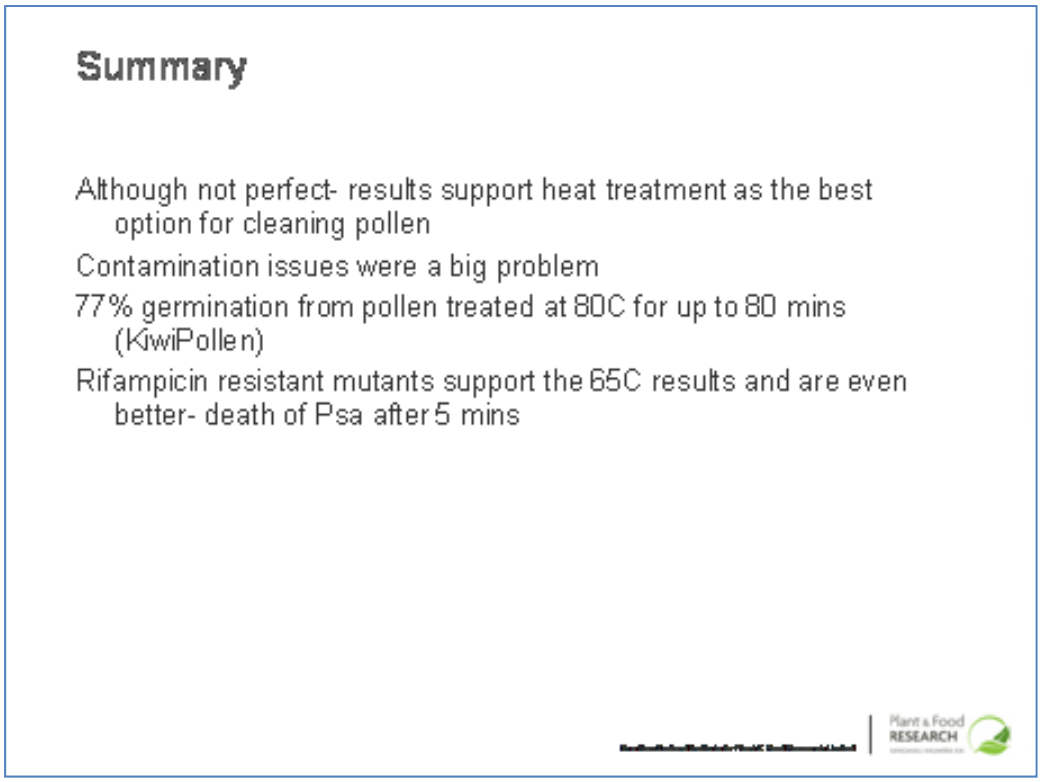


Slide 7. Pollen that had been dried to 4% moisture content (normal pollen is 8% moisture content) was able to survive 70°C for up to 80 minutes. The pollen used in the Ruakura experiments was hydrated prior to heat treatment. Under these conditions the pollen did not survive treatments necessary to kill Psa (70°C for 10 minutes).

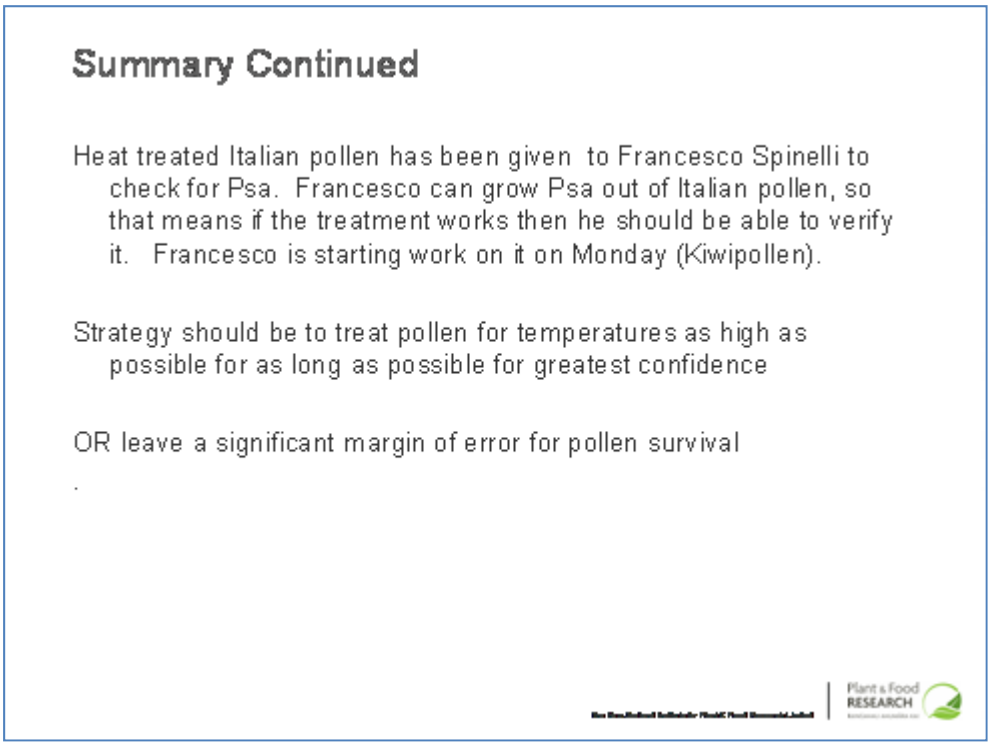


Slide 8. There were some results from Italy that suggested that heat-treated pollen was able to pollinate kiwifruit flowers successfully.

Slide 9



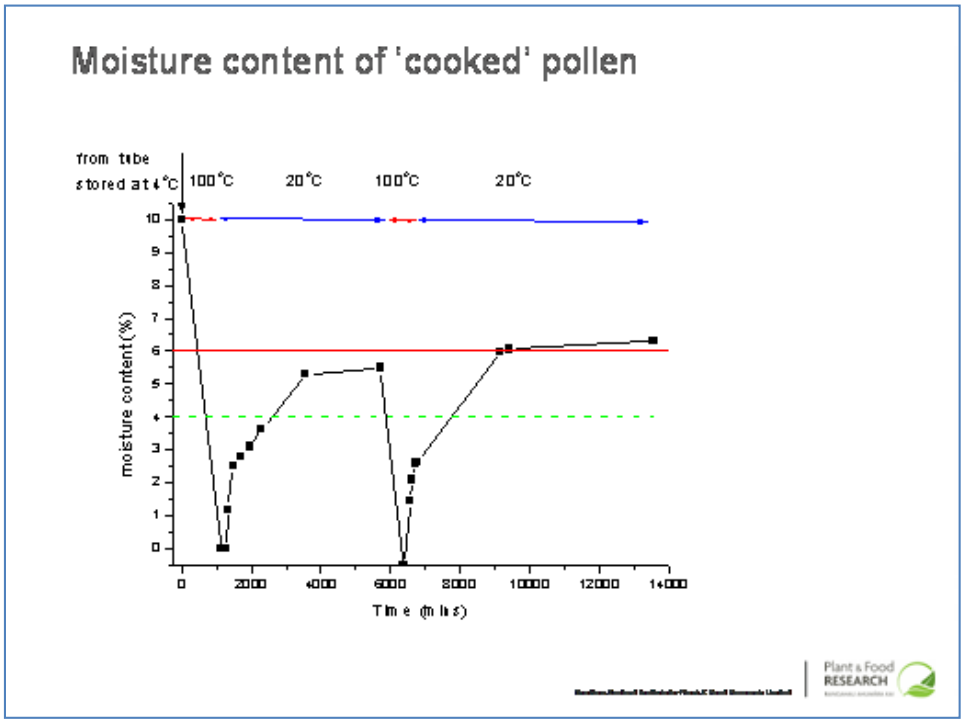
Slide 10



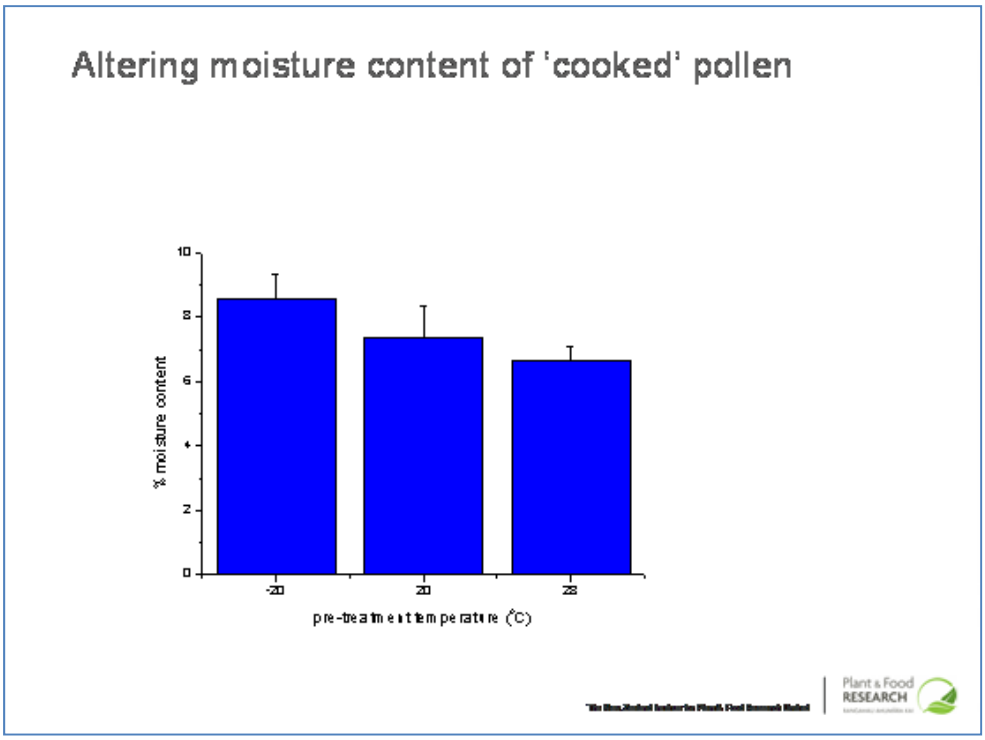
After this work was completed, a series of experiments to develop techniques for further exploring these parameters were conducted in order to undergo a matrix experiment using three different levels of humidity and temperature from 50 to 70°C.

Slide 1

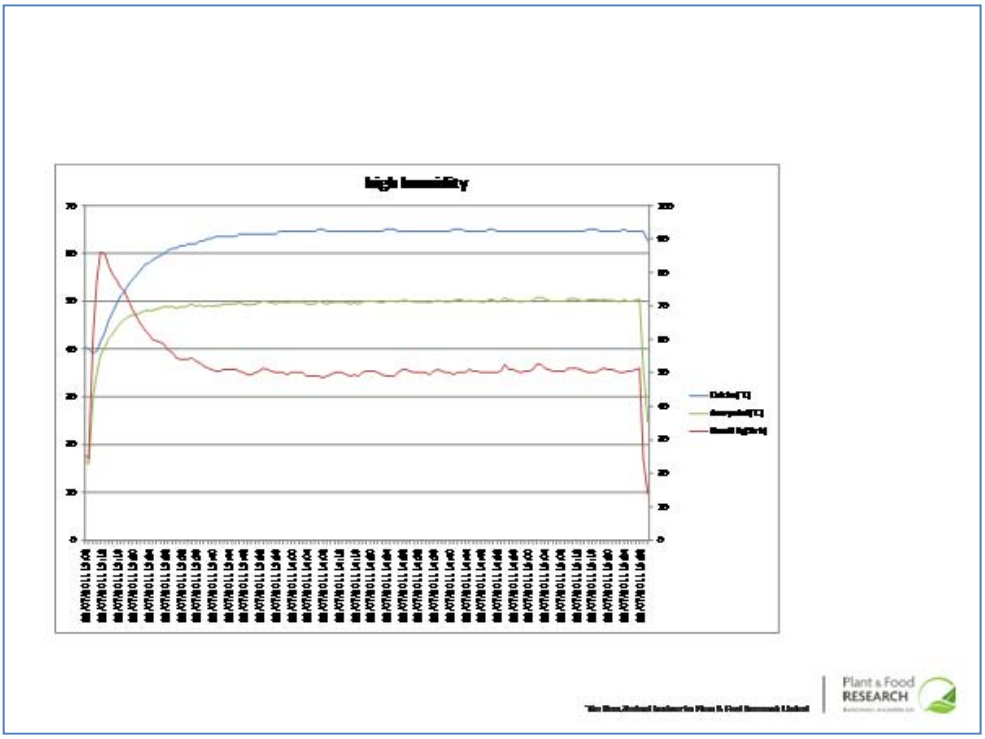
Slide 2 The moisture content of dried, stored pollen was determined, and after it had been twice heated to 100°C ("cooked") to remove bacterial contaminants. Before treatment the pollen had a moisture content of 10%, and after two exposures to 100°C the moisture content was about 6%.



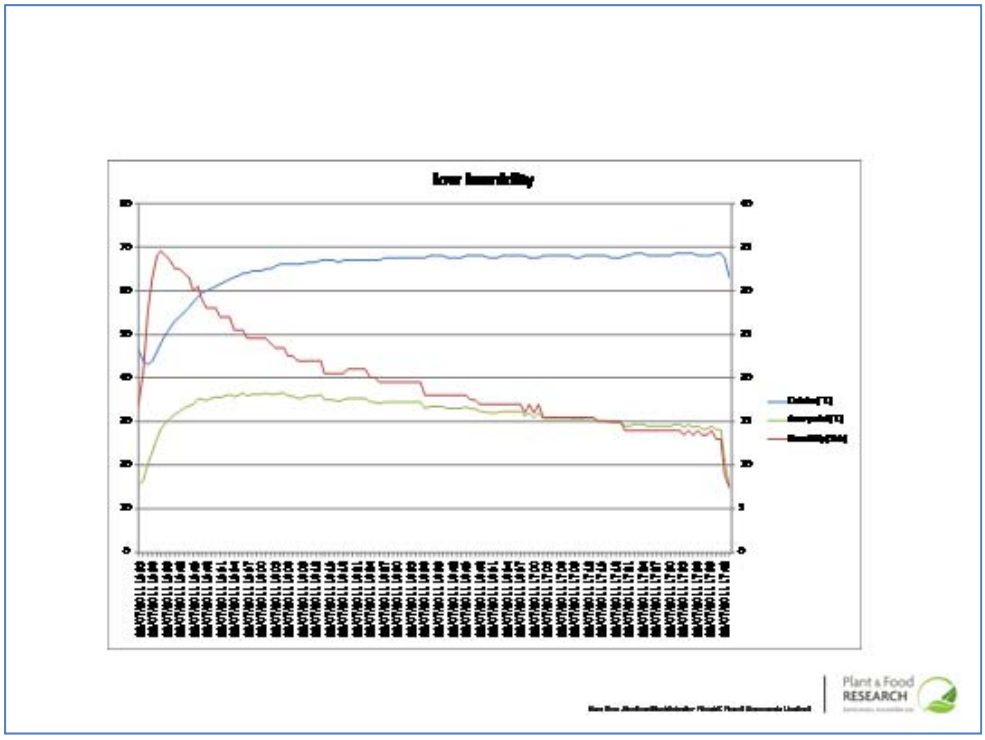
Slide 3 Pretreating cooked pollen by storing at three different temperatures (-20, 20 and 28°C) was able to alter the moisture content slightly.



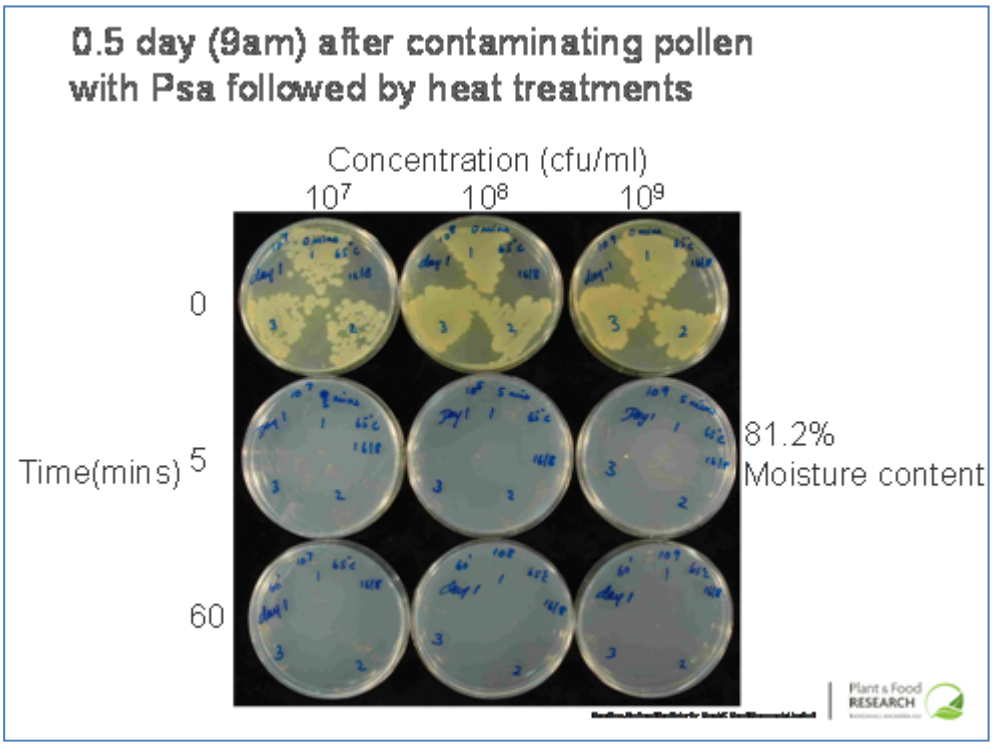
Slide 4 Humidity of 50% could be achieved by placing an open tray of water in an oven set at 65°C.



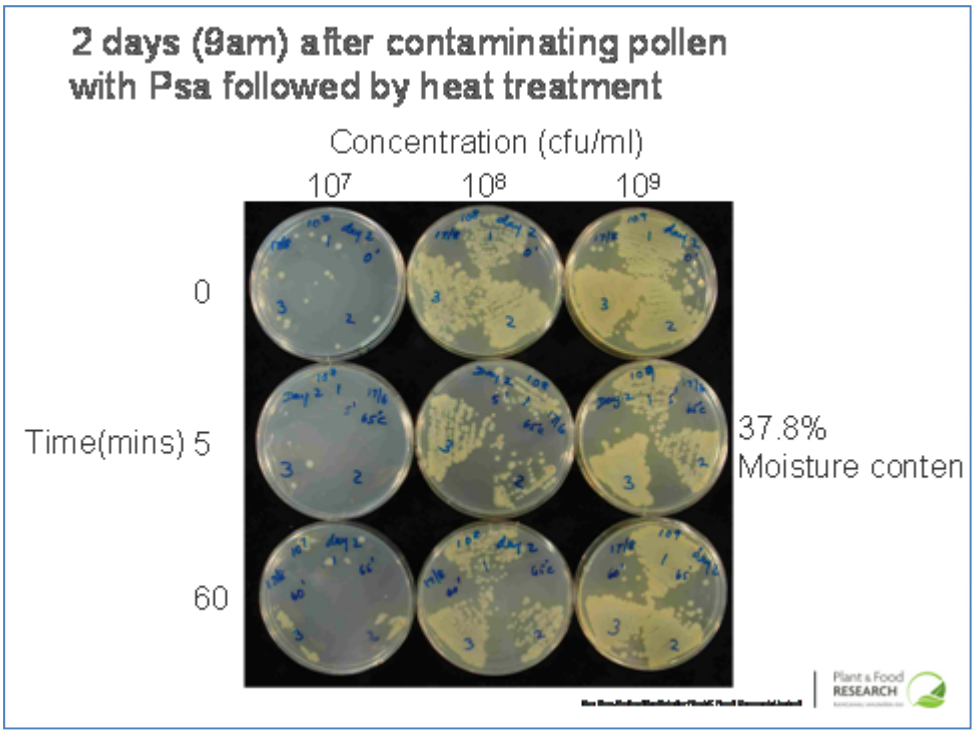
Slide 5 Without the tray of water the humidity was about 15%.



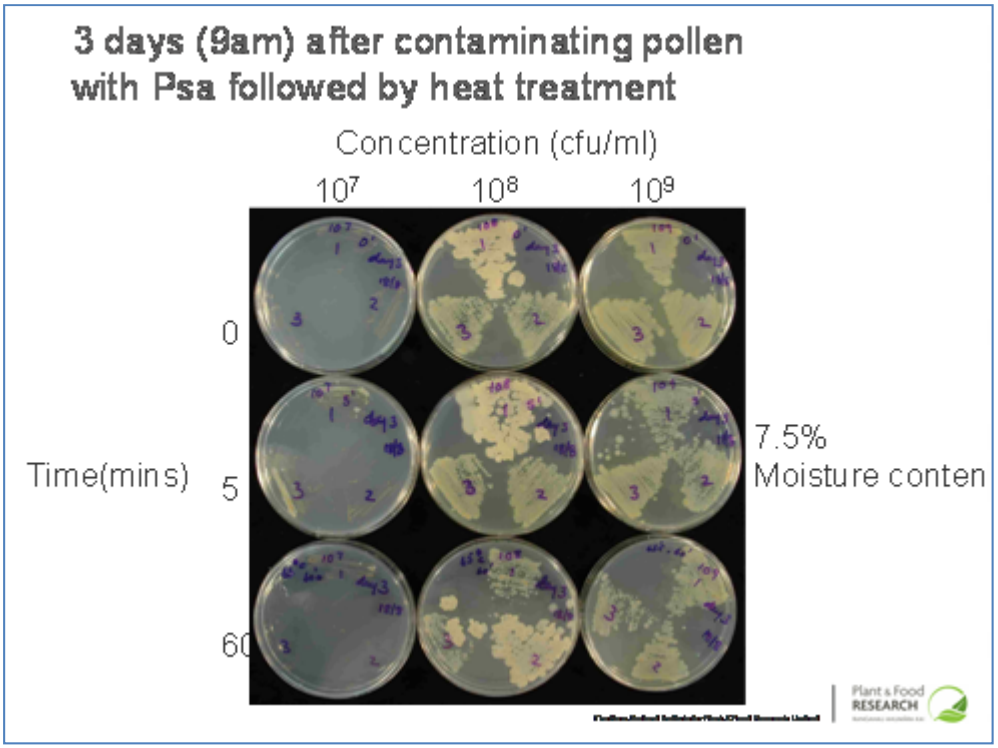
Slide 6 Psa at all concentrations (10^6, 10^7 and 10^8 cfu/ml) was killed after as little as 5 minutes at 65°C, when the moisture content was high (81.2%).



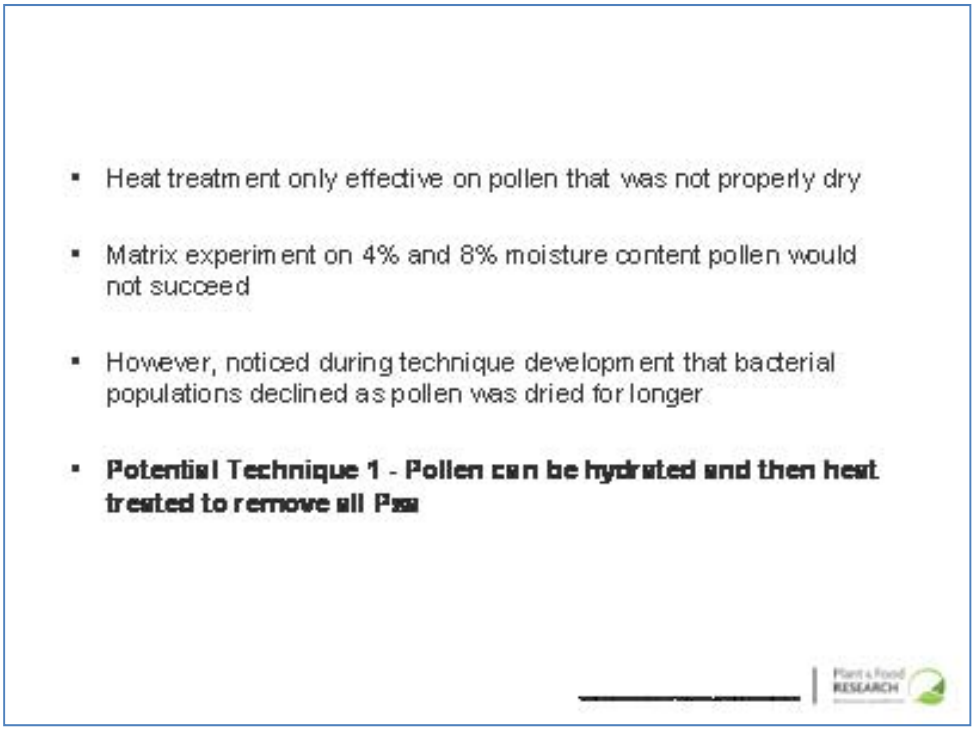
Slide 7 When the moisture content was lower (37.8%) following a further 2 days of drying at 28°C, Psa was not reduced by 65°C at any concentration, but the Psa at 10^7 cfu/ml had been reduced in numbers just simply by dessication because there was some reduction of Psa in the untreated control treatments.



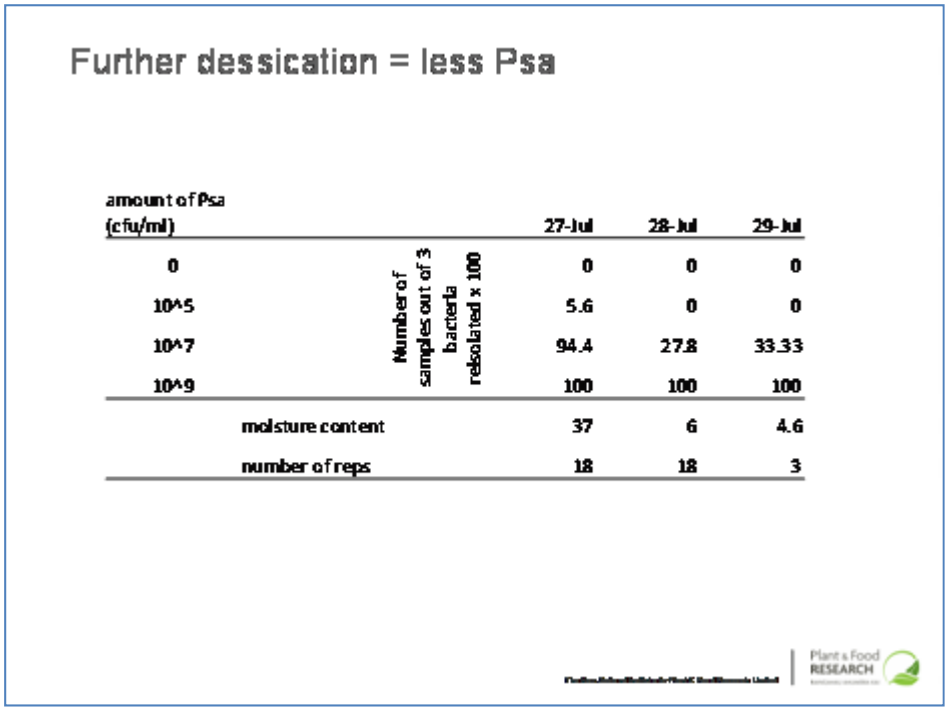
Slide 8 After a further day at 28°C, Psa at a concentration of 10^7 cfu/ml had almost all been killed.



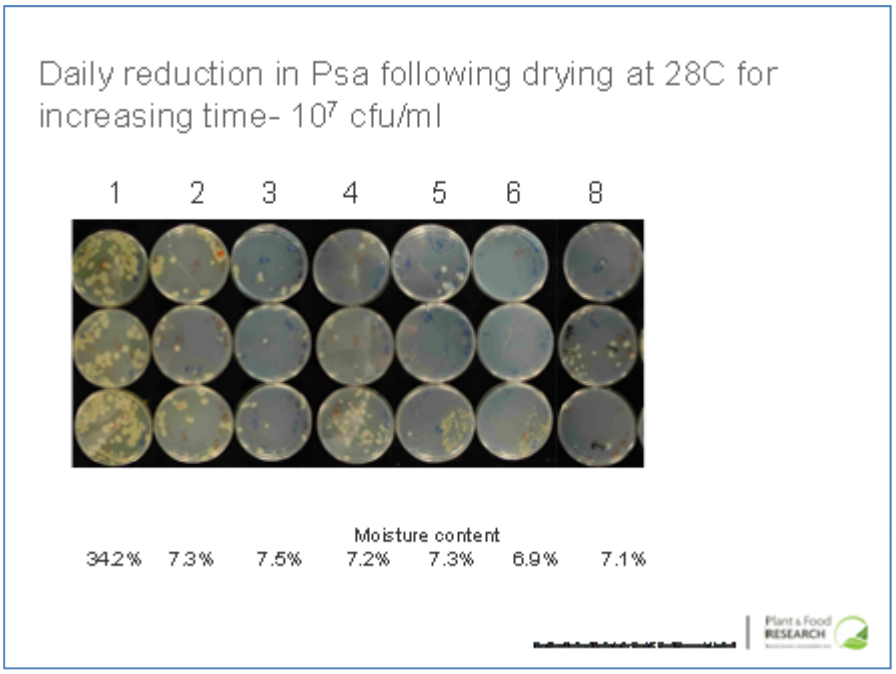
Slide 9. Results showed that if the pollen retained a moisture content of 81% after drying, then the Psa at all concentrations was killed after 5 mins at 65°C. However, once moisture content had decreased to 37.8% Psa survived at all concentrations.



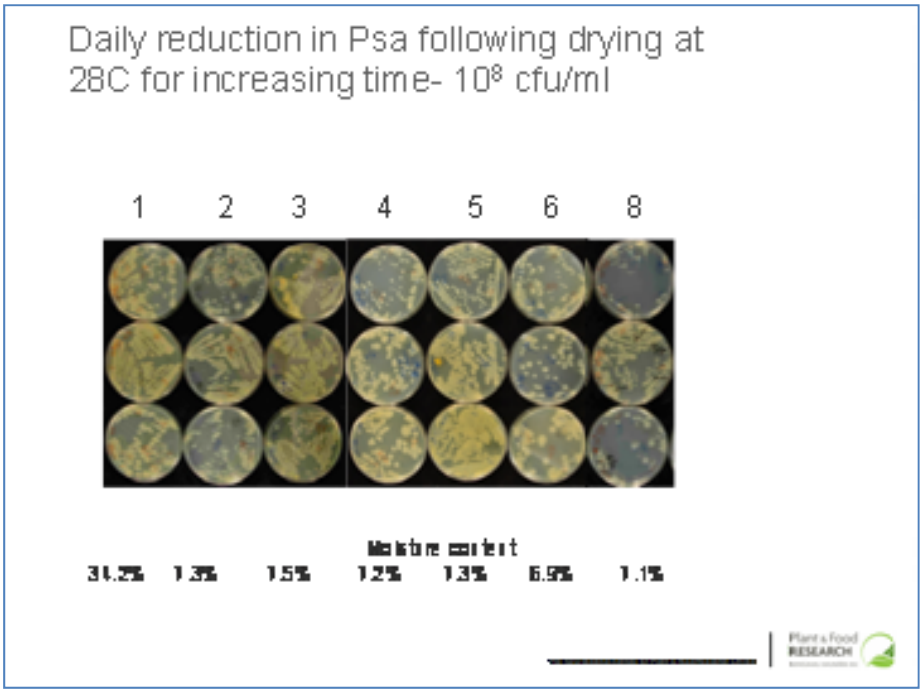
Slide 10 The reduction of Psa at lower concentration by extended drying was repeated, results were similar but total kill was achieved only when bacteria were applied at a concentration of 10^5 cfu/ml.



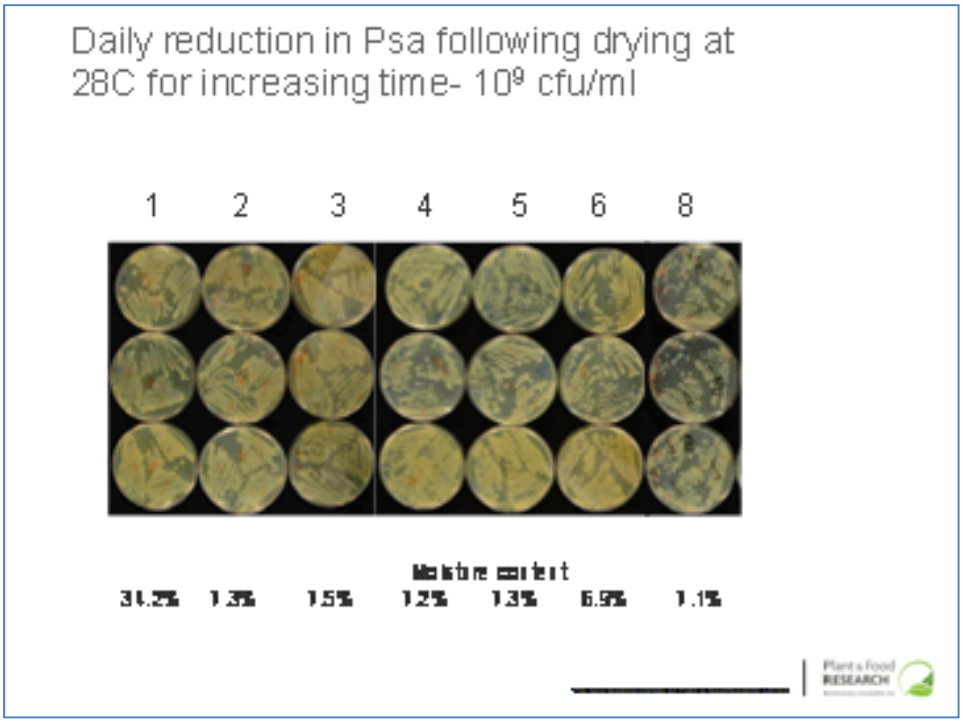
Slide 11 A further repeat using 10^7, 10^8 and 10^9 cfu/ml (next three slides) once again showed a reduction in colony density when the drying time was extended to 8 days. However, total kill was not achieved. There was also some reduction in colony density after 8 days for bacteria at a concentration of 10^8 cfu/ml.



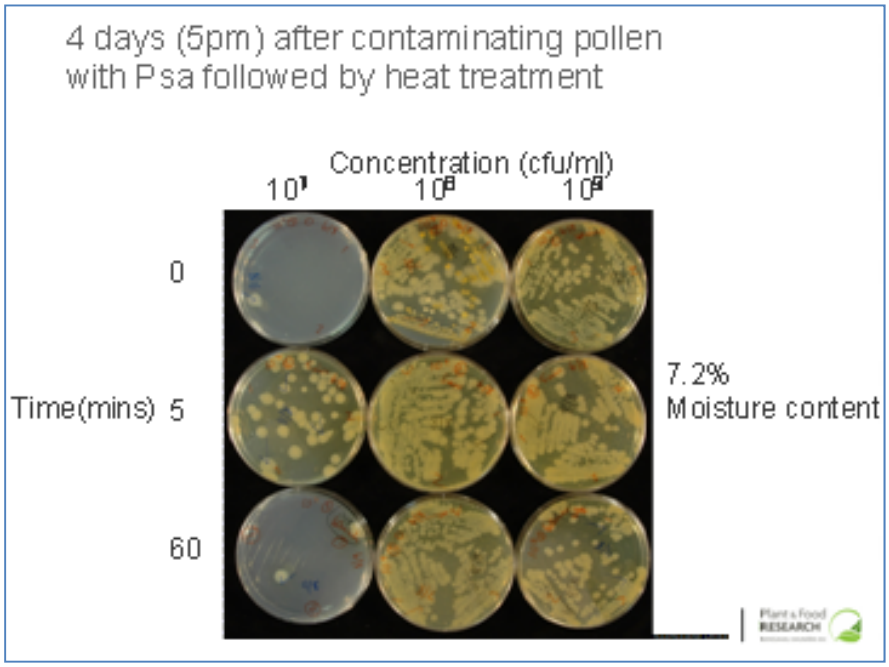
Slide 12



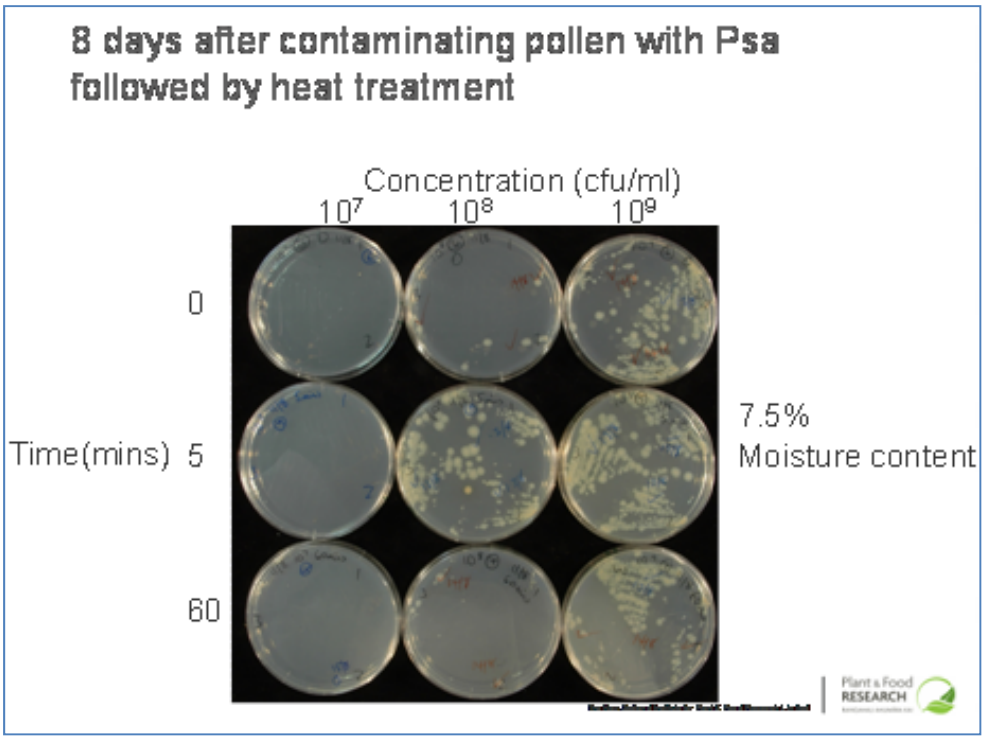
Slide 13



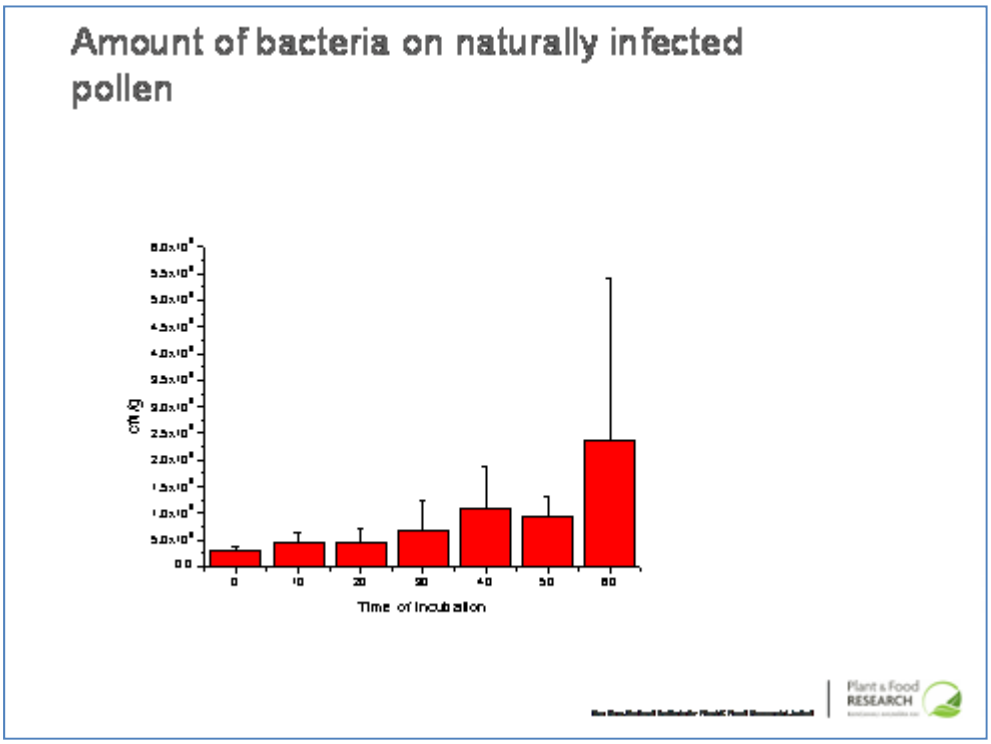
Slide 14 Heat treatment at 65°C for 5 and 60 minutes after 4 days' drying resulted in no further reduction in Psa colony number.



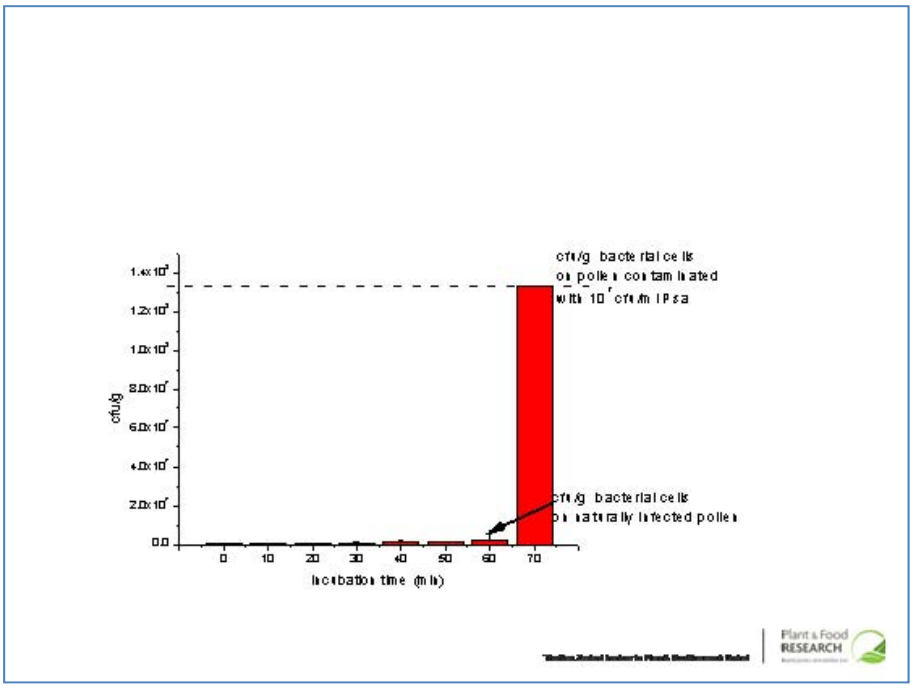
Slide 15 Heat treatment at 65°C for 5 and 60 minutes appeared to result in no further reduction in Psa numbers or colony density.



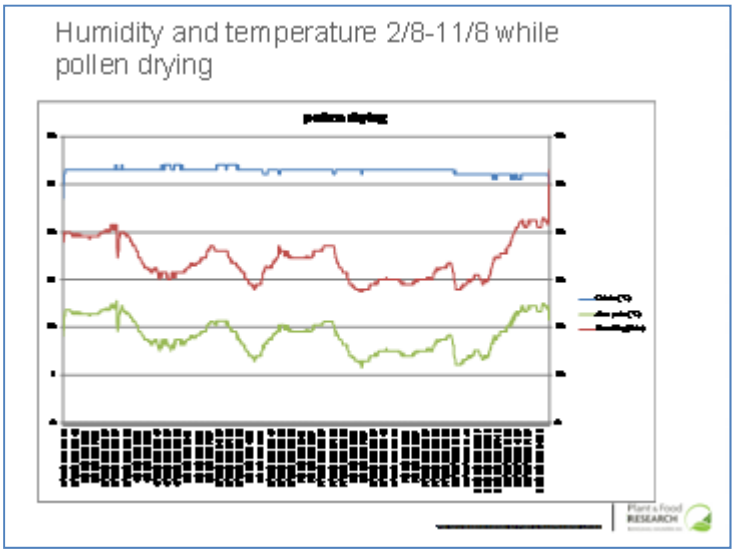
Slide 16 The amount of bacteria on naturally infected pollen was calculated. The bacteria had started to multiply after 60 minutes, but before that time 1 g of pollen was contaminated with about 10^6 cfu of bacteria.



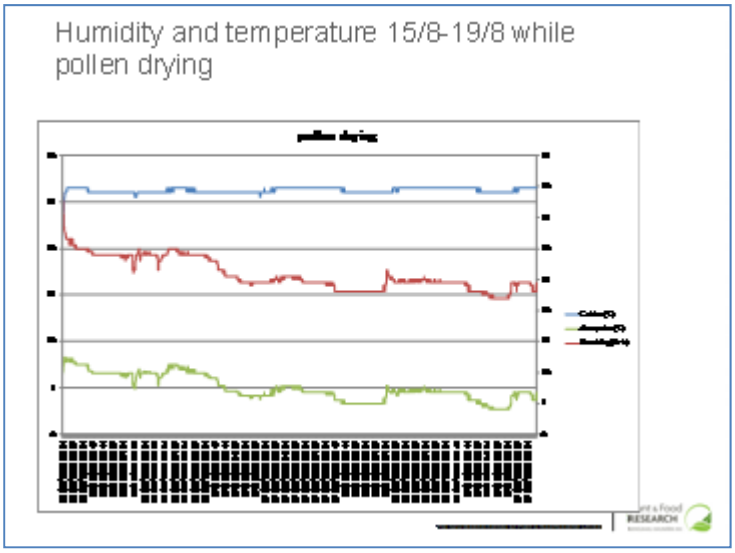
Slide 17 The amount of bacteria on pollen that had been contaminated by us was calculated and compared with the amount of bacteria on naturally contaminated pollen. Artificially contaminated pollen had 100 times as many bacteria as naturally contaminated pollen. However, further sampling would be required to confirm this finding.



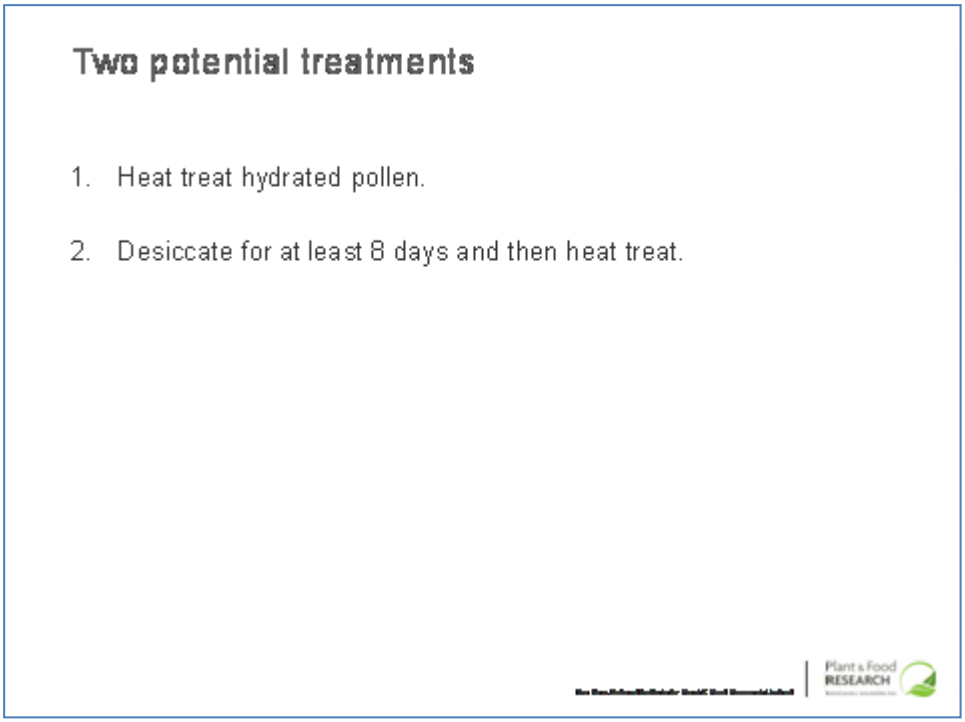
Slide 18



Slide 19



Slide 20



During the course of this investigation the reason for the non-repeatability of the 65°C result was discovered. If the cleaned pollen that was contaminated with Psa had a moisture content of 81% before heat treatment then the heat treatments were effective, but were not as effective when the pollen had a moisture content of 37.8% or lower. Another interesting result from this work was that extended drying also reduced bacterial numbers/colony density.

Slide 21

Work underway

- Repeat and refine the heat treatment of hydrated pollen.
- Repeat and refine the desiccation followed by heat treatment.

Slide 22

Part 3: Heat treating fresh pollen

At this point in the research project, the Pollen Technology Communication Team (PTCT) was formed. In consultation and discussion with the PTCT it was decided to treat fresh pollen because of encouraging reports from Italy that pollen in green anthers could survive 65°C for 60 minutes. Because of the high moisture content of fresh pollen (c. 80%) this showed promise for being able to kill Psa whilst allowing the pollen to survive.

1.5 Methods

To start with various incubators were used, some with a fan and, when required, a trough of water was inserted into the incubator to increase humidity.

For these experiments, 5-10 freshly harvested anthers were added to scintillation vials or glass Petri plates. An aliquot of 10 µl of water as a control and 10 µl suspensions of various concentrations of a rifampicin resistant strain of Psa was added to vials or Petri plates containing anthers. Open or closed receptacles containing anthers were then placed in the incubators, or in some experiments in a water bath, and treated with the described combinations of humidity and temperature for various times. After treatment, anthers from Petri plates were removed into scintillation vials and 500 µl of pollen germination medium was placed in each vial. After an hour of gentle shaking at room temperature (c. 20°C), 100 µl was removed and 10 µl of this placed on Kings' medium B amended with 50 ug/ml rifampicin in Petri plates, and dilutions of 1:10, 1:100, 1:1000 and 1:10000 v/v with sterile dionised water. After at least 48 hours' incubation at 28°C, colonies were counted and results expressed as cfu/ml. After 2 hours' gentle shaking, pollen germination was observed with the aid of the compound microscope, photographed and scored as plus or minus.

At the beginning of the season *A. chinensis* pollen was used, and then, when it became available, *A. deliciosa* pollen was used.

A series of experiments of combinations of humidity and temperature were initially carried out. The pollen was of variable viability, and for this reason only some results are presented.

1.6 Results

A series of experiments using an incubator with a fan and using an incubator with a container of water to increase humidity showed that the higher the humidity, the more effective heat was at killing Psa (Figures 7-12). However, pollen germination was compromised by increasing time at high temperatures (Figure10). Extended times (up to 1200 minutes) at 35°C was able to kill low concentrations of Psa (Figure 13), but when the temperature was reduced to 28°C the Psa survived (Figure 14).

An improved technique where humidity was able to be more accurately controlled was used for the next set of experiments. At this stage *Actinidia deliciosa* pollen became available, which,

although still of variable quality, was of higher viability than the *A. chinensis* pollen used in the previous experiments.

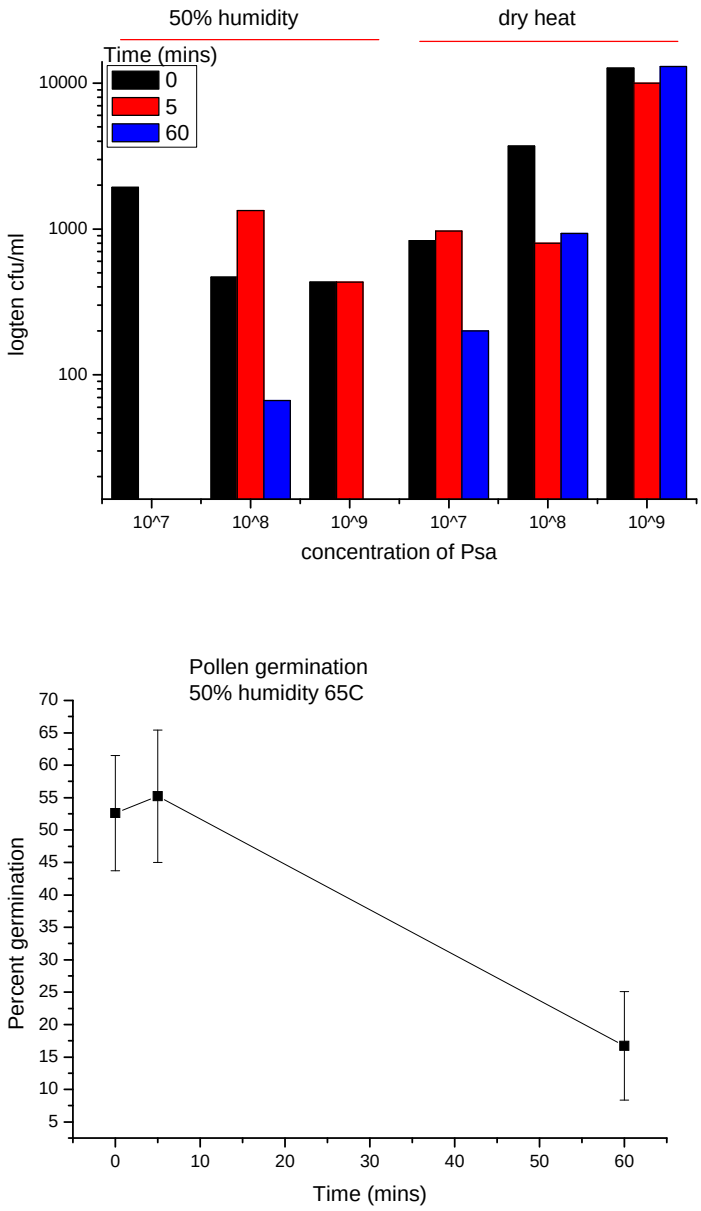


Figure 7: The effect of placing anthers contaminated with three concentrations of *Pseudomonas syringae* pv. *actinidiae* (Psa) in an incubator set at 65**°C** with (50% humidity) and without (dry heat) a container of water on survival of pollen and Psa. This incubator did not have a fan.

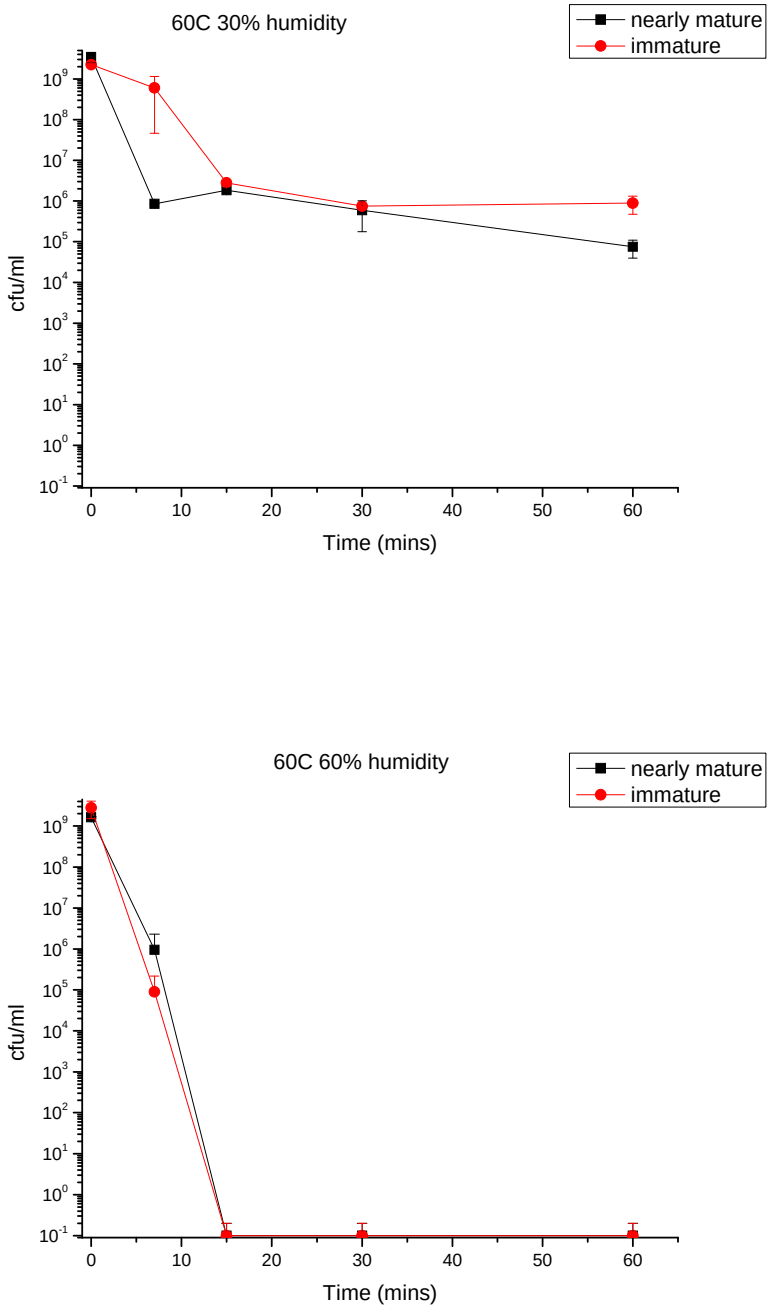


Figure 8: The effect of placing nearly mature and immature anthers contaminated with 1x 10^9 cfu/ml *Pseudomonas syringae* pv. *actinidiae* (Psa) in an incubator with a fan set at 60°**C** with (60% humidity) and without (30% humidity) a container of water on survival of Psa.

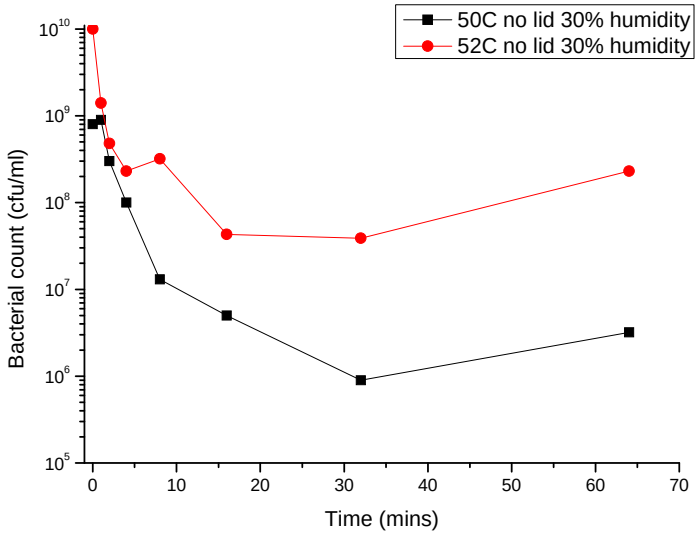


Figure 9: The effect of placing anthers contaminated with 10^9 and 10^{10} cfu/ml of *Pseudomonas syringae* pv. *actinidiae* (Psa) in an incubator with a fan set at 50°C and 52°C on survival of Psa.

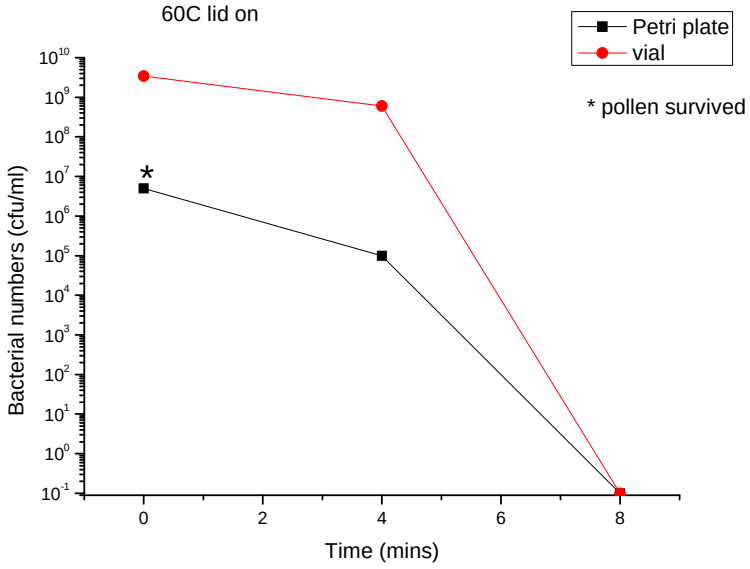


Figure 10: The effect of placing anthers contaminated with 10^7 and 10^{10} cfu/ml of *Pseudomonas syringae* pv. *actinidiae* (Psa) in an incubator set at 60°C with the lid on the scintillation vial (100% humidity) on survival of pollen and Psa.

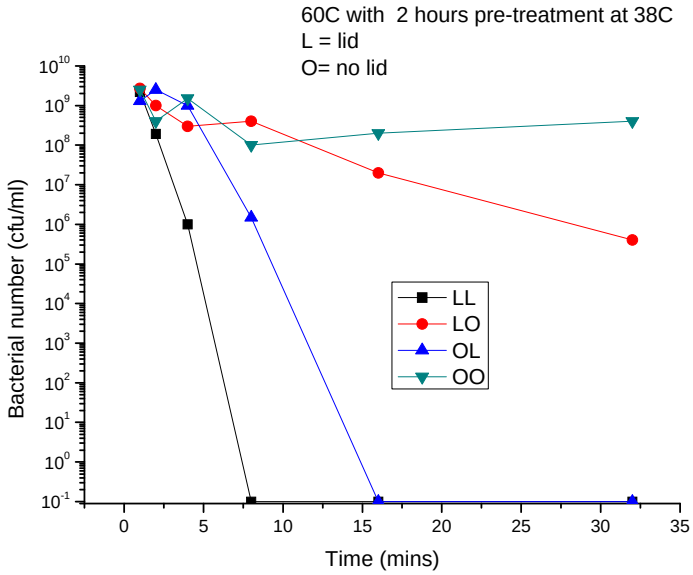


Figure 11: The effect of placing anthers contaminated with 2 x 10^9 cfu/ml *Pseudomonas syringae* pv. *actinidiae* (Psa) in an incubator with a fan set at 38**°C** for 2 hours followed by increasing time at 60**°C** in the same incubator.

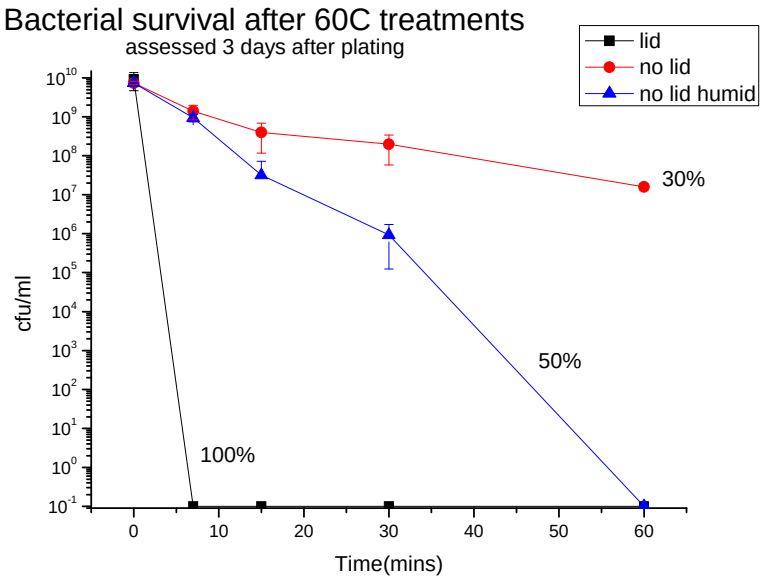
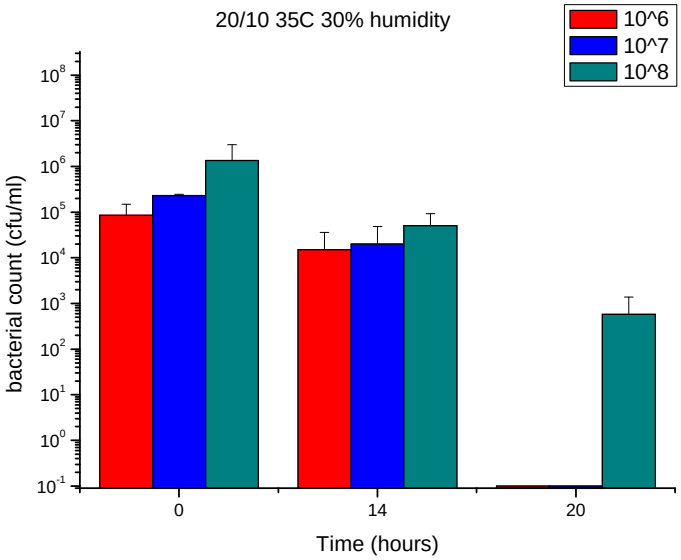
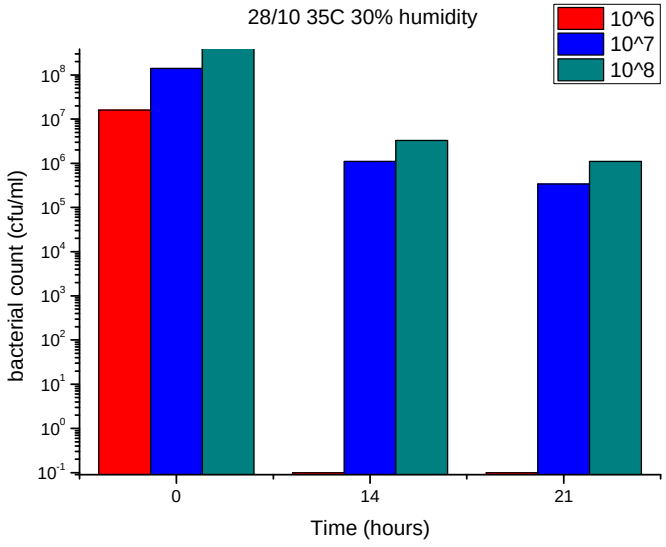


Figure 12: The effect of placing anthers contaminated with 10^{10} cfu/ml *Pseudomonas syringae* pv. *actinidiae* (Psa) in a scintillation vial with a lid (100% humidity) and without (c. 30% humidity) a lid in an incubator with a fan set at 60**°C** and in an incubator without a fan and with a container of water (50% humidity) on survival of Psa.


20/10 35C 30% humidity
10^6
10^7
10^8
bacterial count (cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
0
14
20
Time (hours)


28/10 35C 30% humidity
10^6
10^7
10^8
bacterial count (cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
0
14
21
Time (hours)

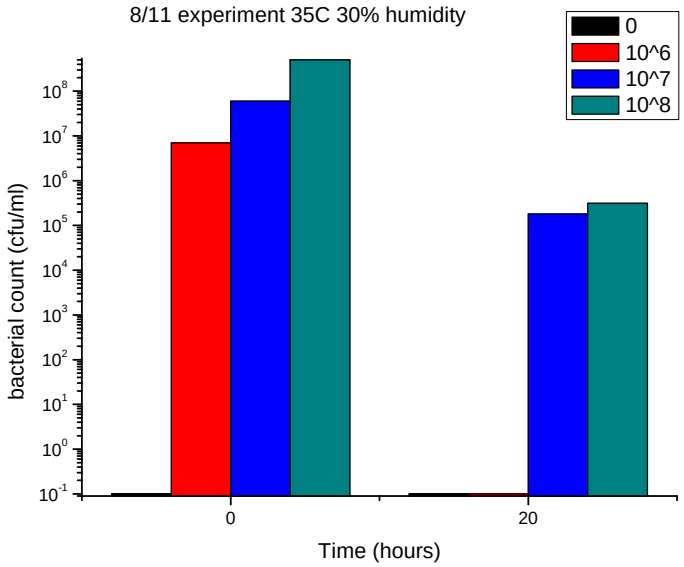


Figure 13: The effect of placing anthers contaminated with 10^6, 10^7 and 10^8 cfu/ml *Pseudomonas syringae* pv. *actinidiae* (Psa) in a scintillation vial without (c. 30% humidity) a lid in an incubator with a fan set at 35**°C** on survival of Psa.

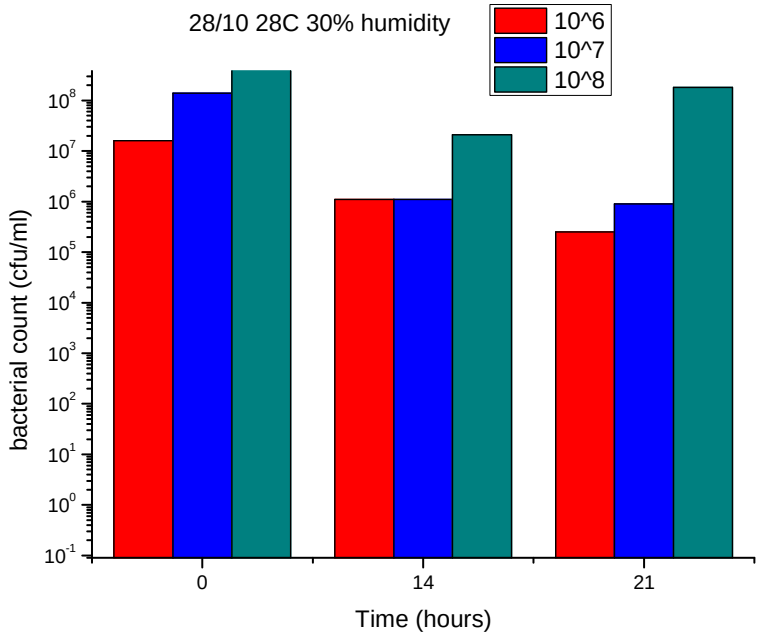


Figure 14: The effect of placing anthers contaminated with 10^6, 10^7 and 10^8 cfu/ml *Pseudomonas syringae* pv. *actinidiae* (Psa) in a scintillation vial without (c. 30% humidity) a lid in an incubator with a fan set at 35**°C** on survival of Psa.

Part 4: Surefruit experiments

In consultation with the Pollen Technology Communication Team a series of experiments were conducted in a modified matrix design using the Surefruit machine developed by Rod MacDonald of Plant & Food Research in order to more closely control humidity and airflow. Temperature, humidity and airflow can be controlled when using this machine. The protocol that was used generated four replicates for pollen viability, but only one for Psa survival. Some experiments were repeated several times to increase replication for Psa survival, but due to time constraints (fresh pollen was only available for a limited time period) some promising treatments were not repeated. The pollen was of variable quality, which made determination of pollen viability difficult at times. The experiments that were conducted showed that treating anthers was a better method than treating buds, and that when the airspeed was reduced to 0.5m/sec, comparable results were obtained using either medium. The results that are reported are those for anthers.

1.7 Methods

For these experiments, 5-10 freshly harvested anthers were added to scintillation vials. 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 a rifampicin-resistant strain of Psa as described previously was added to each of four vials containing anthers. Open vials containing anthers were then placed on a shelf in the Surefruit machine with the described combinations of humidity, airflow and temperature for various times. After treatment, 500 µl of pollen germination medium was placed in each vial. After an hour, 100 µl 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 dionised water. After at least 48 hours' incubation at 28C, colonies were counted and results expressed as cfu/ml. After 2 hours, 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.

It was decided by the Pollen Technology Communication Team (PTCT) that replication was not necessary due to the data being ultimately used to model the responses.

Table 10: Experiments conducted using the Surefruit machine on freshly harvested pollen. The logfiles for these runs are appended.

Date	Temp (ºC)	Humidity (%)			airspeed m/s	buds	anthers
9/11	60	50	70	90	1	+	+
11/11	50	50	70	90	4	+	+
11/11	55	50	70	90	4	+	+
11/11	60	50	70	90	4	+	+

14/11	50	50	70	90	1	+	+
18/11	45	50	70	90	1	+	+
22/11	45	50			1	+	+
22/11	60	50			0.5	+	+
24/11	45	50			1	+	+
29/11	45	50			1	+	+
30/11	35	50			0.5		+
1/12	35	50			0.5		+
2/12	60		30		0.5		+
6/12	60		20		0.5		+
7/12	35		40		0.5		+
8/12	50	50	28		0.5		+
8/12	53	50	25		0.5		+
8/12	55	50	24		0.5		+
9/12	35		60		0.5		+
12/12	35	50			0.5		+
12/12	40	50			0.5		+

Table 11: Combinations of temperature and humidity used to test pollen viability in the Surefruit machine at an airspeed of 0.5m/sec

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

1.8 Results

The first set of experiments using the Surefruit machine showed that pollen was more susceptible to heat damage at high humidities and high airflow (Fig.15-17). If both were reduced, then pollen was able to survive for 12 minutes at 60°C (Figure 17) compared with 1 minute at the same temperature with high humidity and high airspeed (Figure 15). When anthers contaminated with three concentrations of Psa were treated, the pollen was killed at exactly the same time and temperature that killed Psa (Figures 18, 22, 23, 24, 25, 26, 28, 30 and 31) except for one temperature-humidity combination. At 55°C and 50% humidity, the pollen survived for longer than Psa at a low concentration (10^6 and 10^7 cfu/ml) (Figure 27). For confirmation of this result the experiment would need to be repeated. At 35°C pollen survived and Psa was killed at a low concentration (10^5-10^6 cfu/ml) when pollen was treated for 1200 minutes (Fig. 32).

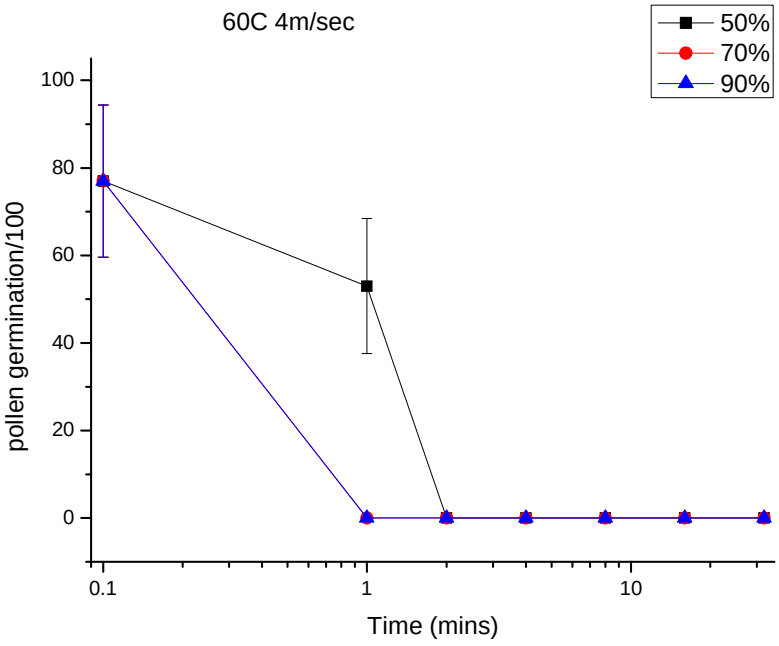


Figure 15: The effect of 4m/sec airspeed and three levels of humidity (50%, 70% and 90%) on kiwifruit pollen viability at 60°C.

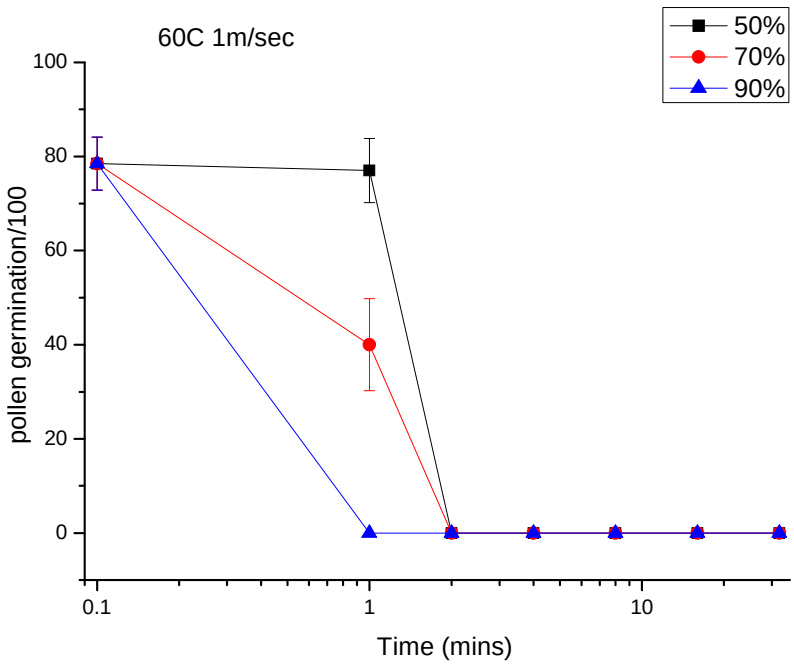


Figure 16: The effect of 1m/sec airspeed and three levels of humidity (50%, 70% and 90%) on kiwifruit pollen viability at 60°C.

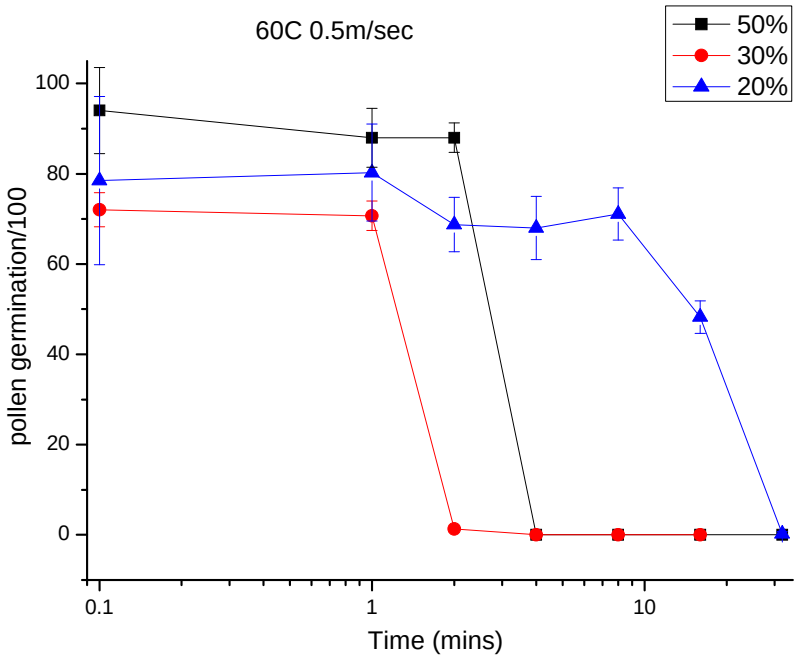


Figure 17: The effect of 0.5m/sec airspeed and three levels of humidity (50%, 30% and 20%) on kiwifruit pollen viability at 60°C.

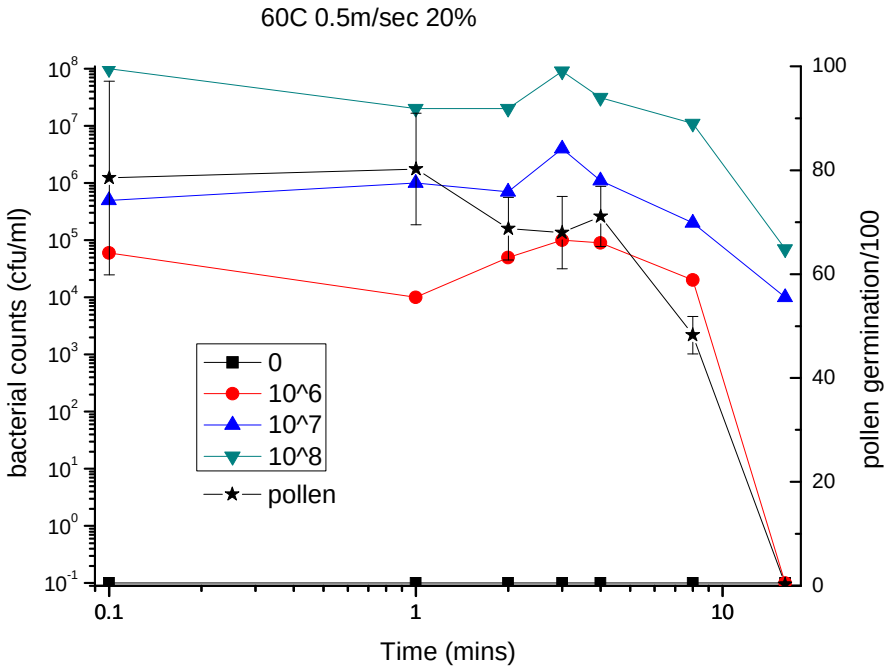


Figure 18: The effect of 0.5m/sec airspeed and 20% humidity on kiwifruit pollen viability at 60°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

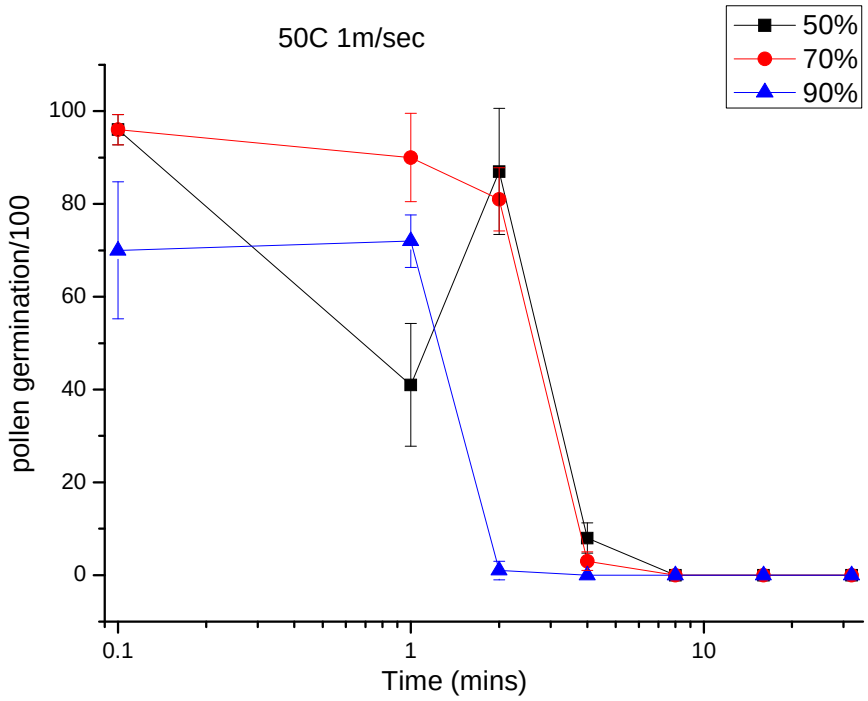


Figure 19: The effect of 1m/sec airspeed and three levels of humidity (50%, 70% and 90%) on kiwifruit pollen viability at 50°C.

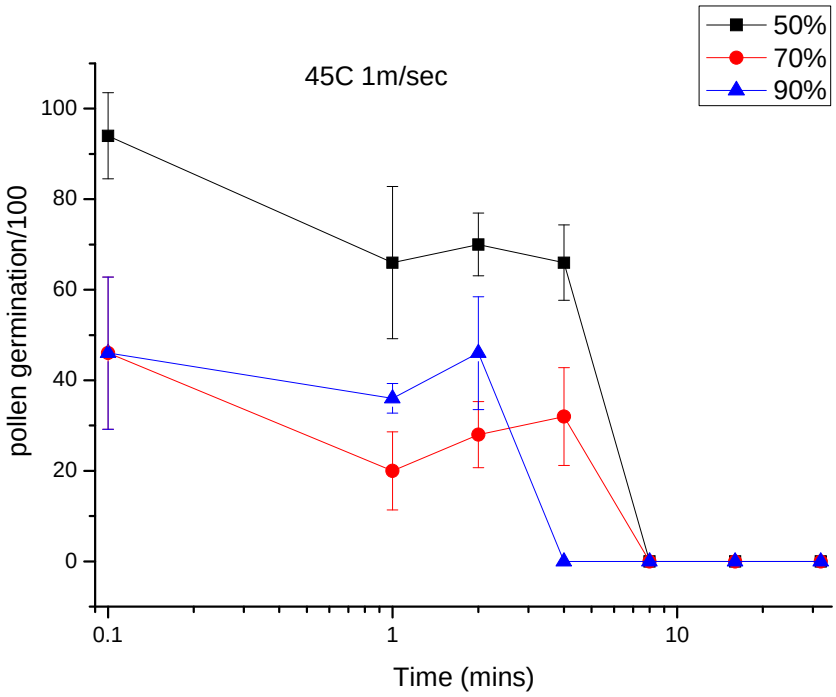


Figure 20: The effect of 1m/sec airspeed and three levels of humidity (50%, 70% and 90%) on kiwifruit pollen viability at 45°C.

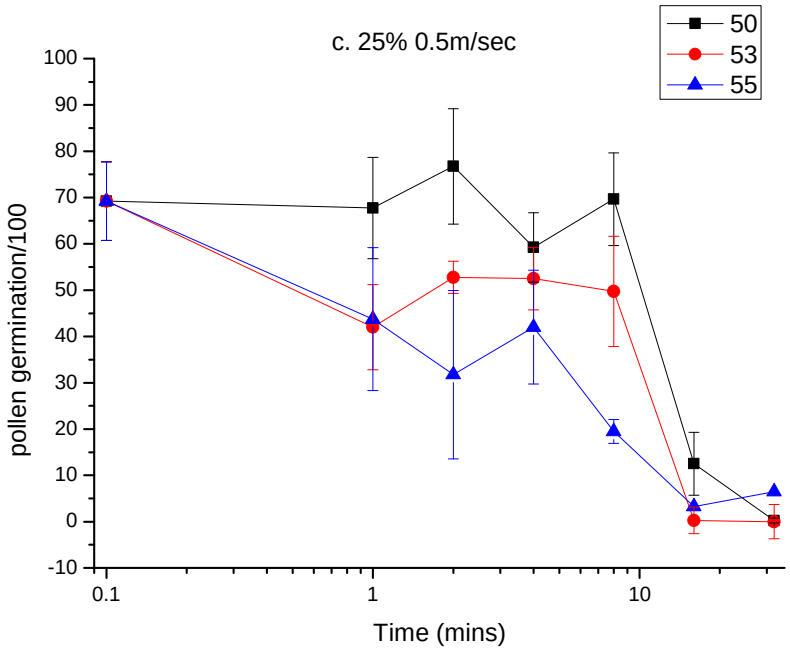


Figure 21: The effect of 0.5m/sec airspeed and c. 25% humidity on kiwifruit pollen viability at 50°C, 53°C and 55°C.

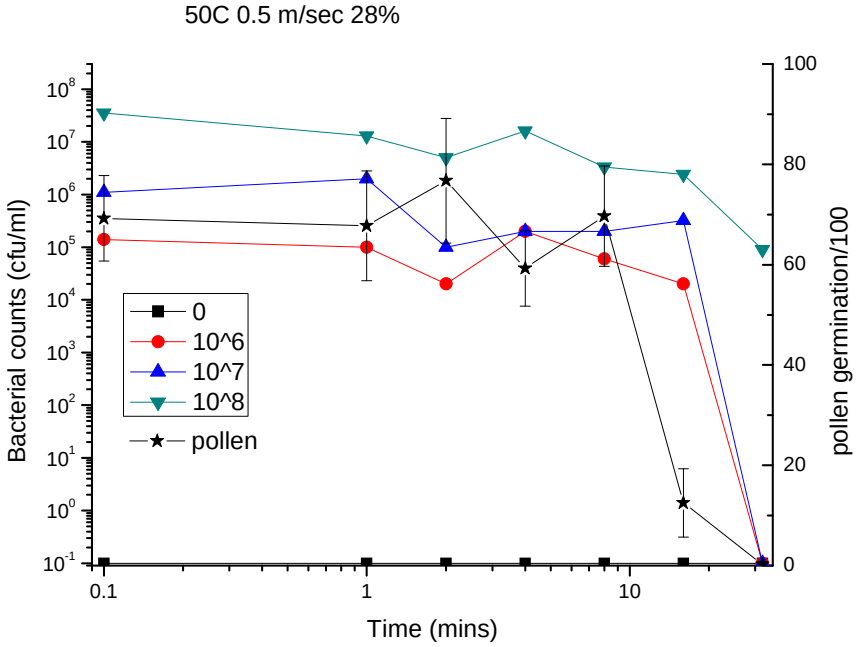


Figure 22: The effect of 0.5m/sec airspeed and 28% humidity on kiwifruit pollen viability at 50°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

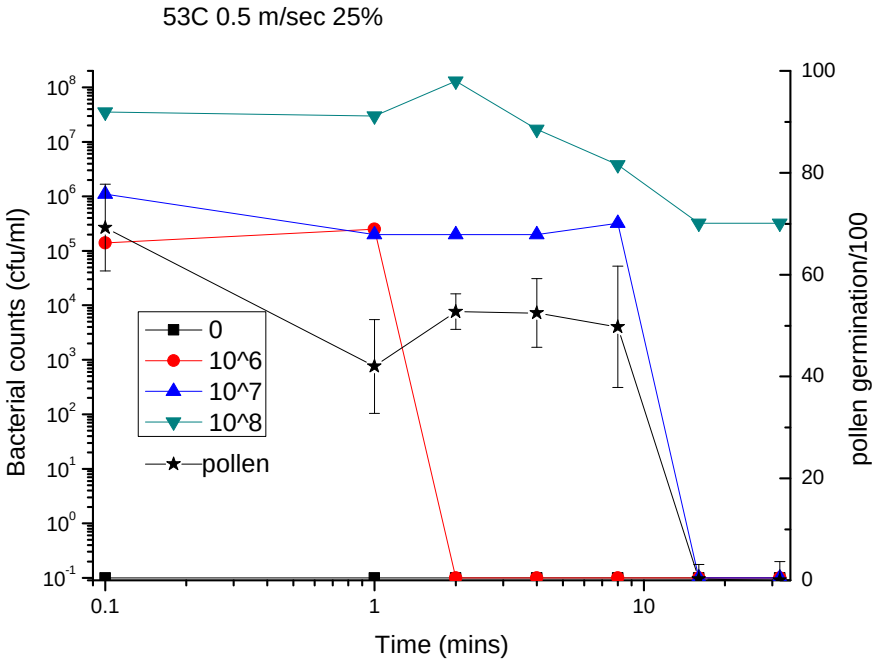


Figure 23: The effect of 0.5m/sec airspeed and 25% humidity on kiwifruit pollen viability at 53°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

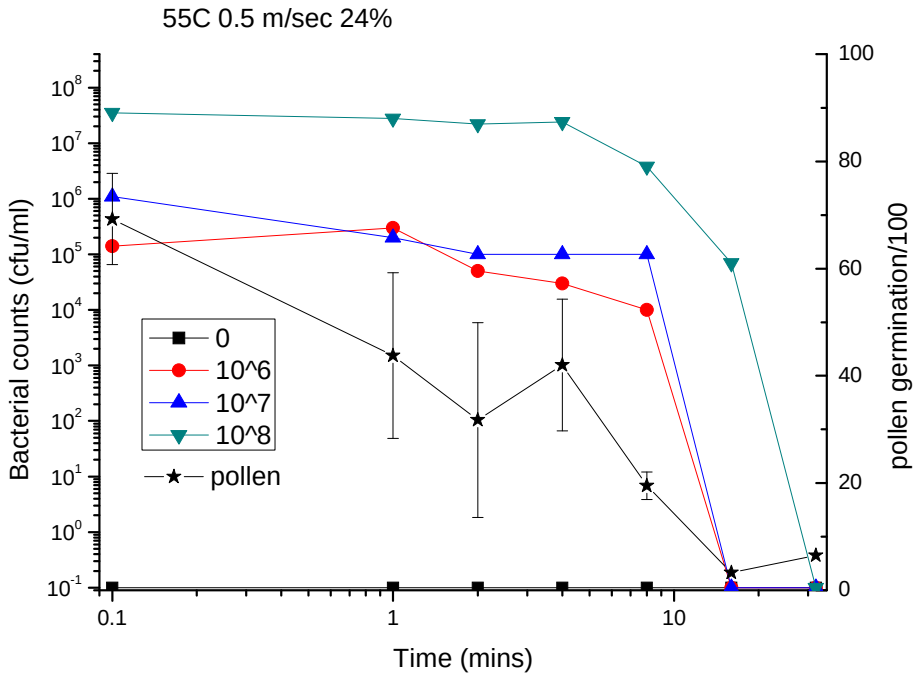


Figure 24: The effect of 0.5m/sec airspeed and 24% humidity on kiwifruit pollen viability at 55°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

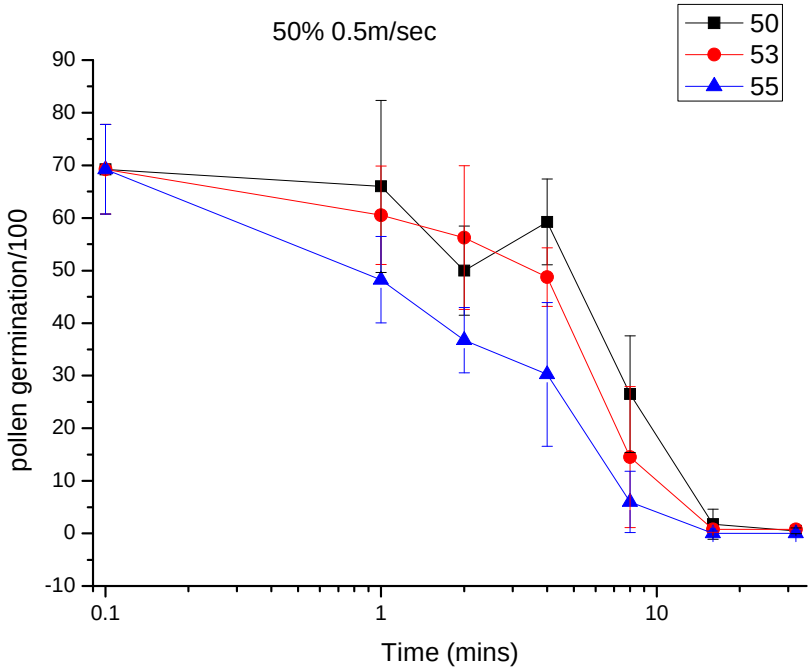


Figure 25: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 50°C , 53°C and 55°C.

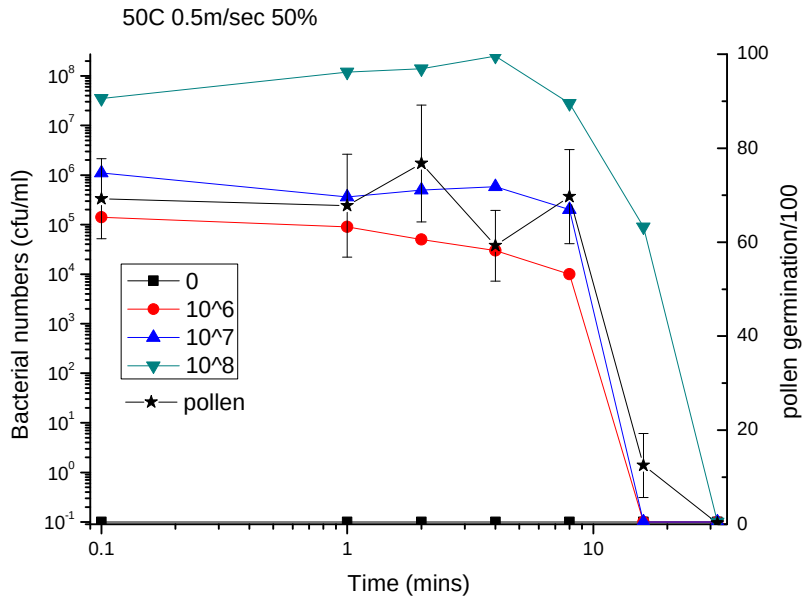


Figure 26: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 50°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

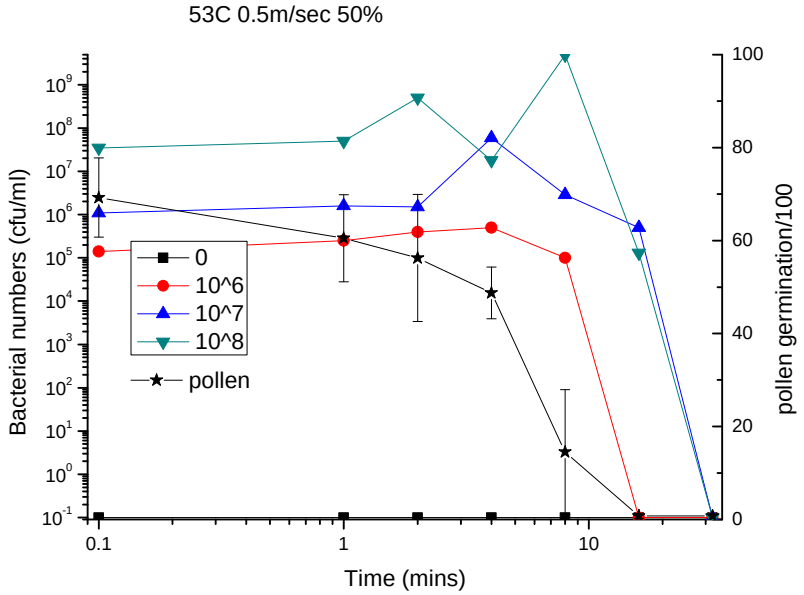


Figure 27: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 53°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

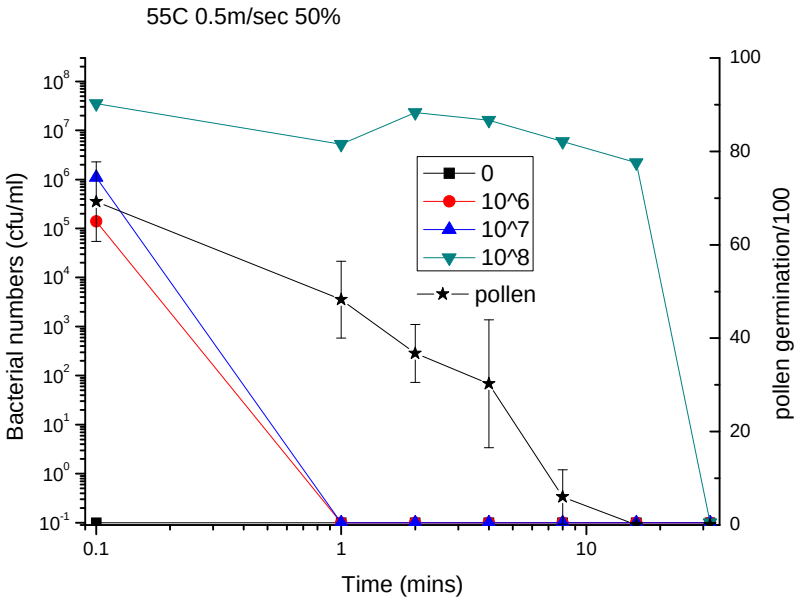


Figure 28: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 55°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

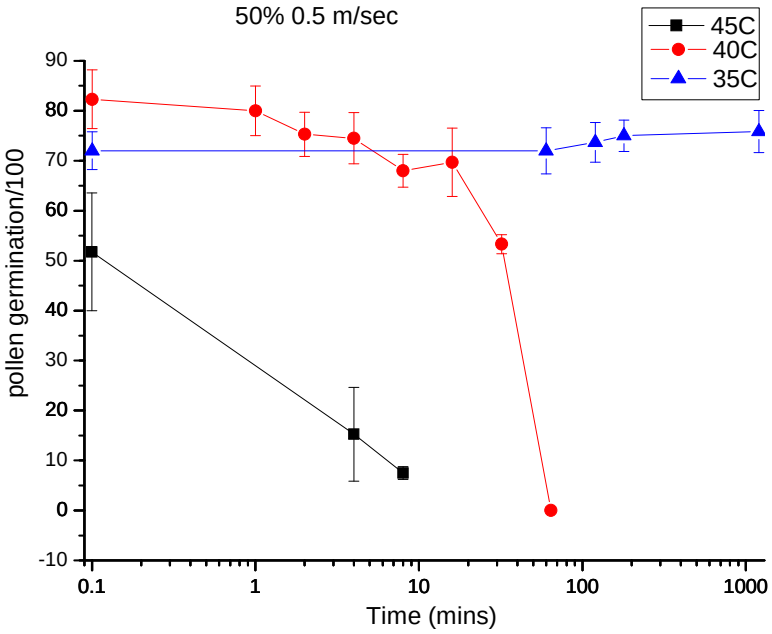


Figure 29: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 45°C, 40°C and 35°C.

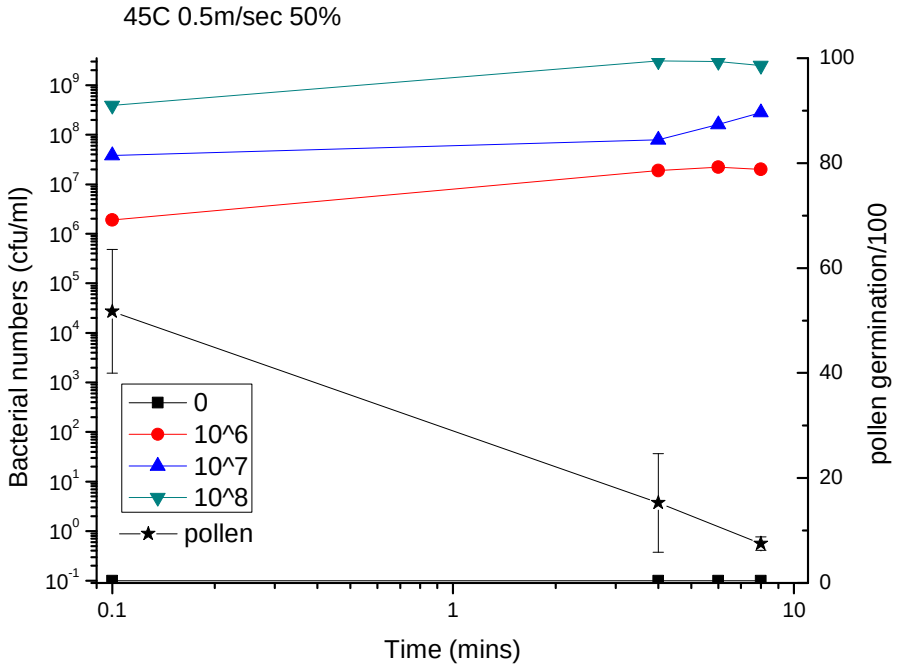


Figure 30: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 45°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

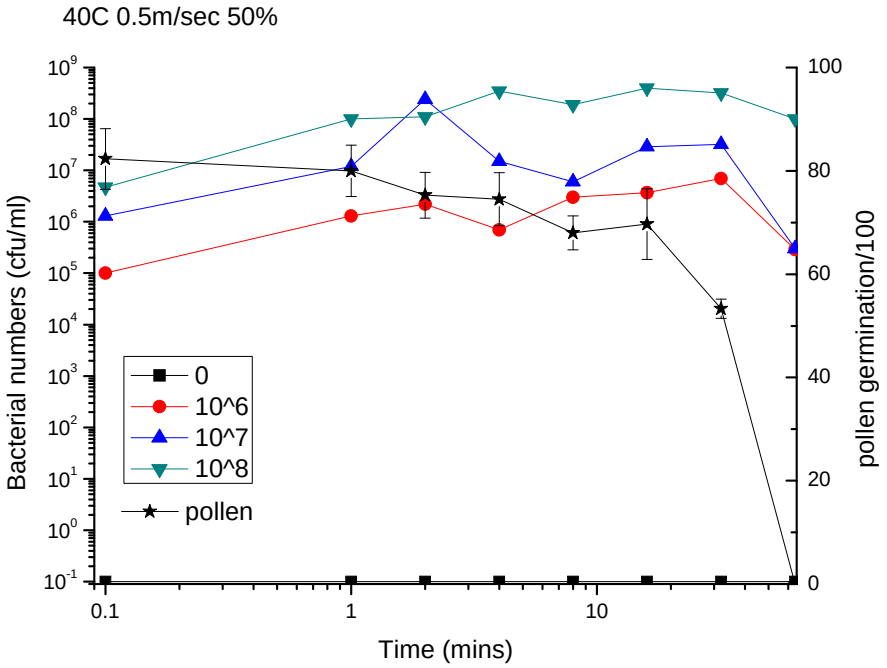


Figure 31: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 40°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

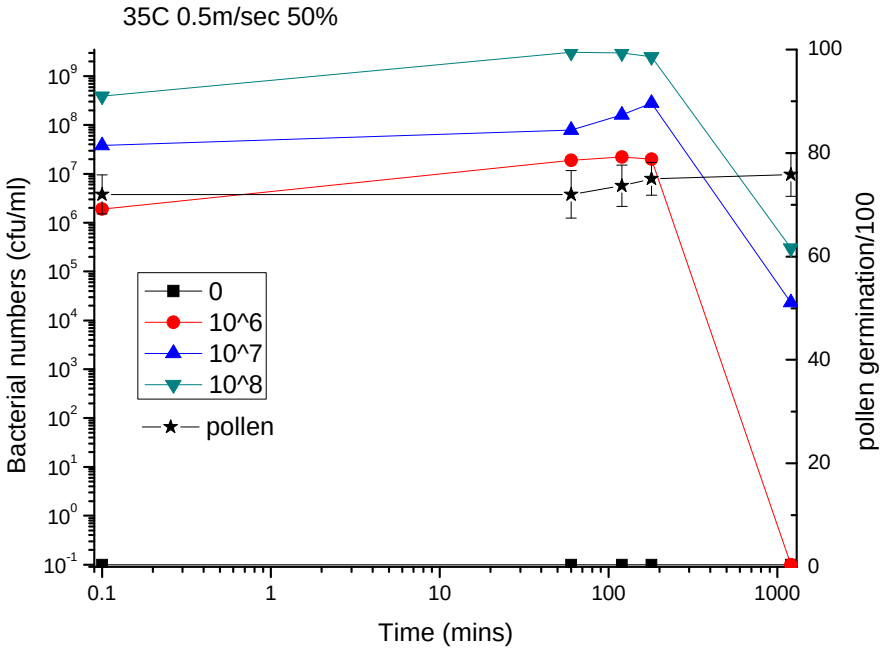


Figure 32: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at 35°C and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

If all these results are plotted on the same graph, and pollen germination adjusted to percentage of germination in untreated controls, then as expected, increasing temperature results in less time that the pollen survives (Fig. 33).

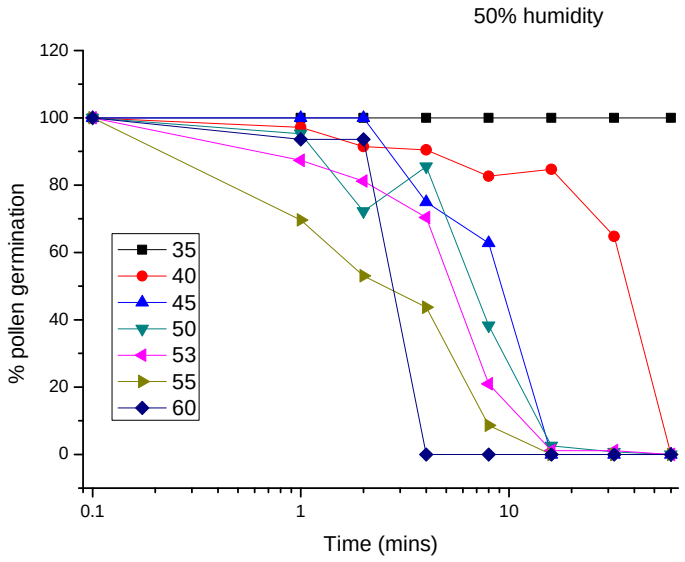
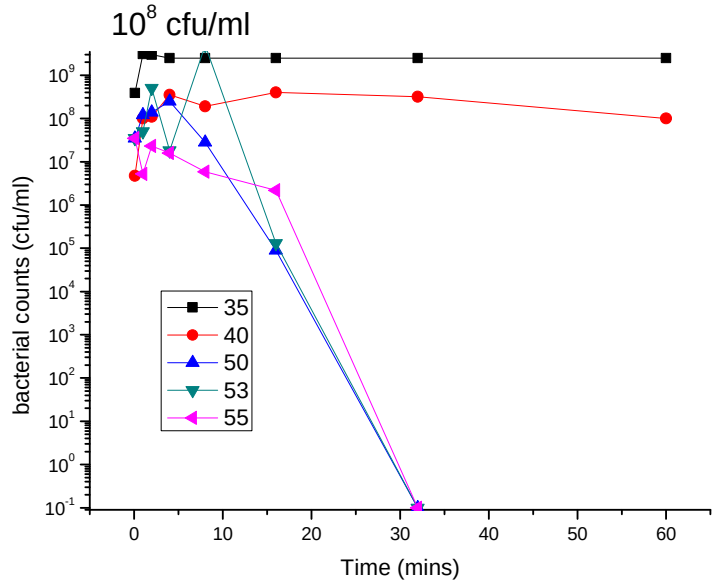


Figure 33: The effect of 0.5m/sec airspeed and 50% humidity on kiwifruit pollen viability at seven different temperatures.

For the bacteria, the same trends apply except that at higher concentrations (10^7 and 10^8 cfu/ml) the bacteria are more resistant to damage by heat (Figure 34).



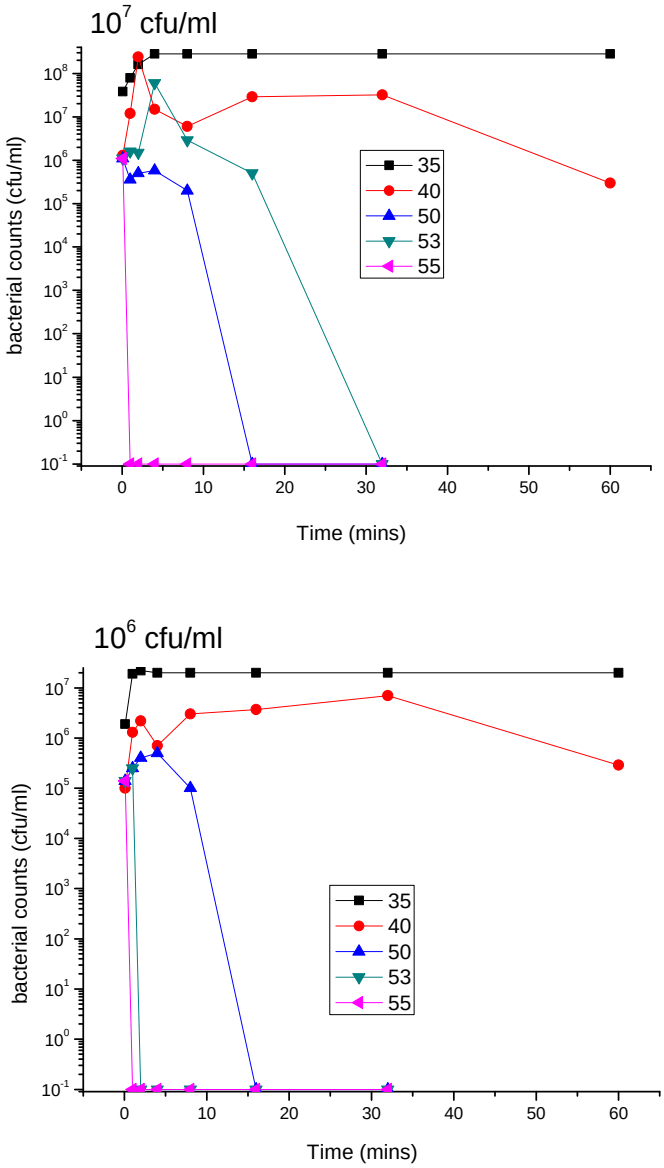


Figure 34: The effect of 0.5m/sec airspeed and 50% humidity at five different temperatures on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

1.8.1 Humidity to break the biofilm

When high humidity was used to attempt to disrupt the resistance of the high concentrations of Psa to heat treatments at 35°C, there was no increase of kill (Figure 35, 36, 37, and 38). However, the pollen remained viable until the humidity was increased to 60%. At this humidity the pollen was damaged.

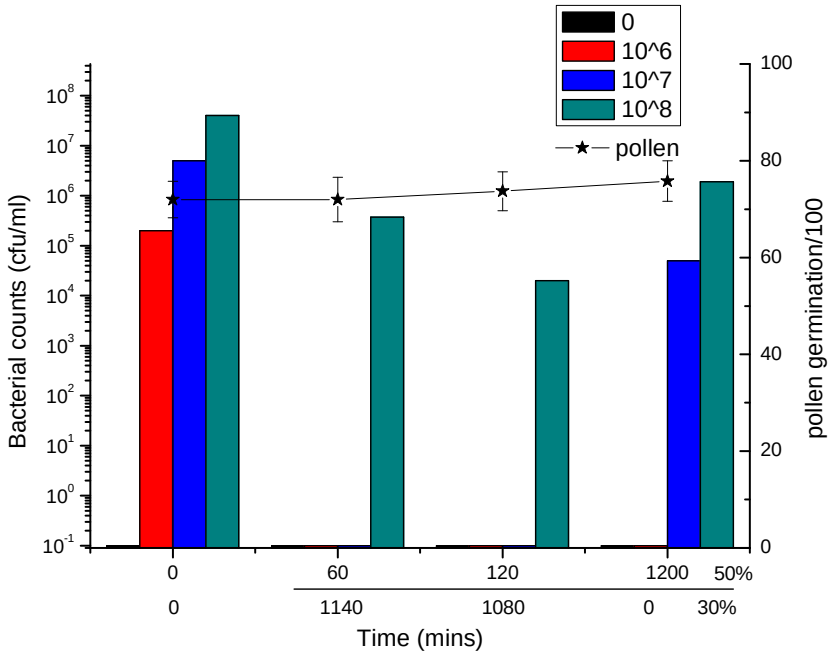


Figure 35: The effect of 0.5m/sec airspeed and 50% humidity followed by c. 30% humidity in a fan incubator on kiwifruit pollen viability at 35**°C** and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

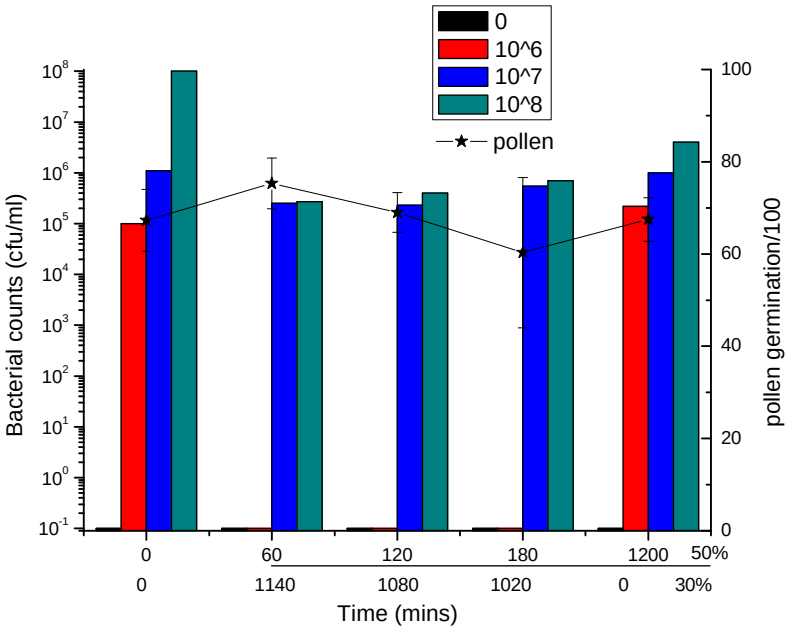


Figure 36: The effect of 0.5m/sec airspeed and 50% humidity followed by c. 30% humidity in a fan incubator on kiwifruit pollen viability at 35**°C** and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

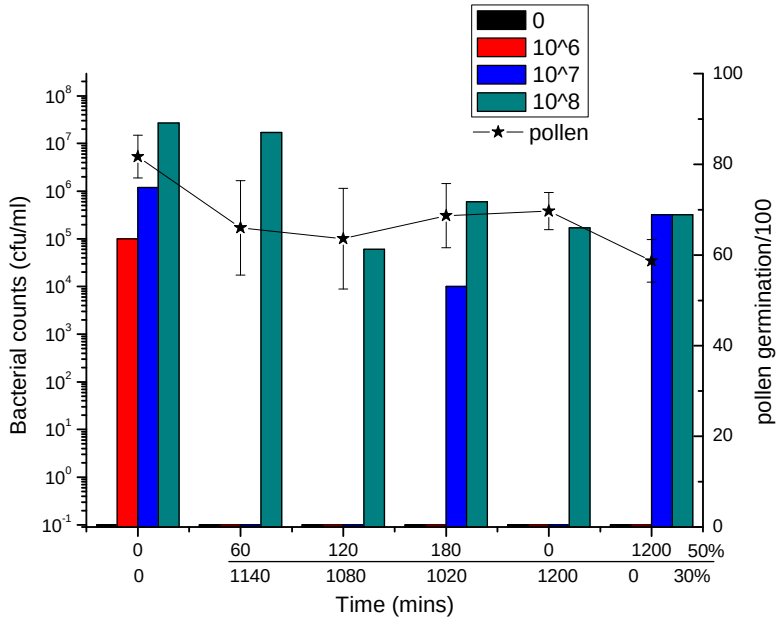


Figure 37: The effect of 0.5m/sec airspeed and 50% humidity followed by c. 30% humidity in a fan incubator on kiwifruit pollen viability at 35**°C** and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

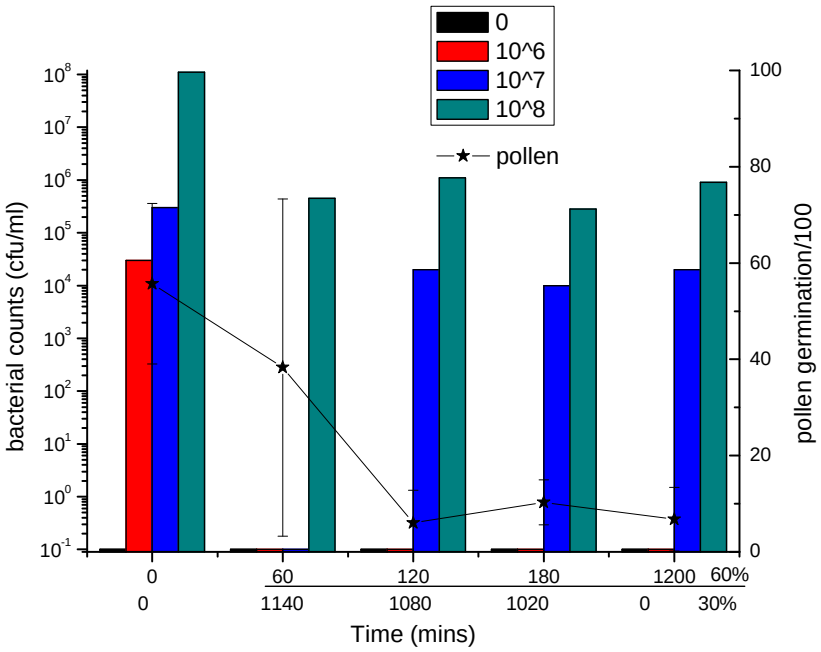


Figure 38: The effect of 0.5m/sec airspeed and 60% humidity followed by c. 30% humidity in a fan incubator on kiwifruit pollen viability at 35**°C** and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

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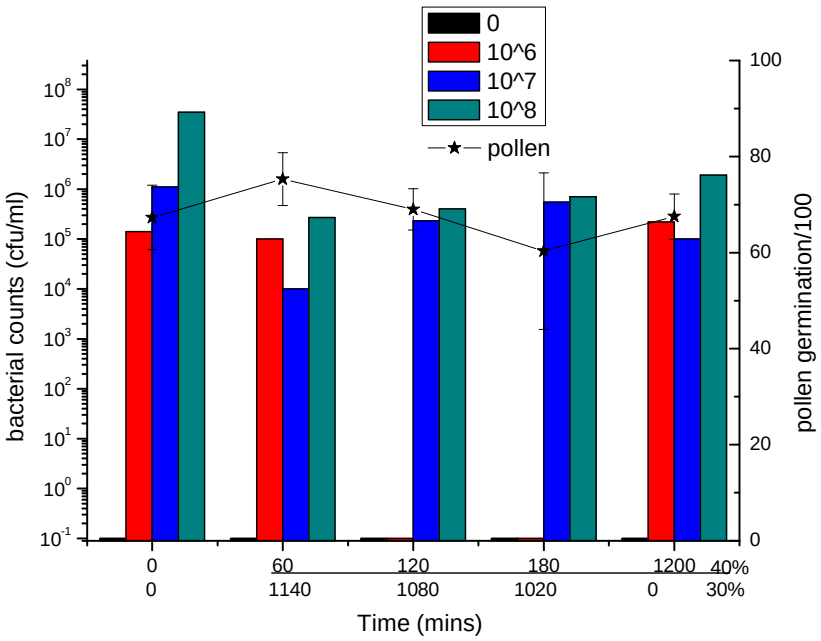

Figure 39: The effect of 0.5m/sec airspeed and 40% humidity followed by c. 30% humidity in a fan incubator on kiwifruit pollen viability at 35**°C** and on survival of *Pseudomonas syringae* pv. *actinidiae* (Psa) applied at three different concentrations (10^8, 10^7 and 10^6 cfu/ml).

2 Discussion

There is a marked difference in temperature response of Psa on pollen compared with Psa in sterile distilled water. This may be due to several factors, one of which is that Psa is known to interact with plant surfaces in many ways including the formation of biofilms (Hall-Stoodley 2004). The biofilm state can be described as an aggregation of planktonic (single) bacterial cells into a colony that adheres to a surface. The bacteria in the biofilm use different biochemical pathways to those in the planktonic state which changes their response to environmental factors (An & Parsek 2007; Izano et al. 2008). For this reason, once a biofilm is formed the bacteria can become more resistant to environmental stressors such as dessication and heat. Our results for heat treatment of pollen can be explained by biofilms. It is proposed that a biofilm did not form on pollen contaminated by Psa until the moisture content was less than 81% and more than 37% . Once the biofilm formed, Psa became resistant to heat treatments. Prior to biofilm formation heat treatments were effective for short periods of time on bacterial cells in a planktonic state, even at high concentrations (10^8 cfu/ml). This is proposed as the reason why heat treatments were effective when Psa was in suspension in sterile distilled water, and on pollen with a high moisture content . Pollen was also able to survive heat treatments once it was dried to 8% moisture content, and survival was enhanced by further dessication to 4% moisture content. Unfortunately these treatments also enhanced the survival of Psa.

Of the wet treatments, a quick wash in 95% ethanol showed the most promise for killing Psa whilst retaining viability of pollen. The effect of this treatment on bacterial survival would need to be investigated further before it could be recommended. However, the PTCT made the decision that wet treatments were not practical and/or did not align with current commercial practice and were to be pursued no further.

The freeze/thawing treatments were ineffective at reducing total bacterial contamination on pollen, but it is not known what effect these treatments would have on Psa survival. An investigation of the effect of freeze/thawing on Psa cells at different concentrations is required.

A window of temperature and humidity at which all Psa was killed but pollen survived was not found. Even at a temperature of 55 °C and humidity of 50% at which pollen survived longer than did Psa at low concentrations (10^6 and 10^7 cfu/ml), the time required to kill the pollen was between 4 and 8 minutes. In commercial conditions a small error in timing would result in loss of the pollen. It is possible that a further reduction in airspeed would allow more margin of error at 55°C, but this would need further investigation. This treatment also requires repeating for evidence of robustness.

The most promising treatment was extended time at 35°C at low humidity. Pollen viability was unaffected by this temperature at humidities up to, but not including, 60%. In contrast, Psa was eventually killed when present at low concentrations (10^5 - 10^6 cfu/ml). The combination of 40% and 30% humidity was not as effective at killing Psa, which suggests that the 30% humidity was incorrect . Humidity was difficult to control in the fan incubator and varied in response to ambient humidity. Unfortunately for this crucial experiment the battery in our humidity/temperature logger failed. In every other experiment the more time that the pollen was at 30% humidity, the more effectively Psa was killed.

Further investigation of the effect of reducing humidity, increasing airspeed, and increasing the duration of treatment at 35°C on pollen viability and Psa survival is recommended. Further investigation of the effect of temperatures above 28°C and below 40°C is also recommended.

The Surefruit machine has the capability of testing the effect of ethanol as a vapour in combination with temperature and humidity, and, because short exposure to 95% ethanol did not reduce pollen viability, it may be worth investigating this treatment further. However, the effect of short exposure of Psa to 95% ethanol would need to be determined before proceeding further. 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 a gaseous form. It is also being used in clinical trials to treat patients with cystic fibrosis, for which the causal organism of secondary infections leading to fatalities is another species of *Pseudomonas* (*Pseudomonas aeruginosa*) (University of Southampton 2012). 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).

These results suggest that the mechanism for loss of viability of Psa at 35°C was dessication. If this is correct, then at this temperature Psa was unable to survive moisture loss unless present at high concentrations. In contrast, Psa was able to withstand moisture loss at 28°C even at low concentrations. This suggests that the mechanism that enables Psa at low concentrations to survive dessication at 28°C was disabled at 35°C.

3 Conclusions

- Although Psa on contaminated pollen was killed by short durations of high temperatures at high humidity, pollen did not survive these same treatments.

- Extended drying of pollen at 35°C and a humidity of 30% did kill Psa when present at a concentration of 10^5-10^6 cfu/ml. If Psa is present in symptomless orchards on pollen at these concentrations, or lower, then this treatment will clean pollen.

- If a drop of Psa at a concentration higher than 10^6 cfu/ml is deposited on pollen from an oozing canker or elsewhere, this treatment will not kill Psa.

4 Future research

- Investigation of lowering humidity below 30%, extending treatment time beyond 1200 minutes, and increasing airspeed on survival of pollen and Psa at 35°C is recommended.

- Combining extended drying at 35°C with the introduction of ethanol or nitric oxide as a vapour may be a fruitful avenue for further research.

- Further investigation of the effect of temperatures between 28 and 40°C on the survival of pollen and Psa is suggested.

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6 Acknowledgments

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7 Appendices

Appendix 1: Mean pollen germination out of 100 after increasing time of treatment at different airspeeds, temperatures and humidity.

Temperature	Airspeed	4m/sec						1m/sec						0.5m/sec											
(°C)	Humidity	50%		70%		90%		50%		70%		90%		50%		30%		28%		25%		24%		20%	
	time(mins)	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
60C	0	77.00	17.40	77.00	17.40	77.00	17.40	78.50	5.63	78.50	5.63	78.50	5.63	94	9.52	72.0	3.77							78.5	18.65
	1	53.00	15.45	0.00	0.00	0.00	0.00	77.00	6.83	40.00	9.80	0.00	0.00	88.00	6.53	70.70	3.27							80.25	10.72
	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	88.00	3.27	1.30	0.00							68.75	6.02
	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							68.00	6.98
	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							71.08	5.78
	16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							48.25	3.59
	32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									0.25	0.50
55C	0													69.25	8.50							69.25	8.50		
	1													48.25	8.23							43.75	15.44		
	2													36.75	6.18							31.75	18.17		
	4													30.25	13.67							42.00	12.28		
	8													6.00	5.80							19.50	2.58		
	16													0.00	0.50							3.25	0.00		
	32													0.00	0.50							6.50	0.00		
53C	0													69.25	8.50					69.25	8.50				
	1													60.50	9.38					42.00	9.22				
	2													56.25	13.67					52.75	3.50				
	4													48.75	5.57					52.50	6.73				
	8													14.50	13.40					49.75	11.90				
	16													0.75	0.50					0.25	2.87				
	32													0.75	0.00					0.00	3.70				
50C	0							96.00	3.27	96.00	3.27	70.00	14.79	69.25	8.50			69.25	8.50						
	1							41.00	13.22	90.00	9.52	72.00	5.66	66.00	16.35			67.75	10.94						
	2							87.00	13.61	81.00	6.83	1.00	2.00	50.00	8.49			76.75	12.45						
	4							8.00	3.27	3.00	2.00	0.00	0.00	59.25	8.18			59.25	7.50						
	8							0.00	0.00	0.00	0.00	0.00	0.00	26.50	11.09			69.67	10.00						
	16							0.00	0.00	0.00	0.00	0.00	0.00	1.75	2.87			12.50	6.81						
	32							0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.58			0.25	0.50						
45C	0							94.00	9.52	46.00	16.81	46.00	16.81	51.75	11.79										
	1							66.00	16.81	20.00	8.64	36.00	3.27												
	2							70.00	6.93	28.00	7.30	46.00	12.44												
	4							66.00	8.33	32.00	10.83	0.00	0.00	15.25	9.39										
	8							0.00	0.00	0.00	0.00	0.00	0.00	7.50	1.29										
	16							0.00	0.00	0.00	0.00	0.00	0.00												
	32							0.00	0.00	0.00	0.00	0.00	0.00												
40C	0													82.30	5.90										
	1													80.00	4.99										
	2													75.30	4.42										
	4													74.50	5.12										
	8													68.00	3.27										
	16													69.70	6.83										
	32													53.30	1.89										
	64													0.00	0.00										
35C	0													72.00	3.77										
	60													72.00	4.62										
	120													73.67	3.98										
	180													75.00	3.15										
	1200													75.83	4.23										

Appendix 2: Bacterial numbers at three initial concentrations (10^6, 10^7 and 10^8 cfu/ml) after increasing time of treatment at 0.5m/sec airspeed, and at different temperatures and humidities.

Temperature	Humidity	50%				30%				28%				25%				24%				20%			
(°C)	time(mins)	0	10^6	10^7	10^8	0	10^6	10^7	10^8	0	10^6	10^7	10^8	0	10^6	10^7	10^8	0	10^6	10^7	10^8	0	10^6	10^7	10^8
60	0					0.0E+00	3.2E+05	5.0E+06	4.0E+07													0.0E+00	6.0E+04	5.0E+05	1.0E+08
	1					0.0E+00	2.0E+06	2.0E+07	3.0E+07													0.0E+00	1.0E+04	1.0E+06	2.0E+07
	2					0.0E+00	1.0E+05	6.5E+05	2.0E+06													0.0E+00	5.0E+04	7.0E+05	2.0E+07
	4					0.0E+00	0.0E+00	0.0E+00	2.7E+05													0.0E+00	1.0E+05	4.0E+06	9.0E+07
	8					0.0E+00	0.0E+00	0.0E+00	2.0E+04													0.0E+00	9.0E+04	1.1E+06	3.1E+07
	16					0.0E+00	0.0E+00	0.0E+00	0.0E+00													0.0E+00	2.0E+04	2.0E+05	1.1E+07
	32																					0.0E+00	0.0E+00	1.0E+04	7.0E+04
55	0	0.00E+00	1.4E+05	1.1E+06	3.5E+07													0.0E+00	1.4E+05	1.1E+06	3.5E+07				
	1	0.00E+00	0.0E+00	0.0E+00	5.2E+06													0.0E+00	3.0E+05	2.0E+05	2.8E+07				
	2	0.00E+00	0.0E+00	0.0E+00	2.3E+07													0.0E+00	5.0E+04	1.0E+05	2.2E+07				
	4	0.00E+00	0.0E+00	0.0E+00	1.6E+07													0.0E+00	3.0E+04	1.0E+05	2.4E+07				
	8	0.00E+00	0.0E+00	0.0E+00	5.9E+06													0.0E+00	1.0E+04	1.0E+05	3.8E+06				
	16	0.00E+00	0.0E+00	0.0E+00	2.2E+06													0.0E+00	0.0E+00	0.0E+00	7.0E+04				
	32	0.00E+00	0.0E+00	0.0E+00	0.0E+00													0.0E+00	0.0E+00	0.0E+00	0.0E+00				
53	0	0.00E+00	1.40E+05	1.10E+06	3.50E+07									0.0E+00	1.40E+05	1.10E+06	3.50E+07								
	1	0.00E+00	2.50E+05	1.60E+06	5.00E+07									0.0E+00	2.50E+05	2.00E+05	3.00E+07								
	2	0.00E+00	4.00E+05	1.50E+06	5.00E+08									0.0E+00	0.00E+00	2.00E+05	1.30E+08								
	4	0.00E+00	5.00E+05	6.00E+07	1.80E+07									0.0E+00	0.00E+00	2.00E+05	1.70E+07								
	8	0.00E+00	1.00E+05	2.90E+06	4.90E+09									0.0E+00	0.00E+00	3.20E+05	3.80E+06								
	16	0.00E+00	0.00E+00	5.00E+05	1.30E+05									0.0E+00	0.00E+00	0.00E+00	3.20E+05								
	32	0.00E+00	0.00E+00	0.00E+00	0.00E+00									0.0E+00	0.00E+00	0.00E+00	3.20E+05								
50	0	0.00E+00	1.40E+05	1.10E+06	3.50E+07					0.0E+00	1.4E+05	1.1E+06	3.5E+07												
	1	0.00E+00	9.00E+04	3.60E+05	1.20E+08					0.0E+00	1.0E+05	2.0E+06	1.3E+07												
	2	0.00E+00	5.00E+04	5.00E+05	1.40E+08					0.0E+00	2.0E+04	1.0E+05	5.0E+06												
	4	0.00E+00	3.00E+04	5.80E+05	2.50E+08					0.0E+00	2.0E+05	2.0E+05	1.6E+07												
	8	0.00E+00	1.00E+04	2.00E+05	2.80E+07					0.0E+00	6.0E+04	2.0E+05	3.3E+06												
	16	0.00E+00	0.00E+00	0.00E+00	9.00E+04					0.0E+00	2.0E+04	3.2E+05	2.4E+06												
	32	0.00E+00	0.00E+00	0.00E+00	0.00E+00					0.0E+00	0.0E+00	0.0E+00	9.0E+04												
40	0	0.00E+00	1.0E+05	1.3E+06	4.7E+06																				
	1	0.00E+00	1.3E+06	1.2E+07	1.0E+08																				
	2	0.00E+00	2.2E+06	2.4E+08	1.1E+08																				
	4	0.00E+00	7.0E+05	1.5E+07	3.5E+08																				
	8	0.00E+00	3.0E+06	6.0E+06	1.9E+08																				
	16	0.00E+00	3.7E+06	2.9E+07	4.0E+08																				
	32	0.00E+00	7.0E+06	3.2E+07	3.2E+08																				
	64	0.00E+00	2.9E+05	3.0E+05	1.0E+08																				
35	0	0.00E+00	1.9E+06	3.8E+07	3.9E+08																				
	60	0.00E+00	1.9E+07	7.9E+07	3.1E+09																				
	120	0.00E+00	2.2E+07	1.6E+08	3.0E+09																				
	180	0.00E+00	2.0E+07	2.8E+08	2.5E+09																				
	1200	0.00E+00	0.0E+00	2.3E+04	3.0E+05																				

Appendix 3: Mean pollen germination out of 100 after increasing time of treatment at 35°C and 0.5m/sec airspeed and at different humidities.

			Surefruit humidity							
	Humidity		60%		50%		40%		30%	
Repeat	time(mins)									
	30%	Surefruit	mean	sd	mean	sd	mean	sd	mean	sd
1	0	0	55.7	16.7	72.0	3.8	67.3	6.7	62.8	10.4
	1140	60	38.3	35.0	72.0	4.6	75.3	5.5	60.0	7.3
	1080	120	6.0	6.8	73.7	4.0	69.0	4.3	58.3	13.9
	1020	180	10.3	4.7			60.3	16.3	64.8	5.6
	960	240							70.8	3.8
	900	300							59.3	9.0
	0	1200	6.8	6.6	75.8	4.2	67.5	4.7	57.8	4.5
2	0	0			67.3	6.7				
	1140	60			75.3	5.5				
	1080	120			69.0	4.3				
	1020	180			60.3	16.3				
	0	1200			67.5	4.7				
3	0	0			81.7	4.7				
	1140	60			66.0	10.4				
	1080	120			63.6	11.1				
	1020	180			68.7	7.1				
	1200	0			69.7	4.1				
	0	1200			58.7	4.7				

Appendix 4: Bacterial numbers at three initial concentrations (10^6, 10^7 and 10^8 cfu/ml) after increasing time of treatment at 35°C and 0.5m/sec airspeed and at different humidities.

			Surefruit humidity											
	Humidity		60%				50%				40%			
Repeat	time(mins)		applied bacterial numbers (cfu/ml)											
	30%	Surefruit	0	10^6	10^7	10^8	0	10^6	10^7	10^8	0	10^6	10^7	10^8
1	0	0	0.0E+00	3.0E+04	3.0E+05	1.1E+08	0.00E+00	2.00E+05	5.00E+06	4.00E+07	0.0E+00	1.4E+05	1.1E+06	3.5E+07
	1140	60	0.0E+00	0.0E+00	0.0E+00	4.5E+05	0.00E+00	0.00E+00	0.00E+00	3.70E+05	0.0E+00	1.0E+05	1.0E+04	2.7E+05
	1080	120	0.0E+00	0.0E+00	2.0E+04	1.1E+06	0.00E+00	0.00E+00	0.00E+00	2.00E+04	0.0E+00	0.0E+00	2.3E+05	4.0E+05
	1020	180	0.0E+00	0.0E+00	1.0E+04	2.8E+05					0.0E+00	0.0E+00	5.5E+05	7.0E+05
	0	1200	0.0E+00	0.0E+00	2.0E+04	9.0E+05	0.00E+00	0.00E+00	5.00E+04	1.90E+06	0.0E+00	2.2E+05	1.0E+05	1.9E+06
2	0	0					0.00E+00	1.00E+05	1.10E+06	1.00E+08				
	1140	60					0.00E+00	0.00E+00	2.50E+05	2.70E+05				
	1080	120					0.00E+00	0.00E+00	2.30E+05	4.00E+05				
	1020	180					0.00E+00	0.00E+00	5.50E+05	7.00E+05				
	0	1200					0.00E+00	2.20E+05	1.00E+06	4.00E+06				
3	0	0					0.00E+00	1.00E+05	1.20E+06	2.70E+07				
	1140	60					0.00E+00	0.00E+00	0.00E+00	1.70E+07				
	1080	120					0.00E+00	0.00E+00	0.00E+00	6.00E+04				
	1020	180					0.00E+00	0.00E+00	1.00E+04	6.00E+05				
	1200	0					0.00E+00	0.00E+00	0.00E+00	1.70E+05				
	0	1200					0.00E+00	0.00E+00	3.20E+05	3.20E+05				