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Pollen Disinfection using Ozone

VLS Project No. E 2013-02

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Executive Summary: Pollen disinfection from Psa-V using ozone

With the arrival of Psa we have seen the rapid infection of male vines used in conjunction with all the commercial kiwifruit varieties. This leaves the industry with the possibility of being unable to ensure adequate pollination for the crop. In the case of Hort16A this shortfall was supplemented by artificial pollination using Chieftain pollen however Hayward males are also becoming infected more rapidly than females and we are left in the position of having inadequate pollen available on badly affected orchards. This, combined with finding Psa-V on pollen, has led to a demand to disinfect pollen from Psa-V contamination. Heat disinfection did not work for high Psa-V concentrations so this project was initiated to examine the possible use of ozone.

Ozone is a fast, broad spectrum antimicrobial agent that works effectively against bacteria, bacterial spores, virus, fungi, etc. It is a very strong oxidising agent which attacks cells walls, membranes etc. so no biological defence is possible. It is non-residual since the end product of the reaction is oxygen and it also has a finite lifetime in air – again breaking down to oxygen. Unfortunately the nature of its chemical attack also poses a threat to pollen. The aim of this project is to try and ascertain whether there exists a treatment window in which the bacteria is killed and the pollen remains viable.

There are technical difficulties in such work since qPCR cannot currently be reliably used to separate live from dead Psa-V and plating Psa-V can be problematic in the presence of naturally occurring microbiological populations.

Of necessity this project also required some study of spiked and naturally occurring Psa-V infections on pollen. We found that Psa-V contamination was highly variable in both systems even though spiking entailed mixing of pollen with a Psa-V in solution followed by drying. We subsequently observed significantly more variability in the Psa-V kill rate than in the pollen kill rate which we attribute (largely) to this intrinsic infection variability.

It is known that temperature, humidity, ozone concentration and exposure time all affect the ultimate efficacy of ozone. Given the size of this study we were not able to examine all these factors so temperature and humidity were kept constant at 15°C and 75-80 % RH respectively. Since ozone efficacy depends on both concentration and exposure time we plotted results on a combined concentration x time (*ct*) scale which effectively collapsed data from a wide range of times (3-180 min) and ozone concentrations (0.3-10 ppm). In order to conveniently examine both pollen viability and Psa mortality we expressed pollen viability and Psa mortality as percentages so all data could be easily combined. Pollen viability was expressed as a percentage of its initial batch value as batches naturally vary and our focus was on the effect of ozone.

Pollen viability data showed less scatter than the Psa mortality. Viability was unaffected at low *ct* values but dropped to ≈ 90% of its initial viability at about $ct = 500$ ppm.min. The kill rate of Psa was more variable however overall there was no clear distinction between Psa on plates and Psa on pollen (either spiked or naturally occurring) The kill rate rose to >90% at a *ct* value of ≈ 150 ppm.min. Thus there appears to be a window $150 < ct < 500$ where pollen remains almost unaffected and the kill rate of Psa is very high.

After any such initial study there remain numerous questions which need to be explored further if the goal is a reliable commercial disinfection process. These include: temperature and humidity mentioned above, the influence of variability and the effect of the absolute level of Psa-V in pollen on Psa-V mortality, practical issues around hardware and some technical/microbiological issues around Psa detection at high mortality rates.

Pollen Disinfection using Ozone

Aim

The aim of this project is to explore the possibilities for cleansing Psa-V infected kiwifruit pollen using ozone gas sterilisation. The key objective is to determine whether there exists a "window" of time and concentration in which ozone gas can kill Psa-V without unduly affecting pollen viability.

Background

There is a considerable apprehension in the kiwifruit industry as to the possibility of pollen spreading Psa-V and consequently there is a strong desire to "cleanse" pollen so it may be guaranteed free of Psa-V regardless of its provenance. This need is exacerbated with the recent widespread infection of Hayward males which brought about a shortage of pollen in many orchards last season.

For many years artificial pollination has played an important role in kiwifruit production and the advent of Psa, and its ravages of Hayward males last season, have only served to increase its importance. Flowers are harvested, dried and milled for pollen which is subsequently sprayed on female flowers. It is thus a potential vector for spreading Psa-V. Previous attempts at cleansing pollen through various methods, including heat, have proven either inconclusive or unreliable. In particular, when pollen is infected with high concentrations of Psa (10^6 cfu/ml and higher) it has not proved possible to eliminate it using heat.

One of the difficulties associated with pollen cleansing are the lack of reliable tools to detect, and subsequently assess the viability, of Psa-V in the presence of natural micro-faunal populations. Until this project there had been little study of live, naturally infected pollen, and consequently, as part of this work, we undertook to try and optimise an effective method for detection of Psa-V in infected pollen using qPCR (taqman assay) using both spiked and naturally infected pollen. Viability confirmation of Psa-V through culturing has been enhanced by the use of a Psa-V specific chromogenic media (Aitken media).

Biocide efficacy is the capability of a chemical (liquid or gas) to inactivate specific challenges of microorganism under defined conditions. The use of gaseous ozone has been tested in various forms for many applications. Ozone is a strong, fast and broad spectrum antimicrobial agent that works effectively against bacteria, fungi and protozoa. Unlike many other sterilising agents ozone is easy and fast to remove after the process and does not leave any chemical residue since the end product of the ozone reaction is simply oxygen. Chemically, ozone is a very strong oxidizer with delocalised electrons which react more readily with other molecules than do localised electrons ($2O_3 + 4e^- \Rightarrow 3O_2$; $\varepsilon^0 = 2.07$ V at 298°K). Inactivation of bacteria is thought to occur by ozone oxidising the fatty acids in the cell membrane and macromolecules like proteins and DNA. The damage caused by ozone is irreversible and causes lysis of the cell wall and the consequently death. It also kills spores and viruses as it oxidises DNA and proteins in spores as well as in viruses.

A variety of chemical, biological and environmental factors determine the efficacy of a particular biocide. These include chemical concentration, pH, temperature, relative humidity, contact time, presence or absence of organic matter, surface or substrate composition, microorganism type, and its tendency toward mono-dispersion or aggregation. In the case of a gas its ability to cross surface boundary layers and to cross phase must also be considered i.e. can it "gain access" to organisms in solution, or when protected by a water film?

The main variables that affect the success of ozone, once it has access to the organism, are contact time and concentration in practice this means it is significantly affected by humidity.

This trial attempted to

1) Develop an experimental system for cleansing Psa-V infected pollen.

2) Explore the operating parameters including humidity, temperature, exposure time, and ozone concentration while measuring the kill rate of Psa-V (desired outcome) and the loss in viability of pollen thorough ozone attack (undesirable outcome).

Materials and Equipment

1) Media used for testing

Aitken media considerably assisted the identification of Psa-V colonies through their morphological characteristics since many other bacteria and fungi do not grow on it. This media was also invaluable for quantification studies conducted on spiked pollen since pollen is densely contaminated with many other bacteria and identification of Psa-V using traditional Kings B media is problematic.

2) Ozone generator

During this trial, we used a high volume generator used for sterilisation of large surface areas and volumes. Its coarse output control was suited to its high volume output and so was not ideal for the work. It had the incontrovertible advantage of being available. The system concentrates oxygen from ambient air and then converts a part of that into ozone. It has the following connections:

- Gas (inlet and outlet)
- Electrical connections (High voltage)

The characteristics of this generator and its rudimentary control system governed the experimental design required to maintain constant low ozone levels for our experiments.

3) Ozone Meter

Ozone was monitored by an Aeroqual UVH photometer. This meter has an internal pump which pulls through air at 0.4 L/min. Ambient levels were monitored continually during the experiments and were recorded every 1 min for subsequent calculations.

A problem with this meter during this project required its return to the factory, cleaning and recalibration which extended the duration of the project.

Temperature and humidity were monitored indepentently and logged every 5 min.

4) Prototype Design

Our prototype design was based on an open rotating, stirred drum powered by a 12v motor through a gear box.

Factors considered for the prototype design are as listed below.

1) Maximum aeration of pollen with gas of the measured ozone concentration
2) Choice of materials to have minimum impact on pollen viability

The initial prototype could be improved:

1) Plastic was used for the containing drum which, in combination with the rubbing movement engendered by its rotation, probably caused static electricity generation which exacerbated pollen's natural tendency to clump. We believe clumping to be unhelpful in achieving maximal biocidal activity of the gas.
2) A second issue was the loss of pollen from the open mouth of the container. This was undesirable (especially when using infected pollen) and also presented a challenge to the

filtration system of our ozone meter.

The initial prototype was replaced with an improved version aimed at overcoming some of the perceived limitations encountered with the protoytpe:

1) The plastic drum was replaced by glass. Glass was chosen as it is pollen friendly and may also reduce static built-up and consequently clumping. We also included two drums to allow multiple pollen samples to be exposed simultaneously.
2) Stirring was increased by several innovations
3) The new glass drums had a much higher length to diameter ratio and the entire drum was tilted to reduce the loss of pollen to the atmosphere.
4) The ozone meter inlet was fitted with 5 micron filter to allow close monitoring of the air adjacent to the pollen while avoiding contamination of the meter.

5) Experimental setup

The experiments were undertaken at Oakside. The ozone was supplied to the chamber via Teflon tubing and the meter inlet (again Teflon) was placed in the chamber adjacent to the experimental setup. Fans were used in the chamber throughout each experiment to ensure the atmosphere remained well mixed. While we didn't have fine humidity control we endeavoured to maintain high humidity (70 to 90 %). Both the humidity and the temperature were recorded during each trial.

Methodology

Identification of Psa-V in infected pollen using qPCR

A study was conducted to optimise an effective method for detection of Psa-V in infected pollen through qPCR (Taqman assay) using both spiked and naturally infected pollen.Viability of Psa-V was confirmed by culturing.

a) 0.2 g of pollen was tested for each treatment viz. control, treated and spiked pollen.
b) DNA extractions were made on 500 µL aliquot of the supernatant collected after spinning and tested by qPCR using a rapid Psa-V test.
c) qPCR results are given as the replication value (Cq) and the standard detection range for Psa-V detection using our laboratory processes is ≤ Cq 33.
d) Identification of Psa-V by qPCR was conducted on naturally infected pollen before its use in the trial to confirm the presence or absence of Psa-V.
e) Culturing and quantification methods confirmed viability and concentration of infection of Psa-V.

Preparation of Psa-V suspension in 0.85 %saline

a) ICMP culture 18800 Psa-V was inoculated in TSB broth and allowed to incubate at 25°C for 48 hours.
b) After incubation, the broth was centrifuged at 4000 rpm for 15 minutes and the supernatant decanted. The cells were re-suspended in 0.85 % saline and allowed to dissolve for 30 minutes.
c) The Psa-V solution was quantified in cfu/mL using serial dilution and plating. The solution was used to inoculate Aitken media for ozone exposure and is referred to as the exposure plate test in this report.
d) The Psa-V solution was also used for artificially spiking pollen for comparison with naturally infected pollen in these trials.

Psa-V Quantification in cfu/mL in infected pollen

a) Throughout this trial 0.2g of pollen was used for quantification of Psa-V by serial dilution, plating onto Aitken media and incubating at 25°C for 2 days.
b) ICMP 18800 Psa-V was used as a reference standard culture for colony comparison of Psa-V. Psa-V colonies are small, roughly circular, smooth and pink in colour. In cases where colonies could not be identified visually, they were checked using qPCR. .

Pollen viability

a) Each sample was tested in triplicate. A control (pollen sample with known viability) was also established. There are 3 steps involved in pollen viability:
   i. Sample preparation. About 0.03g of pollen was weighed using a plastic loop into a beaker to be tested for each replicate.
   ii. Rehydration. The pollen samples were placed in a box lined with moistened paper towel and incubated at 26-28°C. for 2 hours to facilitate complete rehydration.
   iii. Germination. Sugar solution was added to the pollen after rehydration and the sample then placed on a shaker within the incubation chamber. The shaker is set to a rotation of 160 rpm for 3 hours.

Pollen spiking

a) Pollen was heated at 100°C for 8 hours, cooled overnight and then heated again at 100°C for 8 hours. This treatment sterilised the pollen by killing all spore-forming bacteria prior to spiking
b) A known concentration and fixed volume of Psa-V solution was then added to this treated pollen and allowed to stand for 1 hour. This spiked pollen was then incubated on filter paper in petri dishes at 26 - 28 °C overnight, or longer as required. The dried pollen was then scraped off the filter paper and a sample quantified in cfu/mL.

Spiked pollen quantification and repeatability

Inter-sample repeatability was tested by taking a 0.2 g sample from bulk spiked pollen each time the process was used. This sample was re-quantified using an agitation time of 20 seconds to recover maximum Psa-V in the supernatant before dilution.

Intra-sample repeatability was determined by looking at the quantification reproducibility from three samples from the same dilution.

Natural Pollen quantification and repeatability

a) A study was conducted to maximize the recovery of Psa-V from naturally infected pollen using an agitation method and different time intervals.
b) Once the optimal agitation time was found, a repeatability study was conducted to determine the consistency of quantification of small samples of infected pollen taken from a bulk sample of 250g. Quantification of samples was carried out using serial dilution and plating methods and using the highly selective Aitken media.

Exposure plates

a) Quantified Aitken media plates inoculated with Psa-V were exposed in the chamber simultaneously with the pollen cleansing during each trial to determine the biocidal efficacy of ozone at different concentrations and exposure times.

Ozone Trials

Pollen viability was measured on all samples prior to any ozone exposure. Viabilities ranging from 80 to 89 % were measured on pollen used in these trials.

All trials used a combination of exposure times and ozone concentrations to examine efficacy.

Trial 1

Trial 1 was aimed at looking gaining an overview of the effect of various ozone concentrations and exposure time combinations on the survival of Psa-V on plates and on the viability of pollen (Table 1).

Prior to the start of the experiment, plates were prepared, labelled and inoculated with known concentrations of Psa-V solution from neat (8 x 10^6 cfu/mL) down in 8 decadal steps.

The chamber was closed and its humidity raised to 75-85% which was maintained throughout the trials. The ozone was then switched on and when the required level was reached all the samples for a particular run were placed in the chamber. These were then extracted sequentially at pre-determined times. The ozone level was then adjusted and the process repeated with a new set of samples.

At the end of the trial all samples were transported back to the lab for testing. The exposed plates were incubated for 36 hours at 25°C and the pollen samples tested for viability.

Concentration of Ozone (ppm)	Exposure time (Minutes)
0.3	60,120,180
0.6	60,120,180
1.2	60,120,180
2	60,120,180
5	60,120,180
10	60,120,180

Table 1. Ozone concentration and exposure times used to expose plated Psa and to test pollen viability in Trial 1

Trial 2

This repeated the experimental goals of experiment 1 but changed the concentrations and times to target the zone of interest which emerged from the first experiment (Table 2).

Concentration of Ozone (ppm)	Exposure time (Minutes)
3	8,13,33
5	5, 8, 20, 40, 70, 100

Table 2. Ozone concentration and exposure times used to expose plated Psa and to test pollen viability in Trial 2.

Trial 3

Experiment 3 used the same combination of times and ozone concentrations as experiment 2 but introduced both spiked and naturally infected pollen in addition to plates of Psa (as used in the earlier experiments).

Trial 4

This final experiment varied the concentration and time parameters (in response to the previous experiment) again using spiked and naturally infected pollen (Table 3).

Concentration of Ozone (ppm)	Exposure time (Minutes)
8	4,9,13,25,50,120

Table 3. Ozone concentration and exposure times used to expose plated Psa and to test pollen viability in Trial 4

Results

Psa-V Quantification in cfu/mL in infected pollen

A trial was conducted to assess how the recovery of Psa-V from naturally infected pollen was affected by varying agitation times as outlined in Table 4. The same weight of pollen (0.2g) was used throughout so cfu/mL can be directly converted to cfu/gm pollen.

From the results (excluding a statistical outlier at 60s), the recovery looks to be independent of agitation time.

Pollen	Agitation time (s)	Quantification cfu/mL	Quantification cfu/g pollen
S18	20	1×10^2	5×10^2
S18	40	1×10^2	5×10^2
S18	60	5×10^2	2.5×10^3
S18	120	1×10^2	5×10^2

Table 4. Effect of beating time on recovery of Psa=V from naturally infected pollen

Quantification & repeatability of naturally infected pollen

The consistency of repeated sampling of a single line of naturally contaminated pollen (sample S18) is shown in Table 5 and Figure 1.

Despite carefully physically mixing the 250g pollen container before sub-sampling commenced, the measured level of infection varied significantly between successive 0.2 g samples. The result depended on agitation time with 20 s showing significantly higher levels than those from 60 s.

Physically pollen is “sticky” i.e. it tends to clump together and it appears this “granularity” is repeated in Psa levels.

No	Pollen	Agitation time (s)	Weight (g)	Quantification (cfu/g pollen)
1	S18	20	0.2	1.05×10^3
2	S18	20	0.2	4.00×10^2
3	S18	20	0.2	4.50×10^2
4	S18	20	0.2	4.00×10^2
5	S18	20	0.2	5.00×10^2
6	S18	20	0.2	3.50×10^2
7	S18	20	0.2	3.50×10^2
8	S18	20	0.2	5.00×10^2
9	S18	20	0.2	5.00×10^2
10	S18	20	0.2	5.00×10^2
11	S18	60	0.2	5.00×10^2
12	S18	60	0.2	1.50×10^2
13	S18	60	0.2	5.0×10^1
14	S18	60	0.2	5.0×10^1
15	S18	60	0.2	4.00×10^2
16	S18	60	0.2	1.50×10^2
17	S18	60	0.2	5.0×10^1
18	S18	60	0.2	4.00×10^2
19	S18	60	0.2	5.0×10^1
20	S18	60	0.2	1.50×10^2

Table 5. Variability of measured Psa-V levels in replicate samples of naturally infected pollen

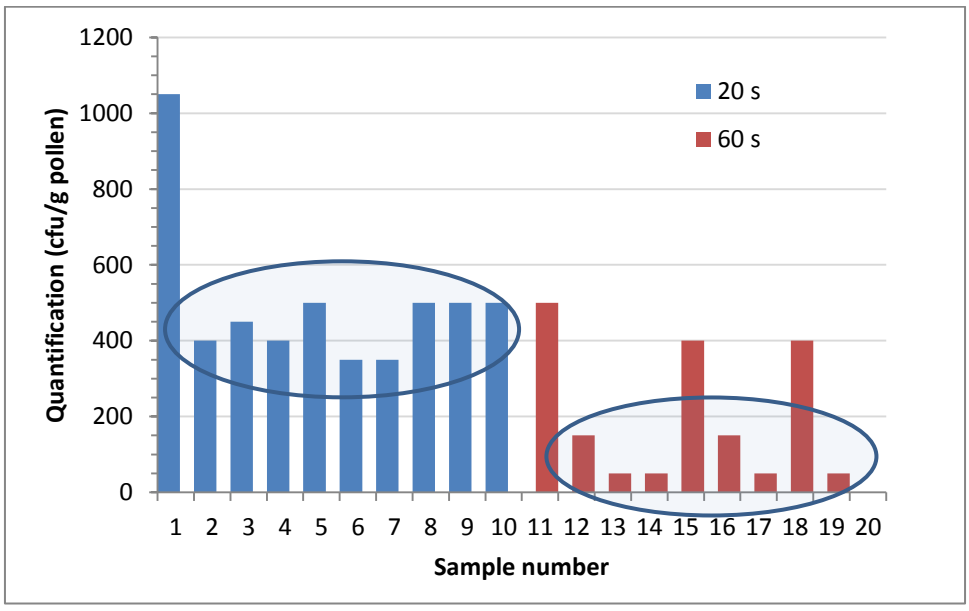

Figure 1. Sample variability when repeatedly sampling a single line of naturally contaminated pollen. Circles highlight the points with outliers removed whose selective stats are given in Table 6.

Sample 1 is statistically and practically an outlier. If it is removed the 20 s mean shifts from 500 to 439 cfu/g pollen and the SD is reduced from 200 to 65 cfu/g pollen. Curiously the 60 s samples form a bimodal distribution with twice as many points around 100 than there are around 450 (which is closer to the 20 s sample average).

	mean (All pts)	SD	Mean selective	SD selective
20s	100	40	87.7[1]	13
60s	39	34	18[2]	10

Table 6. Stats for 20s and 60 s beating times. Selective stats 1 are 20s with first point excluded and 2 60 s low points only.

We will use 20s agitation times for all subsequent work although this dependence is worthy of further exploration.

Spiked pollen

A large sample of spiked pollen was made up by heat steriIising natural pollen, spiking with a volume of 1 x 10^8 cfu/mL Psa solution and then redrying. To test the intra-sample variability a single sample was taken from the spiked pollen and plated 3 times with the results shown in table 7 and Figure 3. The average was 7 x 10^6 cfu/mL (3.5 x 10^7 cfu/g pollen) with an SD of 1 x 10^6 cfu/mL i.e. CV of 14%.

No	Pollen	Weight (g)	Quantification (cfu/mL)
1	SP rep 1	0.2	8 x 10^6
2	SP rep 2	0.2	7 x 10^6
3	SP rep 3	0.2	6 x 10^6

Table 7. Replicate quantification of a single sample of spiked mixed pollen.

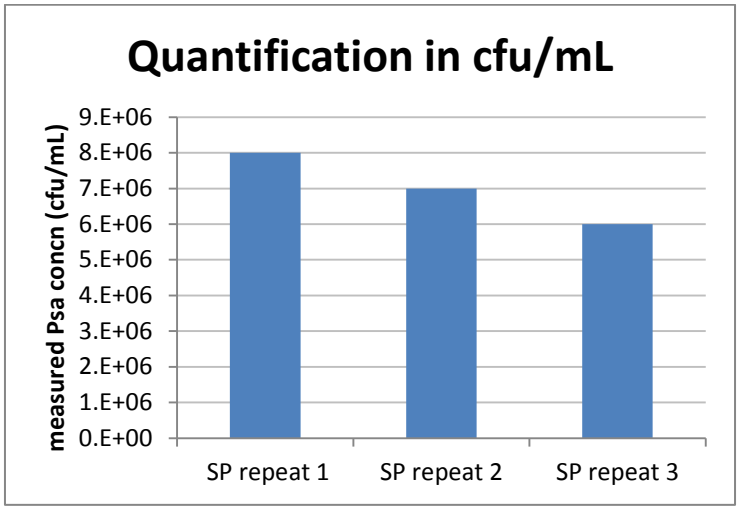

Figure 3. Variability of replicate plating from a single sample of spiked pollen.

Repeatability of Spiked pollen Quantification

Sampling variability was then examined by taking 14 separate samples from the spiked mixed pollen and quantifying each separately. The results are given in Table 8 and Figure 4 and showed that the apparent level of infection in spiked pollen can vary significantly. Pollen sample SP3 is a statistical outlier compared with the rest of the samples which ranged between 1 x 10^6 and 2 x 10^7 cfu/mL (5 x 10^6 - 9 x 10^7 cfu/g pollen). Again the variability was high: the average with SP3 removed is 1.915 x 10^7 cfu/g pollen with an SD of 1.175 x 10^7 cfu/mL i.e. CV of 61%.

No	Pollen	Quantification (cfu/mL)	Quantification (cfu/g pollen)
1	SP1	8.0×10^6	4.0×10^7
2	SP2	6.0×10^6	3.0×10^7
3	SP3	1.80×10^7	9.0×10^7
4	SP4	4.0×10^6	20.0×10^6
5	SP5	1.0×10^6	5.0×10^6
6	SP6	1.8×10^6	9.0×10^6
7	SP7	5.0×10^6	2.50×10^7
8	SP8	7.0×10^6	3.50×10^7
9	SP9	2.0×10^6	1.00×10^7
10	SP10	3.0×10^6	1.50×10^7
11	SP11	3.0×10^6	1.50×10^7
12	SP12	2.0×10^6	1.0×10^7
13	SP13	6.0×10^6	3.0×10^7
14	SP14	1.0×10^6	5.0×10^6
	Average	**4.84×10^6**	**2.42×10^7**
	SD	**4.41×10^6**	**2.205×10^7**

Table 8. Quantification of independent pollen samples taken from a single sample of spiked and mixed pollen. The outlier is shaded.

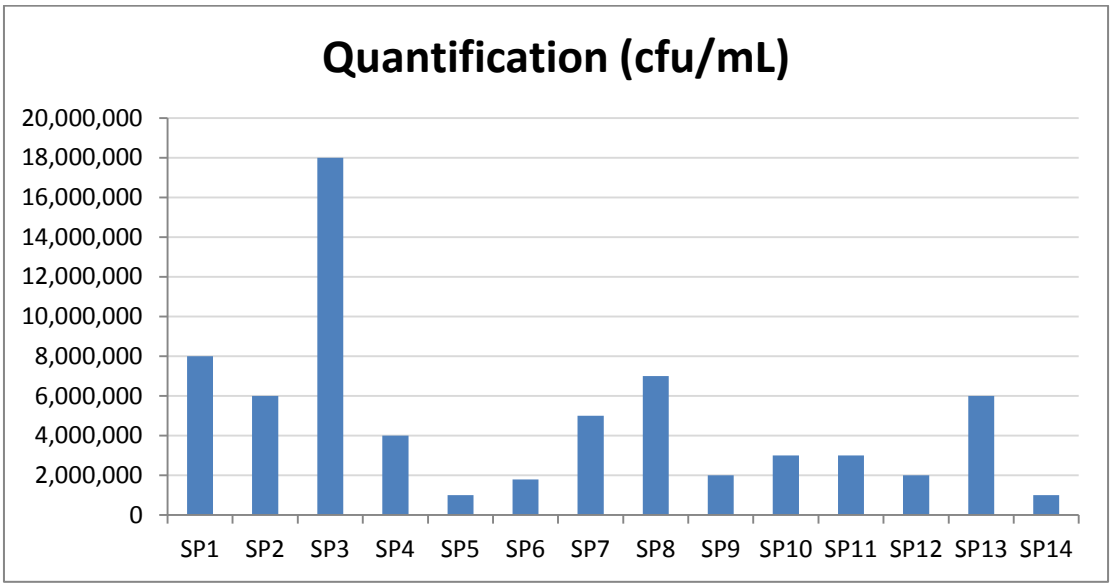

Figure 4. Repeated independent samples from a single batch of spiked and well-mixed pollen from Table 8. cfu/mL is graphed not cfu/g pollen but the look is indentical

Ozone trial 1

The goal of this trial was to look at the ozone kill rate of ***plated*** Psa together with pollen viability for a range of ozone concentrations and exposure times. We are not looking at the kill rate of Psa in Psa infected pollen in this experiment. The trial covered 6 different ozone concentrations combined with 3 different times for each concentration as shown in Table 9.

Pollen

This trial used Pacific pollen which had a pre-trial viability between 80 and 90 %. Once the desired ozone concentration was reached in the chamber, a pollen sample was placed in the chamber and then small sub-samples removed sequentially at the predetermined intervals. Each sample was than split in

3 for replicate quantification (R1, R2 and R3). The times and concentrations were designed to cover a wide range of *exposure* i.e. conc x time (*=ct [ppm.min]*) as previous work led us to expect that, all other factors being equal, kill rate should be proportional to *ct*.

conc	time	ct (ppm min)	Viability % R1	Viability % R2	Viability % R3	Avg Pollen Viability%	Psa cfu/mL	Kill Rate of PsaV	%survival PsaV
0.3 ppm	60	17.35	75	85	83	81.08	$4.0x10^6$	50.0%	50.0%
	120	35.48	84	88	87	86.13	$3.4 x10^6$	57.5%	42.5%
	180	53.24	86	89	85	86.77	$4.1 x10^6$	48.8%	51.3%
0.6 ppm	60	36.11	85	77	90	83.83	$6.0 x10^6$	92.5%	7.5%
	120	72.6	84	82	83	83.00	$4.0 x10^6$	95.0%	5.0%
	180	109.62	87	81	82	83.33	$1.0 x10^6$	98.8%	1.3%
1.2 ppm	60	73.94	88	80	77	81.67	$2.0 x10^6$	97.5%	2.5%
	120	147.08	77	78	78	77.67	$3.0 x10^6$	96.3%	3.8%
	180	219.93	80	81	82	81.00	$2.0 x10^6$	97.5%	2.5%
2 ppm	60	123.02	90	86	86	87.33	$2.0 x10^6$	99.8%	0.3%
	120	245.14	87	87	87	87.00	$2.0 x10^6$	100.0%	0.0%
	180	366.31	82	86	86	84.67	$2.0 x10^6$	100.0%	0.0%
5 ppm	60	297.31	80.3	78.8	80	79.70	0.00	100.0%	0.0%
	120	598.36	70.9	65.9	65.7	67.50	0.00	100.0%	0.0%
	180	899.08	41.8	54.1	57.6	51.17	0.00	100.0%	0.0%
10 ppm	60	598.42	62.9	75.3	71.3	69.83	0.00	100.0%	0.0%
	120	1202.47	58	34	35.7	42.57	0.00	100.0%	0.0%
	180	1804.46	21.4	24.3	28.6	24.77	0.00	100.0%	0.0%

Table 9. Data from Trial 1 showing ozone levels and exposure times along with measured pollen viability, Psa concentration after treatment and calculated kill rate and % survival of Psa on plates. *ct* values are calculated from *measured* concentrations and times rather than the target values shown in the conc. column.

Temperature and RH were recorded through each such trial and activities logged. An example is shown below from Trial 2 in Figure 11. Both showed little variation, temperature was around 15°C and RH in the range 75-80%. Ozone concentration shows some fine variability but remains controlled to within ≈ 0.2 ppm of the set-point.

Ozone concentrations and times were designed to provide a wide range of exposure with some overlap between *ct* values in order to check the validity of the *ct* dependence. Within the scatter of the data we see quite good collapse of the data against *ct* whereas there is no such consistency if we plot the data against either time or ozone concentration separately (Figure 5). The 2nd and 3rd panels of Figure 5 show that, ***at the exposure times we used,*** ozone doesn't affect viability until the concentration rises to 5ppm.

Figures 6 and 7 show the pollen viability from the trial for each treatment plotted again *ct* on linear and logarithmic axes respectively. Since the *ct* values cover more than one order of magnitude most of the points are bunched unhelpfully on the left of the graph when plotted on a linear axis. For this reason we will show *ct* graphs on a logarithmic axis in future .

The horizontal lines at 80 and 90% indicate the viability "range" of the pollen pre-treatment

The variability in viability *before* the ozone treatment is significant. We have shown the data from each replicate on this graph to demonstrate both the considerable scatter and the occurrence of "outlier" values. We expect the scatter is due to

- uncertainty in the viability determination (sampling and counting)
- slightly variable ozone exposure i.e. in ozone kill rate
- inhomogeneity of pollen viability within the sample which might contribute to the variability seen in this determination throughout.

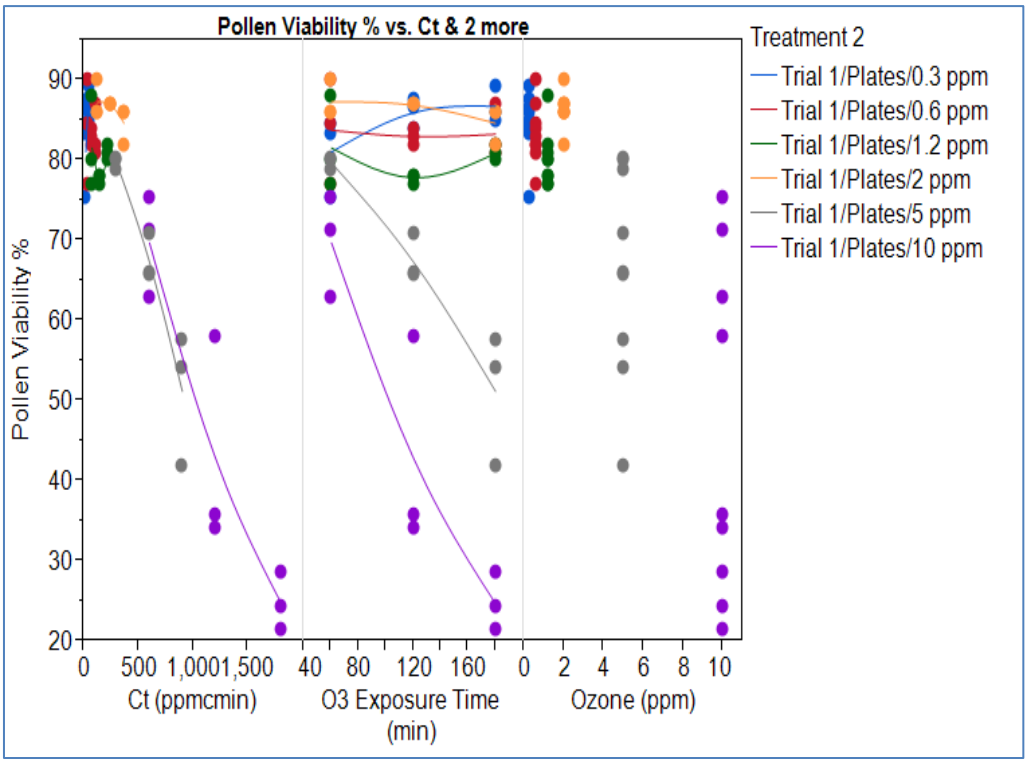

Figure 5. Pollen viability plotted against *ct*, exposure time and ozone. Each point in the vertical series of points of the same colour in the 3rd panel represent different exposure times. It is clear that *ct* provides a consistent basis on to assess the efficacy of ozone in killing pollen.

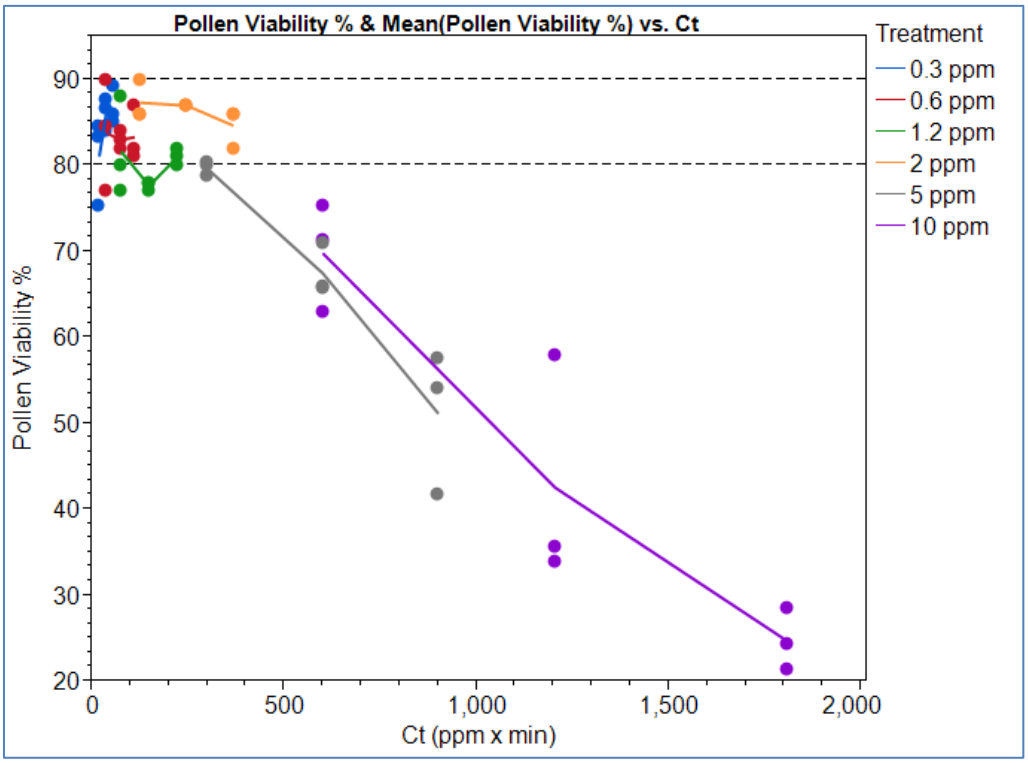

Figure 6. Pollen viability (%) for various ozone concentrations and times plotted against *ct* on a linear *ct* scale (see text). Each rep is shown as a point to provide a complete picture of the experimental scatter in the data. The dashed horizontal lines (80 and 90%) show the viability of the pollen used.

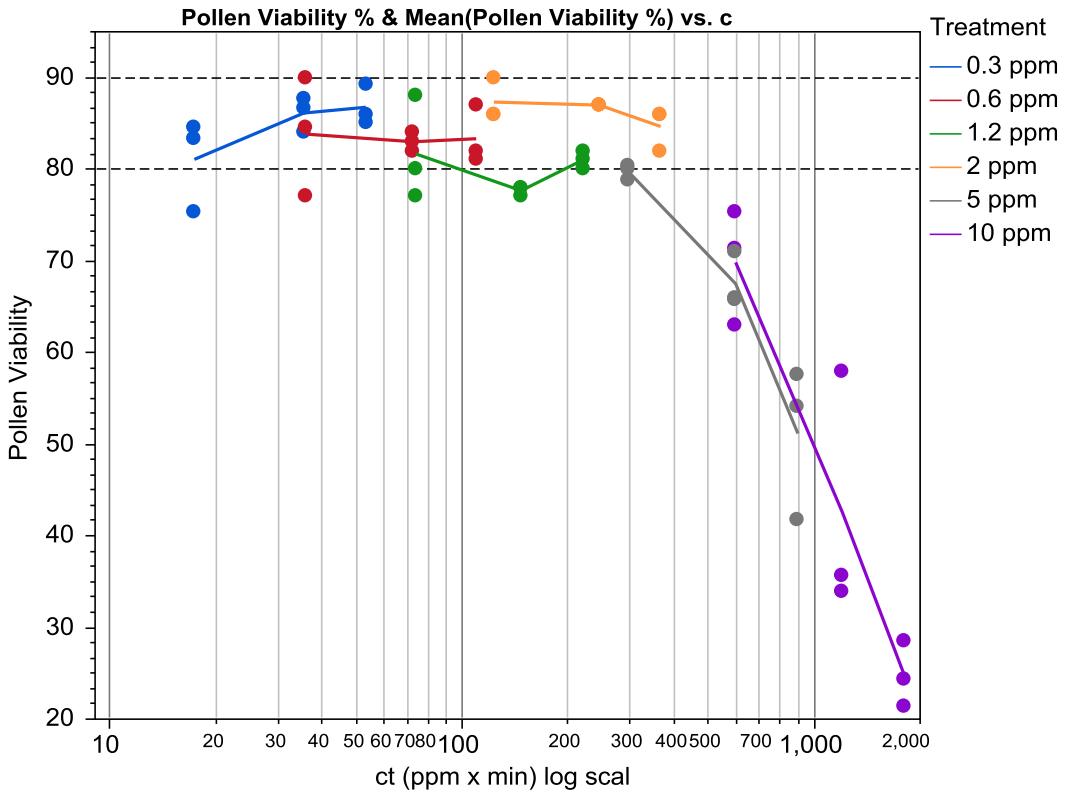

Figure 7. Pollen viability (%) for various ozone concentrations and times plotted against *ct* on a logarithmic scale (see text). Each rep is shown as a point to provide a complete picture of the experimental scatter in the data. The dashed horizontal lines (80 and 90%) show the initial viability of the pollen used.

This variability is clearly seen in Figure 7 and has a range of ~ 12% in viability.

Within this uncertainty we can say:

- Pollen viability was not affected by ozone for *ct* values up to ≈ 400.
- Above this threshold viability dropped from 85 to 20% as *ct* rose to 2000 ppm.min.
    - This looks to be approximately a linear drop when plotted on the linear/log axes i.e. the rate of drop is 65/[log(500)-log(2000)] = -108 %Viability per log(*ct)*

Note that this Figure shows the *absolute* viability - the *treatment* viability is calculated by dividing this by the "average" initial pollen viability i.e ≈ 85%. The scatter in this *initial* viability will be reflected in the calculation of *treatment* viability so some points will be over 100% however the shape and scatter will remain unchanged.

Psa_V

Figure 8 shows the Psa survival data for this trial. Clearly Psa numbers were greatly affected by any ozone concentration over 0.3ppm. We used the initial concentration of Psa combined with the survival numbers to calculate the %kill for each treatment (Table 9, Figure 8) from:
%kill = 100* [initial concn – final concn]/(initial concn)

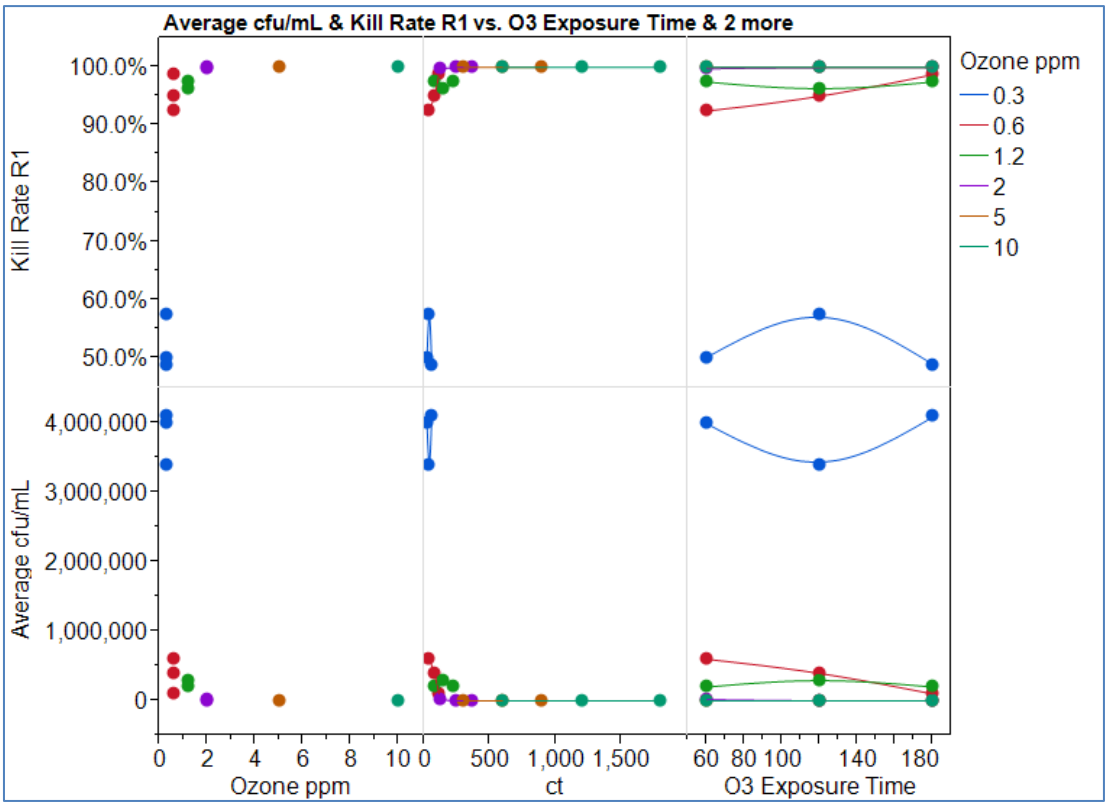

Figure 8. Psa survival for the trial one treatments. The lower panel shows the quantification result plotted against ozone concentration, ct and exposure time (linear scales). The upper panel shows the Psa kill rate (see text) against the same variates.

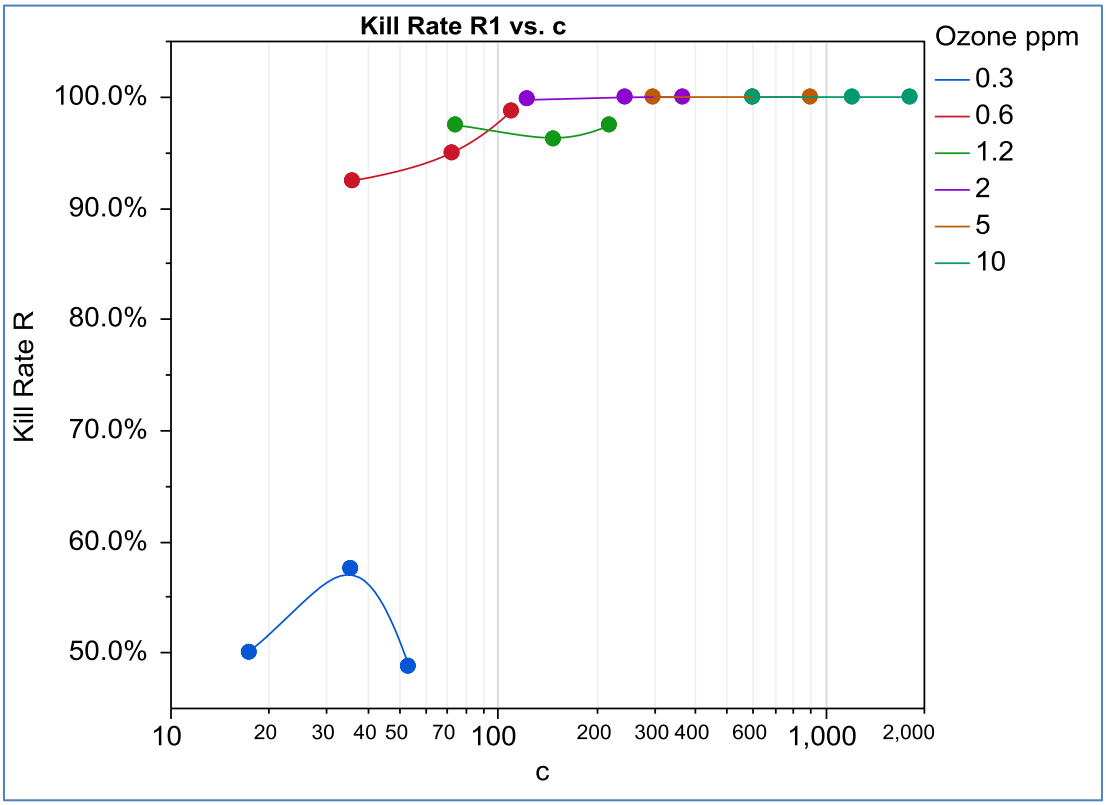

Figure 9. %Psa Survival against ct on a log scale.

If we plot the %kill against *ct* on a log scale it shows the spread in *ct* values and the satisfactory overlap arising from the various time x concentration values for *ct* >100. For *ct* values in the range 35 - 70 there

is poor agreement between the 0.3 and 0.6 ppm ozone concentrations: kill rates of ≈55% and 93% respectively. Clearly this needs more examination – is the *ct* concept breaking down or are the errors (or scatter) in calculating the % Psa kill rate causing this large range?

The advantage of presenting the data in this way is that it allows us to combine both the pollen viability data and the Psa kill rate data attributable to ozone on the one graph in a helpful way (Figure 10).

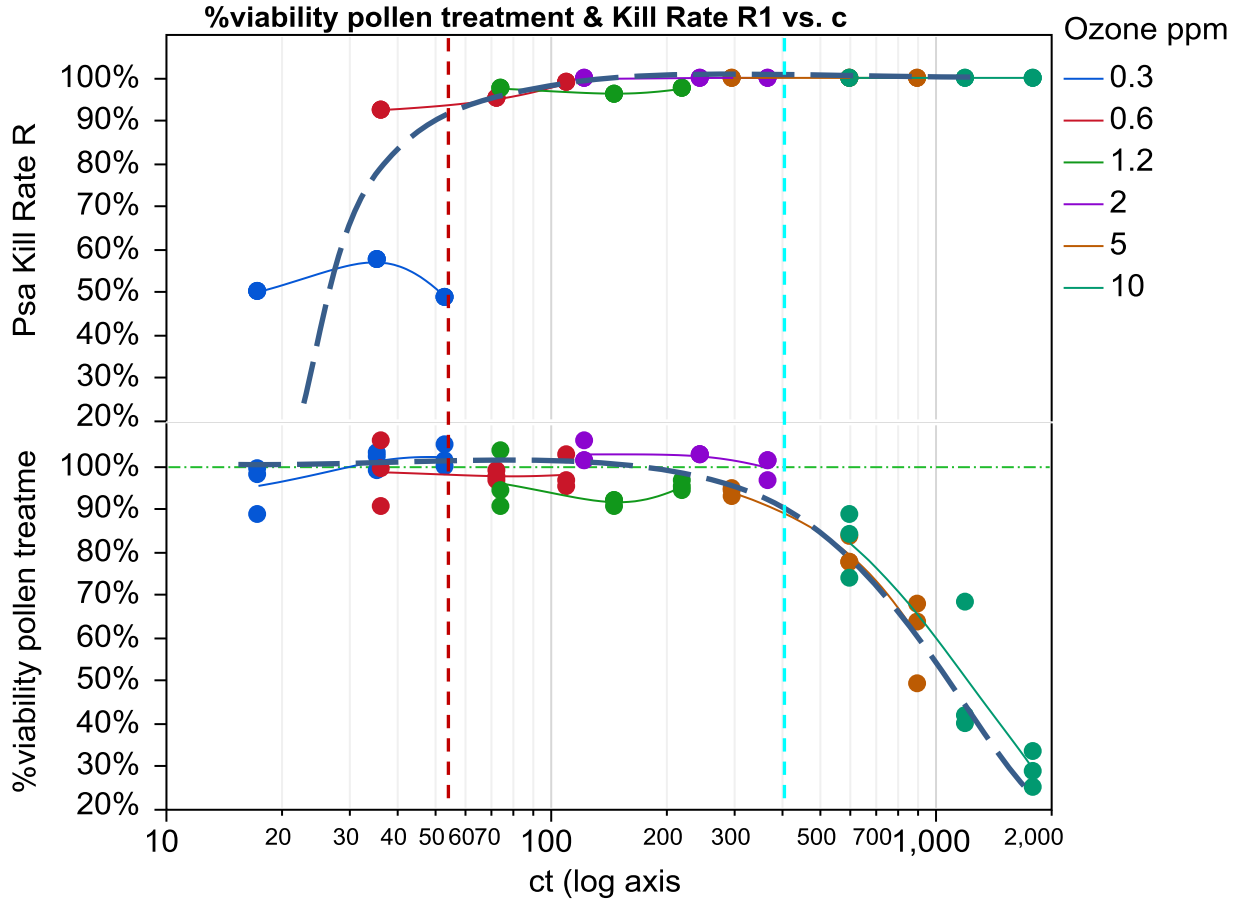

Figure 10. Psa kill rate (top panel) and pollen viability (lower panel) for various ozone levels. The dark blue dashed lines were fitted by eye through the point s(see text) and the vertical dashed lines correspond to the 90% shoulder points for Psa kill rate and pollen viability

In both panels in Figure 10 I have added a (dashed) curve by eye which generally follows each data set (acknowledging the issue discussed above at low *ct* values for Psa kill rate) together with 2 vertical lines corresponding to the 90% threshold for each data curve. The upper pane shows the Psa survival so to the right of the red dashed line less than 10% of the Psa was left alive. In the lower pane to the left of the turquoise line more than 90% of the initial pollen was still viable i.e. between the lines most of the Psa was killed and most of the pollen was viable (90% thresholds). ***In the range 55 < ct < 400 it appears pollen survives ozone while the ozone kills the Psa***.

Ozone trial 2

Trial 2 was carried out using 3 ppm and 5 ppm ozone to create a range of low *ct* values from 14 to 504 (Table 10) since there was a paucity of data in this range in trial 1 and it was the region which showed more variation when plotted against *ct* (Figure 10, top). This trial also tested the robustness of the *ct* concept since we used very different time/concentration combinations to create similar *ct* values: 10x the ozone concentration combined with shorter exposure times.

The concentration of cultured Psa-V in this trial was 1 x 10^7 cfu/mL.

Batch S18 (14/11) of infected chieftain pollen was used for this trial which had an original viability of 70-75%.

Pollen

Figure 11 shows the temperature, humidity and ozone concentrations recorded from this experiment along with an annotation of the experimental activities.

conc	time	ct (ppm .min)	Viability R1 (%)	Viability R2 (%)	Viability R3 (%)	Avg Pollen Viability (%)	Psa (cfu/mL)	KillRate ofPsaV (%)	survival PsaV (%)
3 ppm	8	24.84	74.6	64.3	66.7	68.53	6.5×10^6	35.0	65.0%
	13	39.82	57.5	66.66	50.6	58.25	3.0×10^6	70.0	30.0%
	33	99.18	79.19	79.94	82.8	80.64	1.0×10^6	90.0	10.0%
5 ppm	3	14.26	69.5	74.3	65.9	69.90	3.0×10^6	70.0	30.0%
	9	44.01	81.6	72.3	77.6	77.17	1.0×10^6	90.0	10.0%
	20	99.93	73.6	61.2	68.7	67.83	1.0×10^6	90.0	10.0%
	40	202.35	76.8	75.8	77.2	76.60	1.0×10^6	90.0	10.0%
	70	351.52	70.2	60.1	67.8	66.03	0.03×10^6	99.7	0.3%
	100	504.16	66.6	76	67.8	70.13	0.01×10^6	99.9	0.1%

Table 10. Pollen viability and Psa kill rate when exposed to different concentrations of ozone. All reps are shown.

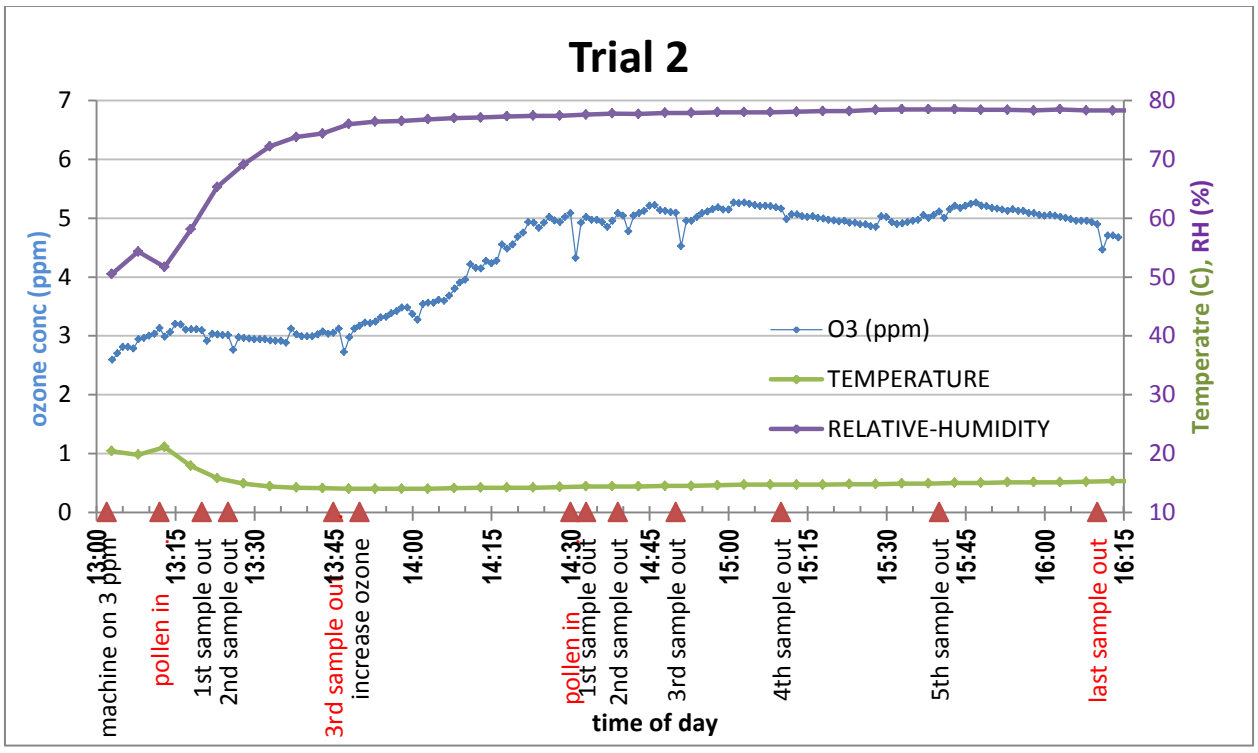

Figure 11. Ozone (left hand axis), temperature and relative humidity (right hand axis) through the 3 and 5 ppm ozone concentrations in trial 2. Time is shown on the lower axis along with annotations of experimental procedure through the trial. There is little perturbation caused by removing samples.

Figure 12 shows the pollen viability data in comparison to trial 1. We have included the representative line from trial 1 in both panels in Figure 12 although it has been displaced to fit the different initial viability of the pollen used in this experiment which was ≈ 72%. Encouragingly the representative line from the first trial fits this second data set reasonably well however we note that this data shows more scatter than the first trial. The 3ppm ozone concentration in particular shows considerable scatter and lacks any "reasonable" trend with *ct*. Without any other obvious reason, we wonder whether the Psa

infection in this pollen caused the increased scatter in viability. This was the first infected pollen used to date.

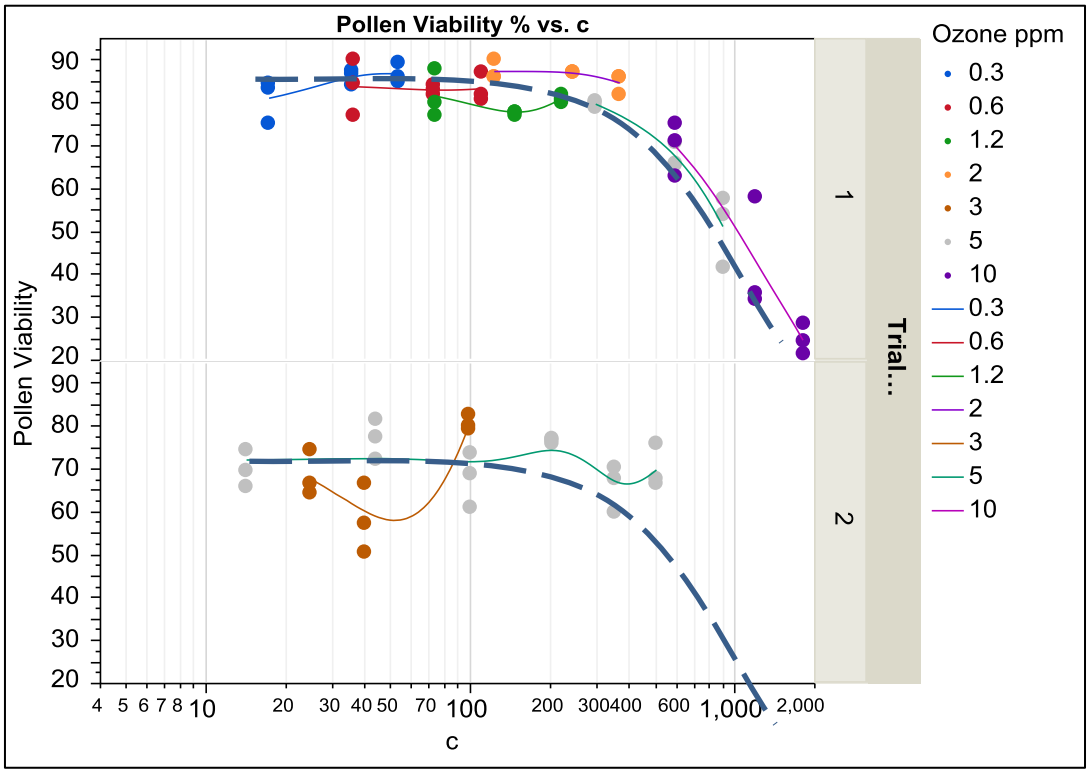

Figure 12. Pollen viability data from Trial 1 (top panel and trial 2 (lower panel) showing all reps plotted against ct on a log scale. The dashed from figure 10 is repeated on these 2 panels.

Psa-V

The Psa results are shown in Figure 13 again including the data and curves from Trial 1 for comparison.

The lowest *ct* value (*ct*=14) appears to be an outlier with the surprisingly high kill rate of 70%. This was exposed for only 3 minutes. Excluding this point we see the *ct* parameterisation fits the data well. This further vindicates the *ct* relationship since this trial used ozone concentrations 10 x those used in trial 1 to achieve the same *ct* values and the data is still well represented by the initial trial curve when plotted against *ct*.

The representative line from trial 1 also describes the form of data well – although it could perhaps shift a little to the right.

Given the curves from trial 1 represent this data well it follows that the same zone with high Psa kill and high pollen viability is also supported by this data.

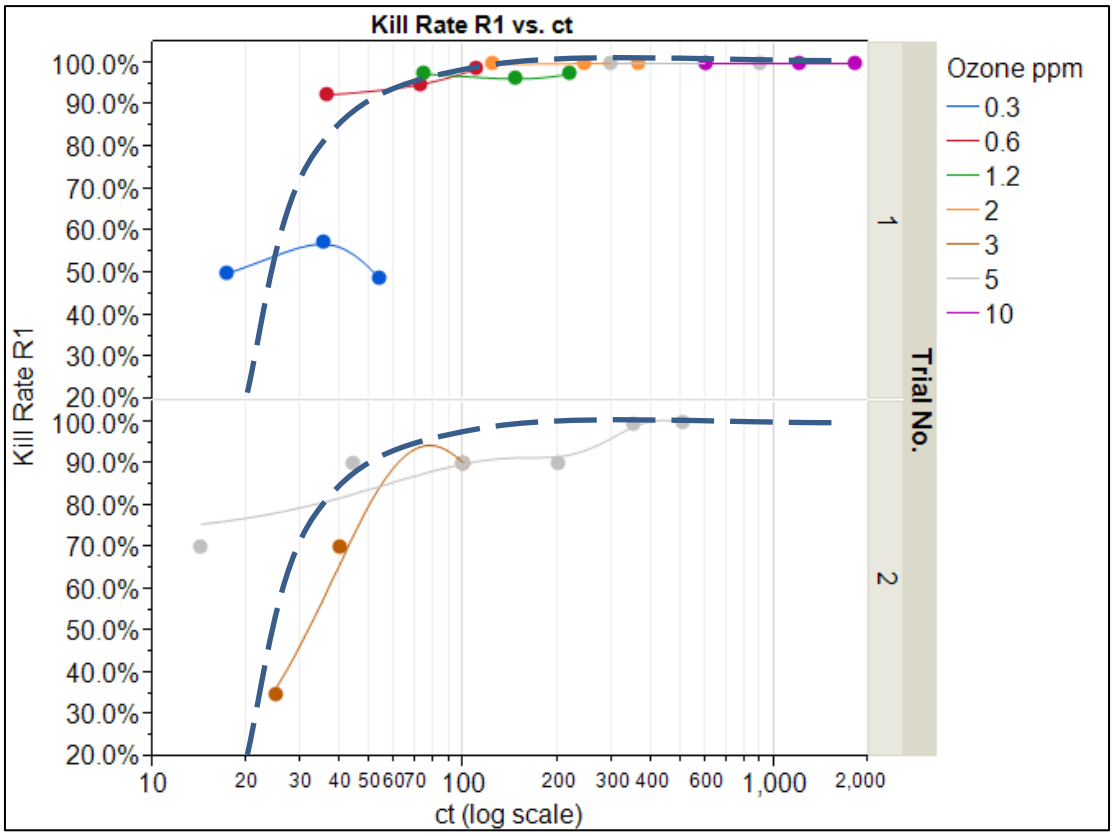

Figure 13. Psa kill rate for trial 1 (top panel) and Trial 2 (lower panel). Two points are on top of each other at *ct* = 100 in the lower panel. The dashed lines are those introduced in Trial 1.

Trial 3

Trial 3 included Psa on plates as in the earlier trials along with naturally infected pollen and spiked pollen in the examination of the kill rate of ozone. It used the same pollen (S 18 batch 14/11, viability 70-75%), ozone concentrations and exposure times as trial 2. The treatments and results are shown in Table 11. Viability data is only available for naturally infected pollen since spiked pollen has been heat treated to ensure sterility before spiking. As for the earlier trials *ct* is calculated from actual monitored ozone concentrations not from the target concentration.

			Plates 4.0 x 10^8 cfu/mL			Spiked Pollen: 5.0x10^5 cfu/g			Natural Pollen: Psa 2.0 x 10^3 cfu/g pollen				
conc ppm	time min	*ct*	Psa cfu/mL	PsaKill Rate (%)	%survival PsaV	Psa cfu/g pollen	KillRateof PsaV	%survival PsaV	Pollen Viability %	Psa cfu/g pollen	PsaKill Rate (%)	% survival PsaV	comment
3	8	24	3.0 x 10^8	25.0	75.0%	1.0x10^4	98	2	75	50	97.5	2.5	≤10 colonies
	13	39	2.0 x 10^8	50.0	50.0%	1.0x10^4	98	2	80	50	97.5	2.5	≤10 colonies
	33	105	2.5 x10^7	93.8	6.3%	5.0x10^3	99	1	77.3	50	97.5	2.5	
5	3	26	3.5 x 10^8	12.5	87.5%	1.0x10^4	98	2	83.4	100	95.0	5.0	
	9	42	1.5 x 10^8	62.5	37.5%	5.0x10^3	99	1	78.3	50	97.5	2.5	≤10 colonies
	20	107	1.0 x 10^7	97.5	2.5%	4.5x10^3	99.1	0.9	82.6	50	97.5	2.5	≤10 colonies
	40	207	1.0 x 10^7	97.5	2.5%	2.0x10^3	99.6	0.4	75.7	50	97.5	2.5	
	70	354	5.0 x 10^6	98.8	1.3%	1.5x10^3	99.7	0.3	74.5	100	95.0	5.0	
	100	523.	1.5 x 10^6	99.6	0.4%	1.5x10^3	99.7	0.3	75.5	2,000	0.0	100.0	

Table 11. Treatments and results for trial 3. The initial Psa-V concentration is specified in the top box for each sample type.

Pollen

Figure 14 shows the pollen viability data plotted against *ct* with trial 1 data for comparison.

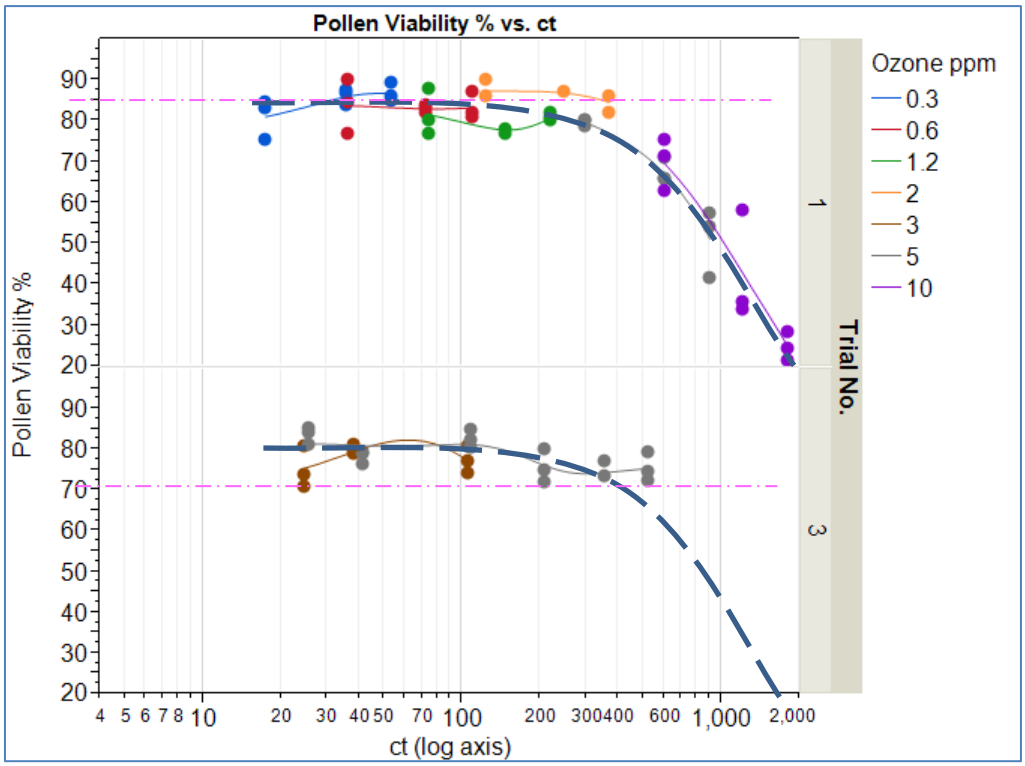

Figure 14. Pollen viability for Trial 3. Dashed lines superimposed from trial 1.

Again the fit is reasonable and it shows the utility of plotting against *ct* as points from the same *concentration* appear on different parts of the curve plotted against *ct.* Whereas the Trial 1 curve plateaus at close the initial pollen viability (85%) the same curve applied to trial 3 fits best at about 80% whereas the initial viability of this pollen was ≈72% (as in trial 2). The 3ppm ozone data seems slightly closer to this level but the only explanation was have for this shift is that a different operator undertook all the viability testing for this experiment.

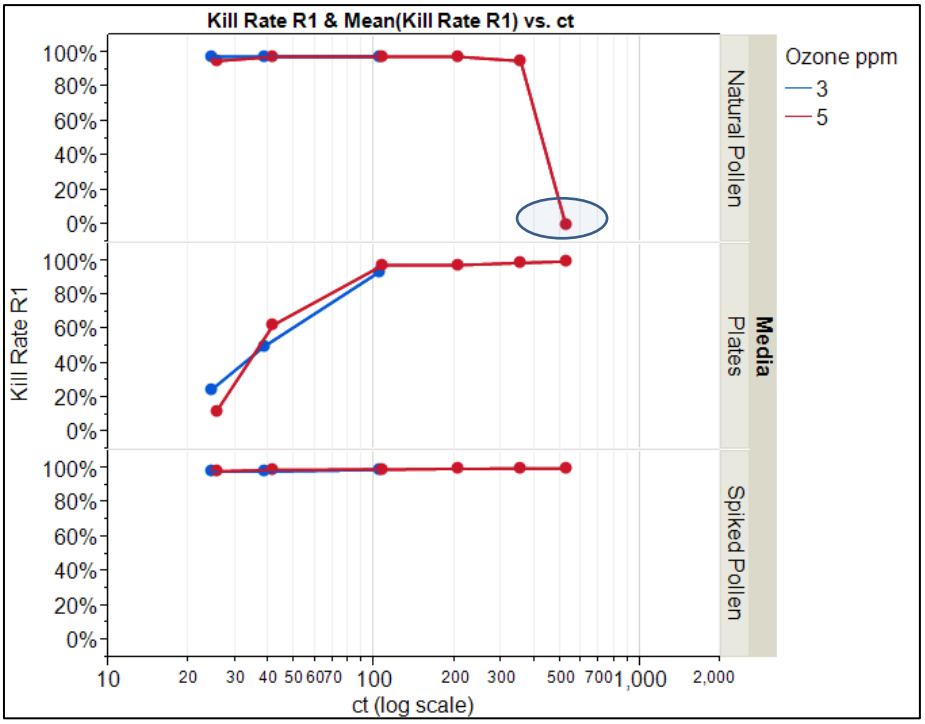

Figure 15. Psa Kill rate for Trial 3 for Psa-V on plates, and in spiked and naturally infected pollen. An outlier in the natural pollen data is circled and omitted from subsequent analyses.

Psa

The reason for the choice of *ct* values in the trial was primarily to look at Psa kill rate as we did in trial 2. Figure 15 shows the Kill rate of Psa on plates (high Psa conc), spiked (medium conc) and naturally infected pollen (low Psa conc).

1. The data at *ct* = 523 for natural pollen is clearly an outlier and seems to be an error since this lone point is completely different from the rest of the data and other trials.
2. The zones of overlap where the same *ct* was created using different ozone concentrations show remarkably close agreement for all Psa sources.
3. Clearly the drop in Psa mortality for *ct* < ≈100 on plates is not reflected in naturally infected or spiked pollen. For both pollen samples all the Psa seemed to be killed at all *ct* values.

If we add the data from the previous two trials and exclude only the single outlier point from trial 3 we still see reasonable agreement in relation to the *ct* "cut-off" below which we get little Psa death. This cut-off is consistent with the original plate data from trial 1 and with the dashed curve we imposed on that data (Figure 16).

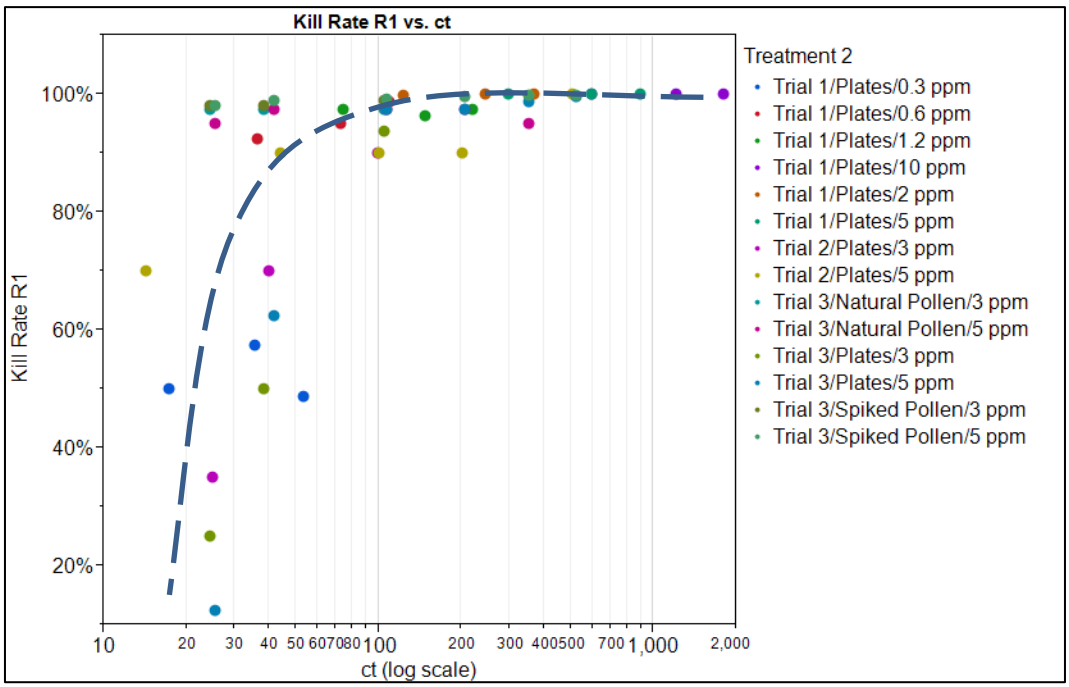

Figure 16. Psa kill rate for trials 1 to 3. One outlier from trial 3 excluded, all other left in. Colours indicate the different treatments.

We note however that both the spiked and naturally infected pollen had considerably lower Psa concentrations than on the plates and these two treatments showed the higher kill rates at low *ct* values. There is certainly the possibility that high Psa populations are "harder" (more difficult?) to kill than low concentrations i.e. there may be an interaction between ozone kill rate and Psa concentration. It is also possible there is a difference in the surface configuration or colony morphology between pollen growing on plates and on pollen.

Trial 4

Trial 4 again used naturally infected and spiked pollen together with Psa on plates. It targeted a slightly wider range of *ct* values than trial 3 while using only one ozone concentration (8ppm) so consequently used some longer exposure times.

It used the same naturally infected pollen as trials 2 and 3 with an initial viability 70-75%.

Pollen

Figure 17 shows the pollen viability data plotted against *ct* and with trial 1 data for comparison. In this case the plateau aligns well with the initial viability of 72% and we note that one of the original operators undertook the quantification in this trial. Only the highest *ct* points (*ct* = 987) deviate from the original curve and appear somewhat high.

conc 8ppm		Plates 7.0 x 10^7 cfu/mL					Spiked pollen 5.0 x 10^3 cfu/g			Natural pollen 5.0 x 10^2 cfu/g pollen					
time min	ct	Psa cfu/mL R1	Psa cfu/mL R2	Avg Psa cfu/mL	KillRate ofPsaV	survival PsaV %	Psa cfu/g pollen	KillRate of PsaV %	%survival PsaV	Psa cfu/g R1	Psa cfu/g R2	Psa cfu/g R3	Avg Psa cfu/g	KillRate ofPsaV	%survivalPsaV
4	33	$2.0x10^7$	$1.9x10^7$	$2.0x10^7$	72.1	27.9	$5.0 x 10^3$	0	100	250	300	250	265	47	53
9	75	$3.4x10^7$	$1.0x10^7$	$2.2x10^7$	68.6	31.4	$5.0 x 10^3$	0	100	150	50	100	100	80	20
13	106	$2.0x10^6$	$6.0x10^6$	$4.0x10^6$	94.3	5.7	$3.0 x 10^2$	94	6	50	65	45	55	89	11
25	204	$5.0x10^6$	$1.0x10^6$	$3.0x10^6$	95.7	4.3	$1.0 x 10^3$	80	20	50	50	50	50	90	10
50	411	$3.0x10^4$	$1.0x10^4$	$2.0x10^4$	100	0.0	$5.0 x 10^2$	90	10	25	15	20	20	96	4
120	987	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$	100	0.0	$2.5 x10^2$	95	5	5	10	0	5	99	1

Table 12. Treatments and results for trial 3. The initial Psa-V concentration is specified in the top box for each sample type.

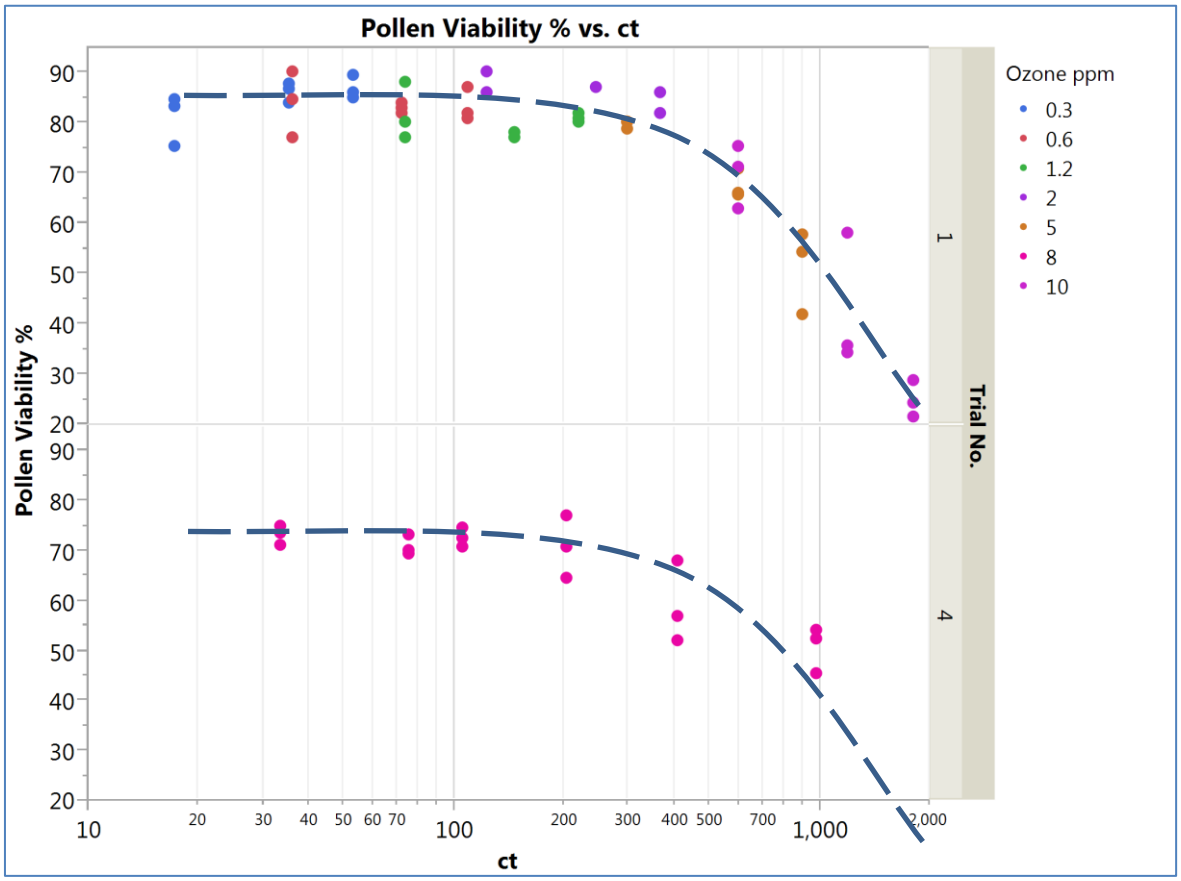

Figure 17. Pollen viability for Trial 4. Dashed lines superimposed from trial 1.

Psa

Figure 17 shows the Kill rate of Psa on plates, spiked and naturally infected pollen for Trial 4 plotted together with trial 3 results for comparison. There are a number of differences between the media:

1. The plate data is similar – both trials show very high kill rates at higher *ct* values but Trial 4 maintains a slightly higher kill rate at lower *ct*.
2. The kill rate of Psa on naturally infected pollen is again complete at high *ct* values but in Trial 3 this high kill rate continued to low *ct* values whereas in trial 4 the kill rate dropped at about the same rate as it did for Psa on plates.
3. The data is more variable for spiked pollen – there are essentially 2 values only:
    a. at high *ct* values there is (again) a very high mortality of Psa
    b. at low *ct* values (33 and 75) the kill rate dropped to 0%. The smooth fitted curve looks very similar to the previous panels but in fact the data has an abrupt step with the lower 2 values showing no mortality. This might be an issue of Psa infection variability in spiked pollen which we identified earlier.

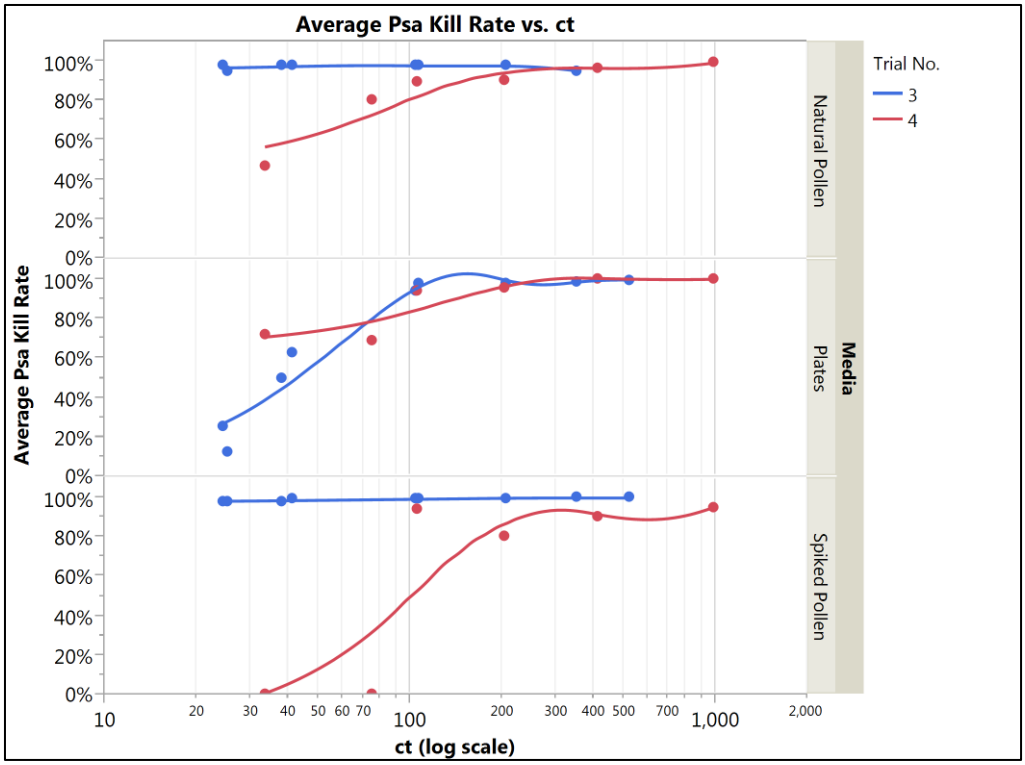

Figure 17. Trials 3 and 4 results for ozone kill rate of Psa on Psa on plates, naturally infected and spiked pollen. The outlier has been removed from trial 3.

Combined data

We can combine all the data for both Psa mortality and pollen viability by scaling the % pollen viability data in the way we introduced earlier i.e. we look at the % viability arising from the ozone treatment only by dividing by the initial viability. Figure 18 shows the combined data in 2 panels with smooth curves fitted to the data as well as the curve fitted by eye to the trial 1 kill rate data (dashed).

We have kept the two variables separate for clarity and we see that there remains a zone $150 < ct < 300$ in which we see very high Psa mortality as well as high pollen viability. The kill rate data on infected pollen from Trials 3 and 4 remained high through lower *ct* values that are below this zone i.e. the zone is a conservative estimate and it may be that ozone will kill Psa on infected Pollen at lower *ct* values than 150.

The fitted curve at the lower end is very different shape than that from Trial 1. Here we have left in all data but the one outlier discussed in Trial 3 and the curve shown is a statistical smooth fit. This has led to the unusual shape of the fitted curve. The lower scatter we have seen in the viability data has led to

the much smoother shape of the fitted curve in lower panel. In this case fitted curve is almost identical to the arbitrary curve we imposed on the Trial 1 data.

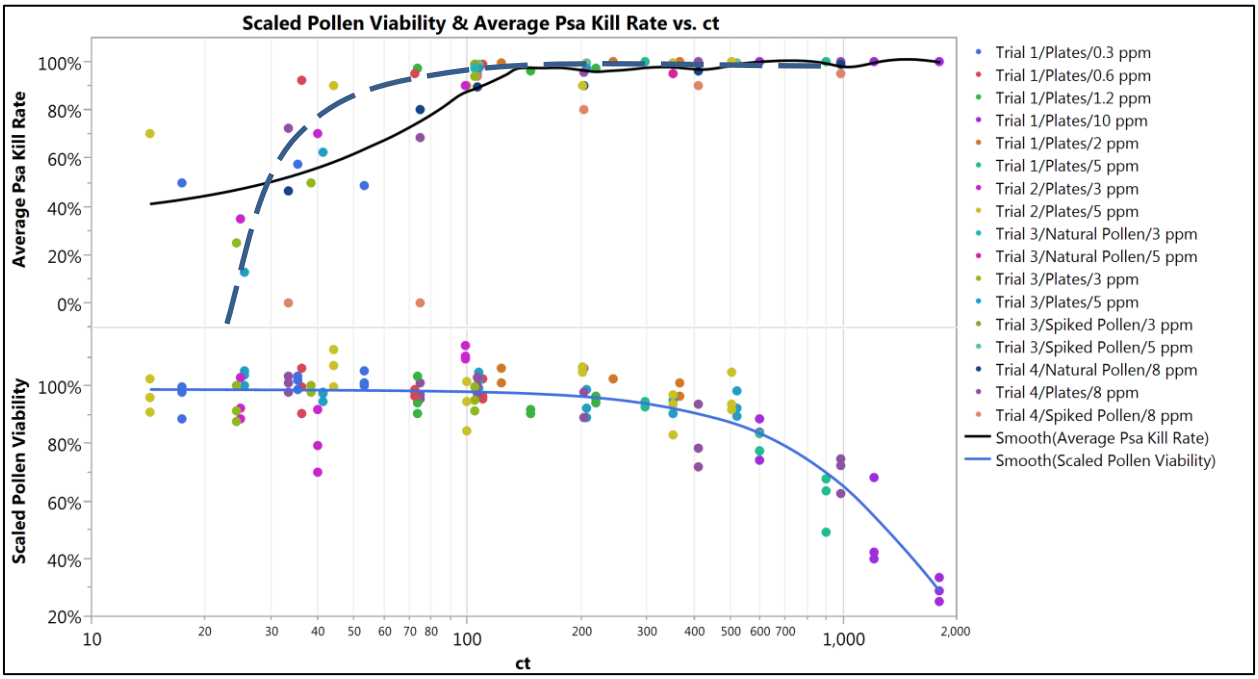

Figure 19. Combined data from all trials omitting only 1 statistical outlier. The sold lines are statistical smoothers and the dashed line in the top panel was fitted by eye to trial 1.

The combined data are shown on one set of axes in Figure 19. The pollen viability curve in this graph (red) is a little more variable than above since its statistical smoothing is now matched to the smoothing of the Psa mortality curve. Largely the waviness simply reflects the clumping of the data – it is simply under-smoothed for the data.

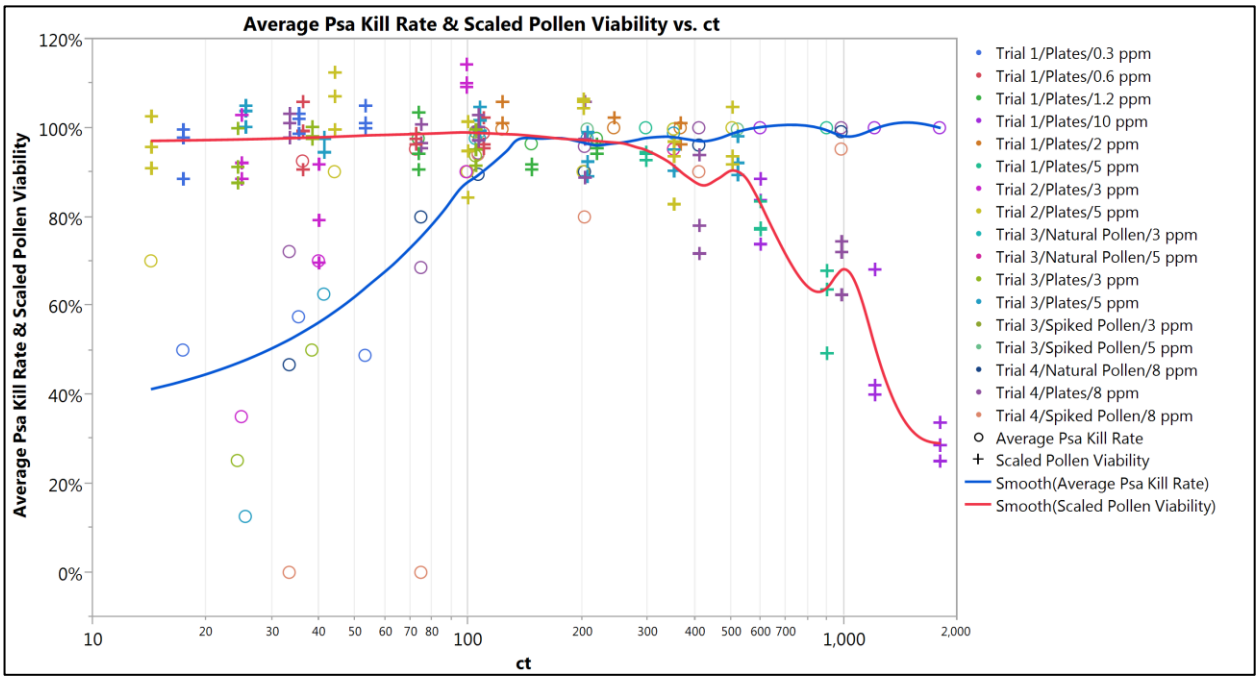

Figure 19. The data bar one outlier for both %pollen viability (red line) and Psa-V kill rate (blue line)

Finally, throughout this report we have used a logarithmic scale for *ct* in order to examine a wider range of values – especially at the lower end. For completeness we show the same data as Figure 19 plotted on a linear scale in Figure 20..

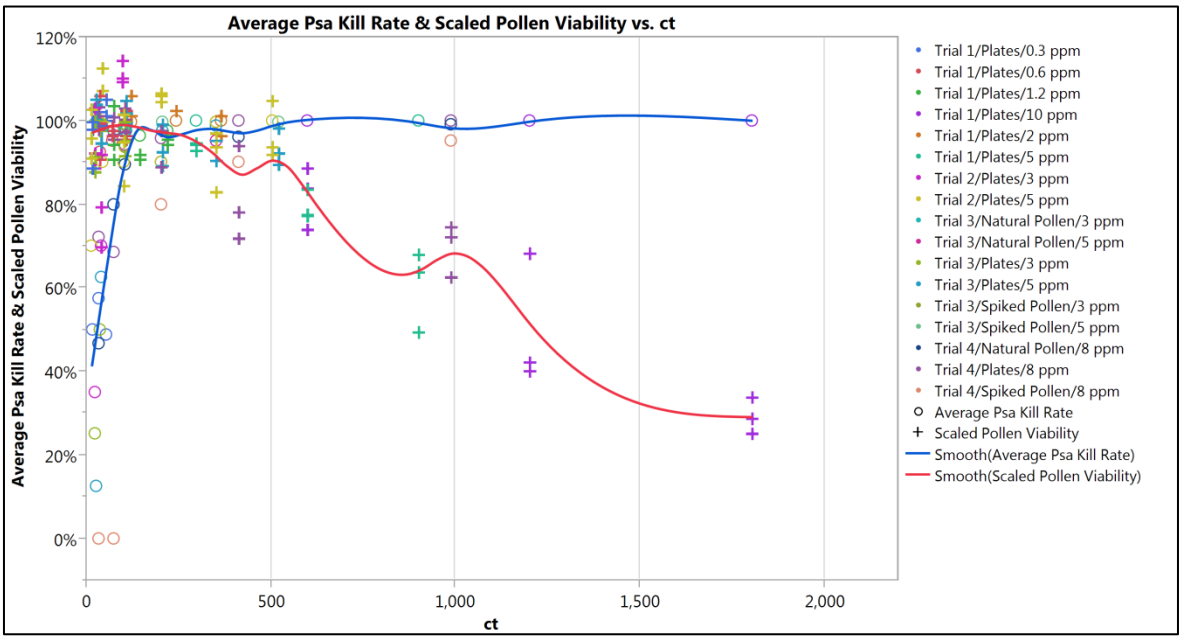

Figure 20. The complete data for both %pollen viability (red line) and Psa-V kill rate (blue line) plotted against *ct* using a linear scale

Summary

Methodology

1) Quantification of Psa-V from pollen seems independent of agitation time.
2) Within naturally infected pollen samples Psa-V a short agitation time seemed to give the best quantification although results were still quite variable even from a well-mixed pollen sample.
3) Quantification from replicate samples from a single batch of spiked pollen also gave vey variable results. For an average of 1.92 x 10^7 cfu/g pollen we had a standard deviation of 1.18 x 10^7 cfu/g pollen i.e. a CV 61% - very high variability.

Ozone trials

4) These trials were undertaken at near constant temperature (16°C) and humidity (78%). We expect changing these conditions will change the ozone efficacy.
5) Combining the two key efficacy variables of ozone concentration and time into a new variable (*ct*) appeared to work well in that it collapsed very different time x ozone concentrations into a single curve for both the effect of ozone on pollen viability and its effect on killing Psa-V.
6) The effect of ozone on pollen viability was very consistent through all our experiments. Viability started to decline at *ct* values > 300 ppm mins.
7) We can take out the effect of natural variations in pollen viability by looking only at the change in viability associated with the ozone experiment.
8) Generally the viabilities measured after the experiment were close to those characteristics of the pollen sample pre-trial. In one trial this was a little different (≈5 %) which we attribute to a new staff member undertaking the quantification.
9) The threshold when ozone was killing 90% of the Psa-V was at *ct* = 150 ppm min.
10) In some trials using infected pollen we had high Psa-V kill rates down to much lower *ct* values (≈40). These data may have been affected by RH or by differences in the level of infection between

plates, spiked pollen and naturally infected pollen. Finding a lower kill rate of Psa-V on pollen with a lower level of Psa-V contamination did not support the argument that higher concentrations will be harder to kill nevertheless this data was fragmentary and the question deserves further study.

11) Overall the experiment showed good consistency in all the pollen viability data and considerably more variability in the Psa-V kill rates at low *ct* values.
12) Given our experimental setup which kept both the air/ozone and the pollen continually well mixed it appears ozone may well provide a useful means of sterilising pollen.
13) More work needs to be undertaken with better temperature, RH and ozone control to confirm this result.

Possible Further Work

1) Some further exploration of agitation times would be valuable to confirm the optimum agitation time for maximum Psa-V recovery.
2) The effects of Temperature and RH on the ozone kill rate of Psa-V and pollen need to be explored – they were largely held constant in these experiments.
3) The variability in Psa-V quantification from spiked and naturally infected pollen invites further study. Spiking at a range of different levels would be one such experiment.
4) Further work looking at quantification at low Psa-V levels is required to try and pin down the kill rate curve. The naturally infected pollen we had did not have very high contamination levels so it normal dilution series used in quantification were not helpful - we were faced with the opposite problem - we needed more sample to give us more resolution in finding Psa-V at low concentrations.
5) From a number of aspects it would be useful to undertake experiments on a wider range of naturally infected pollen.
6) At a practical level we made numerous changes to the equipment we developed and we expect further development would be beneficial.