FINAL PROJECT REPORT

Project Title: PM Viability during postharvest handling of cherry fruit

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Total Project Request: **Year 1:** $ 62,507 **Year 2:** $ 57,987

Other funding sources: None

Budget 1
Organization Name: WSU-IAREC **Contract Administrator:** Hallie Faulk
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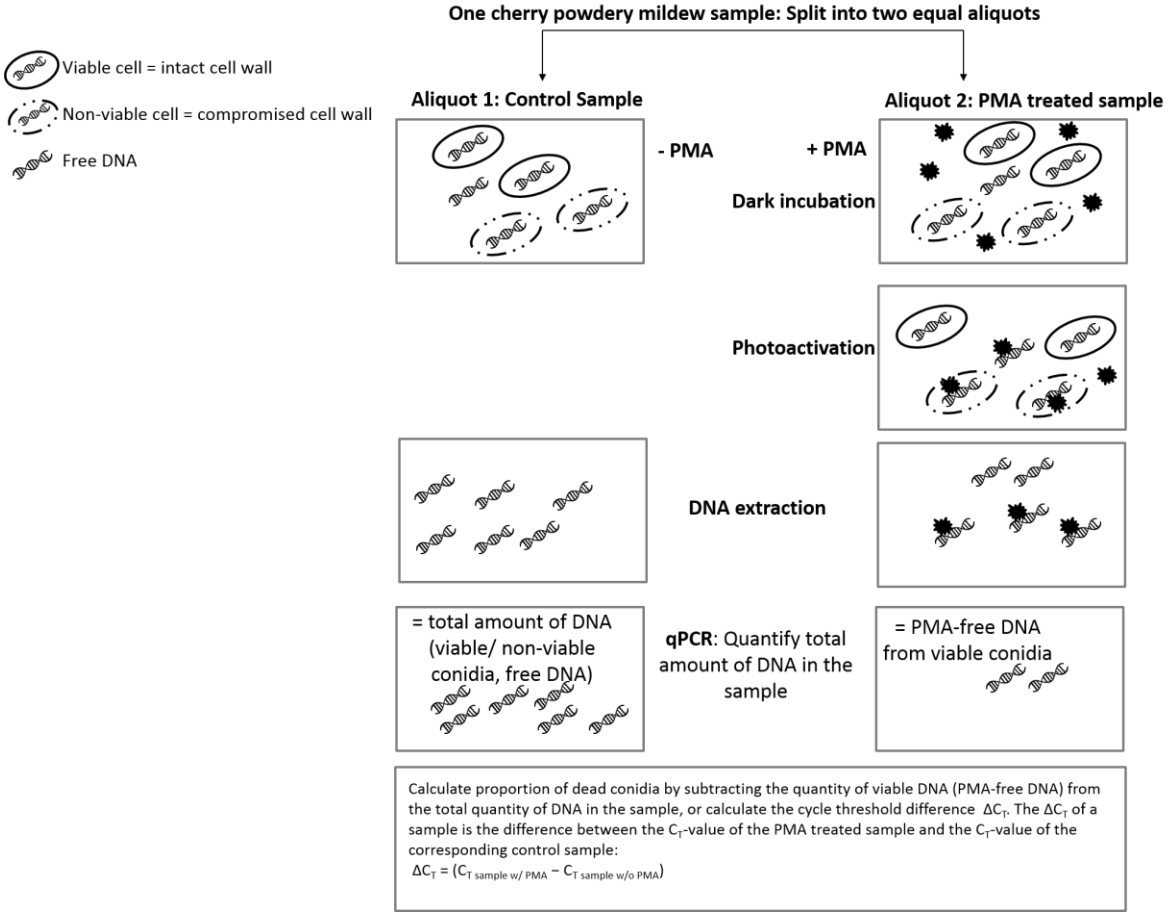
Item	2015	2016	2017
Salaries[1]	$ 34,620	$ 36,005	No-cost extension
Benefits	$ 14,887	$ 15,482	
Wages			
Benefits			
Equipment[2]	$ 5000		
Supplies[3]	$ 6500	$ 5000	
Travel[4]	$ 1000	$1000	
Miscellaneous [5]	$ 500	$ 500	
Plot Fees			
Total	$ 62,507	$ 57,987	

Footnotes:
[1]Associate in research
[2]PMA-Lite™ LED photolysis device, orbital plate shaker, multichannel precision pipettes)
[3]Reagents and material (anhydrous glycerol, DNA extraction kits, qPCR related and general lab supplies, Nitex cloth)
[4]industry wide travel to collect cherry fruit during various post-harvest handling stages
[5] shipping cost of cherry fruit during Washington State off-season to allow extended season research

Objectives

1. Development and validation of a robust viability assay using propidium monoazide (PMA) in conjunction with quantitative PCR to distinguish between viable and non-viable inoculum (conidia) of *Podosphaera clandestina*, the causal agent of cherry powdery mildew.

Recap: Propidium monoazide qPCR (PMA-qPCR) provides an indication of viability based on membrane integrity and the detection of nucleic acids. It can therefore be used for the detection of intact viable cells within a sample. Briefly, the sample which will be analysed is pre-treated with a nucleic acid binding dye (PMA that is considered to be membrane impermeable. During the treatment process the dye is upon photoactivation able to bind to extracellular (free) DNA or DNA from cells with compromised cell walls/membranes. This effectively removes the bound DNA from the sample as it cannot be amplified during the subsequent qPCR. This assay has therefore successfully been used to differentiate between intact and dead cells.
Several methodological parameters may affect PMA efficiency. Among them, 1) the concentration of the dye and 2) the length of photoactivation and of dark incubation appear to be key parameters. The purpose of this objective was to evaluate the efficacy and accuracy of the PMA qPCR procedure to determine conidia viability of cherry powdery mildew (PM). The general PMA qPCR workflow:



2. Quantify and monitor inoculum viability and identifying latent periods on sweet cherry fruit during fruit development and following customary post-harvest handling conditions.

Recap: The occurrence of powdery mildew (*Podosphaera clandestina*) on mature fruit severely limits export opportunities for growers and marketing entities. To date, we have not much insight on powdery mildew viability on harvested fruit, before and after methyl bromide fumigation prior to export, etc. This objective measured powdery mildew viability post-harvest (after methyl bromide fumigation) using the aforementioned PMA viability method.

Significant Findings

- Even if invisible to the eye, the amount of conidia (conidia) found on developing cherry fruit increases significantly week by week as soon as powdery mildew shows up on the leaves (Figure 1). The percentage of conidia that are viable on apparently healthy fruit is small (less than 10%, on average) but increases significantly at the onset of visible infection (up to 60% in 2016). Controlling the invisible increase of conidia mass on developing fruit with preventative fungicides seems imperative.

- Fumigation leads to a significant decrease of conidia viability. However, killing efficacy nearly never reaches 100%. Surviving conidia are capable of producing new infections on susceptible leaves (fresh leaves and fumigated leaves).

- Viability of conidia on fumigated cherries decreased to 0% after 3 days post fumigation (Table 7).

    - ***HOWEVER: The same trend was found for mildew survival on pruned branches. Therefore, it cannot be stated that the loss of viability was due to a delayed effect of the fumigation treatment.***

- Fumigated leaves support powdery mildew growth from both fumigated and fresh powdery mildew colonies. This shows that fumigation leaves a small percentage of viable conidia that can establish new infections if susceptible host tissue is present. Host susceptibility is not reduced by methyl bromide gas.

- Hydrocooling reduced the amount of powdery mildew conidia on fruit surfaces by an average of 56% (range from 44 to 68%). The greatest decrease of viability was achieved through a combination of cold storage and hydrocooling followed by fumigation (Table 6).

Results and Discussion for Objective 1

PMA™ = phenanthridium, 3-amino-8azido-5[3-(diethylmethylammonio)propyl]-6-phenyl dichloride. PMA solution (20mM in H_2O) can be purchased from the manufacturer (Biotium Inc., Hayward, CA). PMA can be diluted in sterile distilled H_2O to produce 2mM and 10mM stock solutions. Stock solutions should be aliquoted and stored in brown (light impermissible) microcentrifuge tubes at -20˚C until needed.

Validation of protocol to distinguish between viable and non-viable *P. clandestina* conidia. The discrimination between viable (live = conidia with an intact cell wall) and non-viable (dead = conidia with damaged cell walls) conidia is one key element of the here described viability qPCR methodology. The proper discrimination has to be validated for the organism of interest. Therefore, a

P. clandestina conidia suspension was freshly prepared and kept at room temperature under constant agitation on a standard laboratory magnetic stir plate. Immediately, aliquots (400µl) were pipetted into sterile 1.5ml light transparent Eppendorf tubes. Tubes were labelled 'Dead-PMA', 'Dead-Control', 'Live-PMA' and 'Live-Control'. *P. clandestina* conidia in tubes with the prefix 'dead' were killed by heat treatment for 2h at 85˚C in a standard laboratory water bath. Loss of viability was also examined by inoculating susceptible sweet cherry leaf disks (cv. Bing) with the heat-killed *P. clandestina* conidia suspensions. Leaf disks were incubated alongside a positive (+ viable conidia) and negative (+ sterile water) control for 21 days at 20°C in a diurnal growth chamber.
All tubes with the suffix 'PMA' were subjected to PMA treatment while tubes with the suffix 'Control' were left untreated. DNA extraction followed by qPCR was performed to calculate cycle threshold (C_T) values. The results of the dead and live conidia examination can be found in Table 1. The change measured in the viable population ("Dead-PMA" minus "Live-Control") is approximately 99.9% indicating that the heating protocol killed all conidia. These results are concordant with results from the leaf disk assay in which no growth had been observed from the heat killed conidia suspension. In order to demonstrate total recovery of conidia and DNA in the test, comparison of "Dead-Control" and "Live-Control" treatments were evaluated. The difference in C_T was small (mean = 1.0), indicating good recovery of all of the conidia/DNA. Finally, the comparison of the "Live-PMA" minus "Live-Control" indicates that a small part of the initial population of conidia were dead before heat treatment. Since *P. clandestina* is an obligate biotroph it can only be cultured on its host. By the time fungal growth is sufficient to carry out experiments (about 21 days), a mixture of old, possibly dead, and young, very viable conidia have formed on the leaves. The results of the "Live PMA" (which reflects the DNA from the intact/viable conidia) minus "Live Control" (which reflects the DNA from all conidia in the suspension) show this natural development. Overall, results indicate that the protocol is very well suited to differentiate between viable and non-viable conidia of *P. clandestina*.

Table 1. Mean cycle threshold (C_T) differences and standard deviation (STD) for viable (live) and heat killed (dead) *Podosphaera clandestina* conidia. Assay conditions: 10 min dark incubation, 20 min photoactivation, 20µl final PMA concentration

	Cycle threshold difference (ΔC_T)		
	Dead PMA - Live Control	*Dead Control - Live Control*	*Live PMA - Live Control*
Experiment 1	8.2	-0.6	3.1
Experiment 2	14.5	3.6	4.6
Experiment 3	10.7	0.04	6.1
Experiment 4	16.1	0.9	3.1
Mean	**12.4**	**1.0**	**4.2**
STD	3.6	1.9	1.5

Final PMA concentration: 20µM (e.g. add 4µl of the 2mM PMA stock solution to 400µl conidia suspension); **Dark Incubation interval:** 10 minutes (room temperature), **Photoactivation interval:** 20 minutes exposure to blue light emitting diodes (LED)

General protocol to evaluate sweet cherry powdery mildew viability on cherry fruit:
Step 1: Fruit sampling and washes. Thirty to forty cherries (per replicate) can be submerged in 500ml sterile water (1 L Pyrex bottles) containing 0.001% Tween20, a mild non-ionic surfactant and a dispersant to help distributing conidia evenly in the liquid. Bottles should be immediately placed on a rotary shaker at 300rpm for 5 to 10 minutes to rinse the fungus of the fruit surface. The resulting wash solution will contain conidia from the sample. Immediate sample processing is recommended.

Step 2: Filtration, PMA treatment, and DNA extraction. Each cherry wash solution is filtered through a 12-micron polycarbonate membrane using vacuum assisted filtration. Filter size (12 micron) is small enough to retain powdery mildew conidia contained within the wash solution and big enough to separate out bacterial and smaller fungal conidia, which will pass through the filter. The filter is removed aseptically with the help of sterile forceps, rolled loosely with the conidia containing side facing inward and placed into an 5mL Eppendorf tube containing 2mL of 0.001% Tween water. The tube is vortex vigorously for 2 min (e.g. on a horizontal vortex) to dislodge conidia from the filter membrane. Remove filter and discard. Vortex tube again and split the liquid into two 1.5mL clear (light permissible) centrifuge tubes (800µl per tube). Label tubes. The first tube will serve as the control sample to measure the total amount of DNA in the sample (see: general PMA qPCR workflow). Place this tube in the freezer until DNA extraction. The second tube will be treated with PMA to determine how many conidia in the sample are viable. Add 8µl of the 2mM PMA stock solution to 800µl conidia suspension under low light to achieve a 20mM final PMA concentration. Gently shake the tube and place in a dark box (or drawer) for a 10 min dark incubation period. Mix by inverting the tube halfway through the incubation period. After the incubation, gently mix the tube again and expose the conidia suspension to blue light emitting diodes (LED) using the PMA-Lite™ LED Photolysis Device (Biotium Inc., Hayward, CA) to activate and permanently cross-link the PMA dye to all accessible DNA. As a direct consequence of the irreversible crosslinkage, the targeted DNA cannot be amplified during the subsequent qPCR step. DNA extractions can be performed using any routine protocol (e.g. the MoBio UltraClean Microbial DNA isolation kit from MoBio Laboratory Inc., Carlsbad, CA) following the manufactures' protocol.

Step 3: qPCR assay.
A primer pair specific for *P. clandestina* and optimized for the use with qPCR was developed previously by Calabro (2007). The forward primer sequence: 5'CTCCACCCGTGTGAACTGA. The reverse primer sequence: 5'GAGGTCATCCAAAATATATGTGT. Quantitative real-time PCR (qPCR) assays can be performed, e.g., on a LightCycler 480 system (Roche, Indianapolis, IN) using 96-well plates in a total reaction volume of 20 µl. Each reaction consists of: 10 µl of SYBR Green mastermix (e.g. PerfeCTa® SYBR® Green FastMix® from Quanta Biosciences, Beverly, MA), 1µl of each primer at 400 nM final concentration, 3µl of PCR grade water and 5µl of template DNA. Each well of the 96-well plate is loaded manually with 20µl of the reaction mix and sealed with an adhesive cover before running the qPCR assay. A positive control with a known concentration of purified *P. clandestina* DNA and a no template control (NTC) are added to each assay for quality control. The qPCR assay is carried out according to the following protocol: Pre-incubation at 95°C for 10 min; 45 PCR cycles at 95°C for 10 s, 58°C (primer specific annealing temperature) for 20 s, 72°C for 30 s with fluorescent data collection; and a cooling period of 10 sec at 40°C. Melting curve analysis of the PCR products is conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artefacts. DNA samples, including unknowns, positive and NTC, are tested in duplicates. Results are recorded only if no signal was detected from the negative control. The instrument software can be used to calculate the threshold cycle (C_T) values using the second derivative method.

Standards are included in each qPCR assay in order to calculate the DNA concentration of the unknown samples. Purity and DNA concentration of the standards can be determined with a NanoDrop spectrophotometer (NanoDrop products Wilmington, DE). Five standards are prepared using a 10-fold dilution series. DNA from each dilution (tested in triplicates) serves as a template to construct a standard curve. The threshold cycle (C_T) values, linear regression coefficient (R^2), line equation and PCR efficiency (E) are calculated with the instrument software. The efficacy of the qPCR should be at least 90%.

Data analysis. At the end of each qPCR run, the amount of DNA per sample and the C_T value for each sample will be calculated by the instrument. C_T values are inversely proportional to the amount of DNA in the sample. Typically, C_T values below 29 cycles show abundant DNA, and C_T values above 38 cycles indicate minimal amounts. To evaluate the effect of PMA treatment on a sample, the cycle threshold difference (ΔC_T) is calculated. The ΔC_T of a sample is the difference between the C_T value of the PMA treated sample and the C_T value of the corresponding control sample: ΔC_T = (C_T sample w/ PMA − C_T sample w/o PMA). In general, a ΔC_T of about 3.6 is equivalent to a 10-fold difference in the concentration of the amplified organism.

Results and Discussion for Objective 2

Invisible conidia on developing fruit.
In 2017, clusters of developing fruit (cv Sweetheart) were removed weekly to assess natural disease pressure (Figure 1). During the duration of the sampling, cherry fruit were not visibly infected with powdery mildew. Hence, the natural disease pressure described in Figure 1 reflects the invisible increase of conidia (expressed as the amount of fungal DNA found on the cherries) up until the visible onset of PM signs in the orchard (June 26 - June 28). With the start of the foliar epidemic (May 24) in the orchard, the amount of conidia found on fruit increased significantly week after week. This increase was only halted during June 14 and June 21, where the amount of conidia found on fruit was significantly similar to each other. The ΔC_T difference between week 0 (May 17) and week 6 (June 28) is 6.0 (26 – 20 = 6.0) which equals an approximate 1000-fold increase in powdery mildew mass. This indicates that there were 1000 times more conidia on fruit on June 28 compared to May 17 (Figure 1). During the invisible infection time, powdery mildew is not active on fruit; viability is less than 10%. Hence, the increase of conidial quantities observed on developing fruit resulted from a continuous deposition of airborne conidia produced on infected leaves. However, most conidia deposited on the fruit do not remain viable. Viability of conidia increases significantly with the onset of visible fruit infection (fungus starts to grow and sporulate).

This confirms our findings from 2016 where the increase of powdery mildew conidia viability on fruit was measured. There was a 168-fold increase in sporulation (expressed as the amount of DNA quantified by qPCR) between June 18 and July 1, 2016 (Figure 2). Only 10% of conidia found on fruit were viable on June 18. This number increased to 62% by the beginning of July (Figure 2). In general, viability on developing cherry fruit was 10% or less on asymptomatic fruit. Once fruit becomes infected, conidia viability increases. Since powdery mildew colonies are dynamic and composed of mycelium and conidia (differing in age) conidia viability never reaches 100%. In times of rapid growth (visible ongoing infections) conidia viability is also greatest. However, even during latent periods (invisible infections), a small percentage of conidia always remain viable (between 2 and 10%). Controlling the increase in conidia through preventative fungicide sprays or manipulation of the orchard environment seems imperative. Spray coverage is key, conidia deposited on fungicide treated surfaces do not attach, and consequently, quantities are reduced.

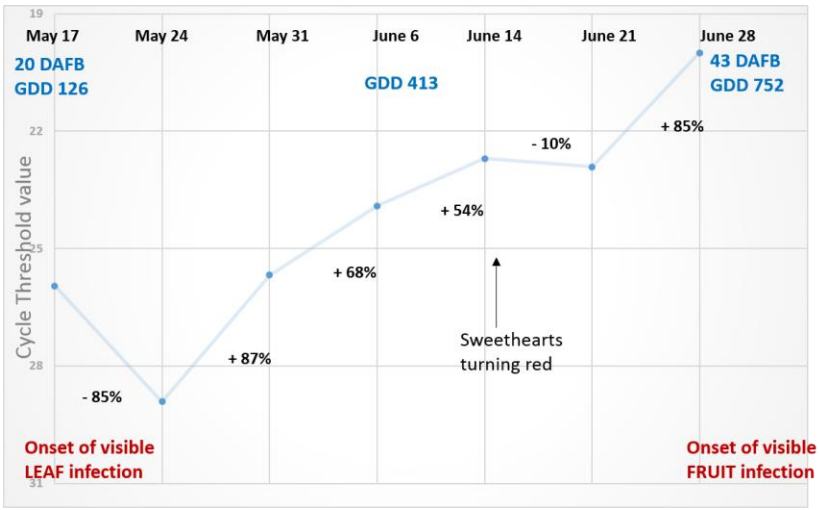


Figure 1. 2017: 6-week increase of conidia on developing Sweethearts at WSU-Prosser. Fruit were asymptomatic for powdery mildew (healthy looking) until June 28. Cycle threshold (C_T) values reflect the average quantity of DNA from *P. clandestina* conidia obtained from 60 cherries (15 cherries per rep, 4 reps). Lower C_T values indicate high amounts of DNA, while higher C_T values mean lower amounts of DNA. The values displayed next to the graph indicate % increase (or decrease) compared to the previous week. The biofix to calculate Growing Degree Days (GDD) was January 1st. DAFB refers to Days after Full Bloom.

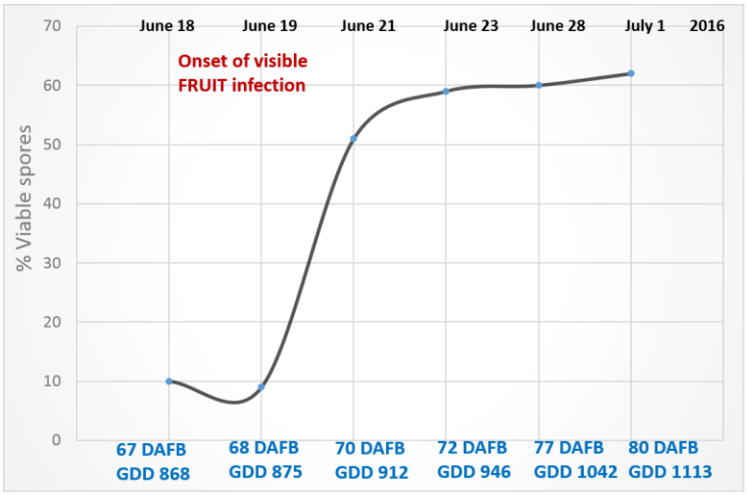


Figure 2. 2-week increase of powdery mildew conidia viability on Sweethearts in 2016. Cherries were grown at WSU (Prosser, Roza station, block D51). The onset of visible infection started between June 19 and June 21, 2016 (GDD 868-875). The onset of foliar infection started May 11 (GDD 399). Biofix for GDD is January 1st.

RESULTS related to Methyl Bromide Fumigation

Hydrocooling and conidial viability. The average time span for cherries to undergo commercial hydrocooling is about 3 minutes. This study investigated the effect of the chlorine found in the hydrocooling water on powdery mildew conidia viability. The results showed that a 24h exposure of powdery mildew conidia to hydrocooling water had no significant fungicidal effect. No decrease in viability was noted. However, hydrocooling is a very forceful event during which the fruit is in contact with the chilled water. We replicated the 3 min hydrocooling process in the lab by simply submerging the fruit in chilled hydrocooling water, moving them gently. As a result of this pre-fumigation fruit wash, quantities of conidia on sweet cherry fruit surfaces was reduced by an average of 56%.

Conidia survival after methyl bromide (MB) fumigation

Fumigation chamber protocol (for fruit intended to ship to Australia):
Chamber Temperature: 47°F (to less than 51°F)
Dosage of MB: 4.5 pounds of MB per 1000 cubic feet or 72 grams/ M^3
Cubic foot volume of chamber: 29,366

In 2016, the effect of fumigation on conidial survival was studied. Fruit were harvested at the experimental orchard (WSU, Roza, block D51) the day of the fumigation and transported to Zirkle Fruit in the late afternoon. Fruit were contained in a perforated, open box or in a single layer on a perforated plastic tray. Fumigation was conducted overnight. The next morning fumigated cherries were retrieved, bagged immediately to prevent cross-contamination and transported to the lab. Fumigated cherries were exclusively handled in a laminar flow hood (positive air flow). Samples were processed the same day. There was significant variation in the reduction of conidia viability observed in bulk processed cherries (cherries in a box). Viability ranged from 81.8% to 98.8% (Table 2 and Table 5). When analyzed layer by layer, cherries sampled from the middle layer (layer 3) had significantly less dead conidia after fumigation (Table 2). In the same study, removing the stem from the cherry prior to fumigation had no significant effect. However, there was a trend that stem removal increased the efficacy of fumigation which is likely due to a sheltering effect of the stem to the conidia. Fumigation was less successful on cv 'Sweetheart' compared to cv. 'Bing' cherries (mean reduction of 87.5 versus 99.3% with stems attached and 86.7 versus 99.6% with stems removed) (Table 2).

Table 2 Efficacy of methyl bromide in reducing powdery mildew viability on cherry fruit fumigated in bulk (box) or single layers (tray)

			Run[a] 1	***Run 2***	***Mean***
Trial	**Cultivar**	**Position in box**	**% dead conidia after fumigation**		
Box - Trial 1	Bing	Random	96.4	97.2	96.8
Box - Trial 2	Bing	Top	96.8	98.0	97.4
		Layer 1	98.5	99.9	99.7
		Layer 2	96.4	89.3	94.0
		Layer 3	92.7	50.5*	81.0
		Layer 4	96.8	99.3	98.5
		Bottom	97.5	99.8	99.3
		Mean	**96.5**	**89.5**	**98.0**
			Run1	***Run 2***	***Mean***
Trial	**Cultivar**	**Stem^**	**% dead conidia after fumigation**		
Tray- Trial 1	Bing	+	94.4	93.6	94.0
Tray- Trial 2	Bing	+	99.6	99.4	99.5
Tray- Trial 3	Sweetheart	+	87.9	87.1	87.5
		Mean	**97.8**	**97.2**	**97.5**
Tray- Trial 2	Bing	-	99.7	99.5	99.6
Tray- Trial 3	Sweetheart	-	87.1	86.2	86.7
		Mean	**94.4**	**93.8**	**94.1**

[a] Each sample was tested in duplicates in two independent qPCR analyses (Run 1 and Run 2). The reported value is the average of three replicates (30 cherries each) per sample (per tray or per layer in the box).
^ The stem was either removed (-) or left attached (+) to the fruit before fumigation.
* Value is statistically different from the others

As can be seen in Table 3, the fungicidal effect of methyl bromide on single cherries varies. For some cherries, none (0%) or few (0.1%) of conidia survived the gas treatment while survival rates on other cherries reached 30%. Since cherries from Experiment 1 and 2 were randomly chosen from a box, higher survival rates may be explained by the position of the cherry in the box as could be seen in Table 2. The same variability was seen for cherries fumigated in bulk (box) where survival ranged from 1% to 16.7% and for cherries fumigated in a single layer on a tray where survival ranged from 3.6 to 12.9%.

Results in Table 5 show the effect of cold storage and hydrocooling followed by fumigation on conidia survival. **The sole process of submerging cherries in hydrocoolant water for 3 min before fumigation reduced the average conidia number by 56%.** Both cold storage and hydrocooling reduced conidia viability even without fumigation. The combination of cold storage, hydrocooling and fumigation resulted in the largest amount of dead conidia (99.8 to 100%) in cherries fumigated on a tray (Table 4) and 92.2 to 99.4% in cherries fumigated in a box (Table 5).

Table 3 Fungicidal effect of methyl bromide viability measured on single sweet cherries

	Experiment 1 [^]	***Experiment 2***[^]	***Experiment 3***[~]
Workflow: Orchard →Fumigation	**Outcome: % dead conidia after fumigation**		
Tray, single layer[a]	96.4	90.6	87.1
Box, multiple layers (bulk) [a]	93.6	99.0	83.3
Cherry 1	**70.3***	98.9	100 [b]
Cherry 2	97.7	99.9	99.9
Cherry 3	92.3	98.3	99.9
Cherry 4	99.8	**72.2***	99.7
Cherry 5	99.3	99.1	95.9
Cherry 6	72.2	99.6	89.3a
Cherry 7	99.3	99.4	91.7
Cherry 8	97.2	99.6	99.1
Cherry 9	99.1	99.6	99.6
Cherry 10	100.0	99.8	n/a
Cherry 11	99.8	99.9	n/a
Cherry 12	99.9	87.9	n/a
Cherry 13	99.9	99.4	n/a
Cherry 14	97.0	97.8	n/a
Cherry 15	99.9	99.9	n/a
Cherry 16	99.5	87.9	n/a
Mean (single cherries)	**95.2**	**96.2**	**97.2**
STD (single cherries)	9.6	7.5	4.1

[a] Mean of three replicates (tray) or five replicates (box). Each replicate contained 30 cherries.

[^] Single cherries were obtained randomly from a bulk sample (fumigated in a box)

[~] Single cherries were fumigated in an open clamshell container, n/a – not available

* Value is statistically different from the others

In 2016, we observed that conidia harvested from fumigated cherries were not able to establish infections on fresh, susceptible leaves even though conidia viability was never reduced to 0%. One

possible explanation could be that the effect of the gas has a delayed effect on membrane integrity. A similar effect has been observed for radiated conidia (e.g. UV radiation) during which the conidia dies but the cell membrane stays intact for a period of time before the cell disintegrates. In 2017, the focus of the fumigation study was to confirm or refute this effect on fumigated conidia. To this end, cherries were fumigated and subsamples were processed for up to 3 days post fumigation starting immediately after fumigation (like in the previous year) and every 24h thereafter. The results are shown in Table 6: Fumigation significantly reduced conidia viability (96.2 to 99.8% conidia dead after fumigation). Viability slowly declined within the following 48h and reached 0% after 72h (3dp fumigation) in all three experiments. However, if methyl bromide fumigation indeed kills all conidia and the % viable conidia detected by the PMA assay are dead conidia with an intact cell membrane but no cell function, then these fumigated conidia should not be able to infect susceptible leaves (as was observed in 2016). To this end, branches with vigorously growing powdery mildew colonies were cut from orchard trees, kept in water and fumigated. Fumigated powdery mildew inoculum was used in leaf disk assays using a) fresh, susceptible leaves, and b) fumigated, susceptible leaves. As a comparison, non-fumigated powdery mildew conidia were used to inoculate a) fresh, susceptible leaves, and b) fumigated susceptible leaves. The four combinations tested were: Fumigated Inoculum/Fumigated Leaves = double negative; Fumigated Inoculum/ Fresh Leaves; Fresh Inoculum/Fumigated Leaves; Fresh Inoculum/ Fresh Leaves = positive control. Powdery mildew grew in all leaf disk assays regardless whether the fungus or leaves were fumigated. ***It can be concluded that fumigation does not kill all the conidia but leaves a small percentage of survivors that are capable of initiating disease if presented with a susceptible host. Also, Fumigation does not affect leaf susceptibility***. A useful reminder: A 99.9% reduction would reduce 1,000,000 conidia to 1,000 conidia. A 99.99% reduction would reduce 1,000,000 down to 1 conidia. A single conidium is enough to initiate an area wide epidemic.

Table 4 Comparative fungicidal effect of stem cold storage and hydrocooling followed by methyl bromide fumigation on powdery mildew viability

Workflow: *Orchard*	*→ Pre-Treatment*			*→ Fumigation*	***Outcome:*** ***How many conidia are still alive?***		
Harvest cherries	**Stem**[a]	**Cold**[b]	**Hydrocooled**[c]	**Fumigated**[d]	**Experiment 1**	**Experiment 2**	**Experiment 3**
					% dead conidia before fumigation		
Control[e]	+	No	No	No	64.1	53.6	78.5
Control	+	**Yes**	No	No			99.9
Control	+	No	**Yes**	No	91.2	82.2	
Control	+	**Yes**	**Yes**	No			99.5
					% dead conidia before fumigation		
Fumigated	+	No	No	**Yes**	90.6	87.1	
Fumigated	-	No	No	**Yes**	99.4	86.2	
Fumigated	+	**Yes**	No	**Yes**	40.0		98.8
Fumigated	-	**Yes**	No	**Yes**	43.8		94.0
Fumigated	+	No	**Yes**	**Yes**		98.2	99.9
Fumigated	-	No	**Yes**	**Yes**		94.0	99.8
Fumigated	+	**Yes**	**Yes**	**Yes**		99.9	99.9
Fumigated	-	**Yes**	**Yes**	**Yes**		100.0	99.8

[a] The stem was either removed (-) or left attached (+) to the fruit before fumigation.

[b] Cherries were stored in a cold room (4°C, 39°F) for 4 to 6 hours before fumigation.

[c] Cherries were submerged in chilled hydrocooler water (39°F) for 3 min before fumigation.

[d] Cherries were fumigated in a single layer on a plastic tray.

[e] Control was harvested, treated and evaluated. Reflects viability of conidia on orchard fruit before fumigation.

Table 5. Efficacy of methyl bromide (alone or in combination with cold storage and hydrocooling) in reducing powdery mildew viability on sweet cherries fumigated in bulk (perforated, open box)

	Experiment 1 *No Pre-treatment*[b]	***Experiment 2*** *No Pre-treatment*[b]	***Experiment 3*** *No Pre-treatment*[b]	***Experiment 4*** *+ Cold & Hydrocooling*[c]	***Experiment 5*** *+ Cold & Hydrocooling*[c]
	% dead conidia before fumigation				
Control[a]	90.1	64.1	50.5	78.5	72.2
Position in box	**% dead conidia after fumigation**				
Top layer	98.0	77	56.4	77.0	100.0
Middle layer	99.1	74	79.8	99.9	99.6
Bottom layer	99.3	94.4	79.8	99.6	98.7
Mean[d]	**98.8**	**81.8**	**72.0**	**92.2**	**99.4**
STD[d]	**0.7**	**11.0**	**13.5**	**13.1**	**0.7**

[a] Control was harvested, treated and evaluated. Reflects viability of conidia on orchard fruit before fumigation.

[b] Cherries went straight from the orchard to fumigation

[c] Cherries were stored in cold storage (39°F) for 6 hours and submerged in chilled (37°F) hydrocoolant water for 3 minutes before fumigation.

[d] Mean of 3 reps with 30 cherries each per layer; combined Mean of 270 cherries per box; STD = Standard Deviation

Table 6. Time-course development of conidial viability after methyl bromide fumigation

	Experiment 1		***Experiment 2***		***Experiment 3***	
	ΔC_T[a]	% dead[a]	ΔC_T[a]	% dead[a]	ΔC_T[a]	% dead[a]
Control, pre-fumigation^	1.5	**61.7**	2.0	**72.2**	1.0	**47.2**
0 dp fumigation*	8.7	**99.6**	5.1	**96.2**	9.6	**99.8**
1dp fumigation	11.4	**99.9**	6.9	**98.8**	11.5	**99.9**
2dp fumigation	9.3	**99.7**	7.9	**99.4**	8.8	**99.6**
3dp fumigation	12.5	**100.0**	11.9	**100.0**	12.5	**100.0**

[a] average of 8 replicates with 30 fruit per replicate. $\Delta C_T = (C_{T\ \text{sample w/ PMA}} - C_{T\ \text{sample w/o PMA}})$. In general, a ΔC_T of about 3.6 is equivalent to a 10-fold difference in the concentration of the amplified organism.

^ Percentage of dead powdery mildew conidia found on a freshly picked and visibly contaminated orchard sample (4 reps, 30 fruit each) before fumigation.

* Fumigated samples were picked up at Zirkle Fruit at the end of the fumigation cycle and processed immediately (0dp), or stored at 4°C for 24h (1dp), 48h (2dp) or 76h (3dp) before processing.

Executive Summary:

Conventional methods to identify Podosphaera clandestina, the causal agent of sweet cherry powdery mildew, are based on cultural and morphological characteristics (phenotype), and quantification is mainly based on spore counting or dilution plating. These methodologies are time-consuming, require individual expertise in fungal taxonomy and are not able to differentiate between live and dead conidia. However, the question whether the fungus is dead or alive bears great importance for cherry powdery mildew research. Propidium monoazide qPCR (PMA-qPCR) provides an indication of viability based on membrane integrity and the detection of nucleic acids. It can therefore be used for the detection of intact viable cells within a sample. In this study the PMA-qPCR methodology was optimized, validated and applied to quantify viable cells of *P. clandestina* in naturally infected samples, after various post-harvest handling (cold storage, hydrocooling) of the fruit and after methyl bromide fumigation.

Conidia can be found on fruit with the onset of foliar powdery mildew. Even if invisible to the eye, the amount of conidia found on developing cherries increases significantly week by week. The fungus is inactive during this invisible time of infection and the increase of conidial numbers on fruit is likely due to a constant deposition of conidia originating from infected leaves. Even if foliar infection is well controlled, or not visible in the orchard, airborne propagules can be carried by the wind from nearby, infected orchards. Or infections can be hiding on the orchard floor (unmanaged root suckers) or high up in the upper canopy (if spray regimes do not cover the upper canopy as well as the lower canopy). The fungus has many ways of coming into an orchard. The fungal inactivity on fruit during the invisible infection period is also shown by the small percentage of viable conidia (less than 10%, on average). Conidial viability on fruit increases significantly when infection switch from invisible to visible. Visibility indicates that the fungus is actively growing. Powdery mildew control has to start at the invisible time of infection. Spray coverage is key.

Fumigation leads to a significant decrease of conidia viability. However, killing efficacy nearly never reached 100% and left a small percentage of survivors that were capable of initiating disease if presented with a susceptible host. The greatest decrease of viability was achieved through a combination of cold storage and hydrocooling followed by fumigation. A three-minute immersion in chilled, hydrocoolant water reduced the amount of powdery mildew conidia on fruit surfaces by an average of 56%. The hydrocoolant water itself had no significant fungicidal effect. Methyl bromide did not affect leaf susceptibility; the fungus was able to grow on leaves exposed to the gas for the length of a fumigation cycle. However, conidial viability decreases with time leaving all conidia dead after 72h (post fumigation). The same pattern of conidial die off was seen in pruned (infected) branches left on the orchard floor. Therefore, it cannot be concluded that the decrease in viability was caused by the fumigation but seems to be the natural course of conidial die off on a non-viable host.