FINAL PROJECT REPORT **YEAR**: 3 of 3 (No cost extension approved in 2018)
WTFRC Project Number: CH-16-104

Project Title: ABC of sweet cherry powdery mildew: adaption, behavior and control

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Total Project Request: **Year 1:** $81,321 **Year 2:** $82,187 **Year 3:** $84,435

Other funding sources: None

Budget 1
Organization Name: WSU-IAREC **Contract Administrator: Tim Palacios**
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Item	2016	2017	2018
Salaries[1]	36,504	37,964	39,483
Benefits[1]	17,522	18,223	18,952
Wages			
Benefits			
Equipment			
Supplies[2]	25,000	25,000	25,000
Travel[3]	1000	1000	1000
Miscellaneous[4]	1295*		
Plot Fees			
Total	81,321	82,187	84,435

Footnotes:
[1]Associate in Research
[2]Molecular supplies (DNA extraction, sequencing costs, PCR and qPCR related chemicals, primer development), Nitex mesh for *in vivo* studies, general supplies for greenhouse and laboratory (petri dishes, agar), fungicides
[3]Sampling trips through Washington and Oregon State
[4]Geneious Software license, international shipping of DNA samples

Recap of original objectives

1. **Adaptation:**
   a. Isolate and characterize cherry powdery mildew populations in commercial orchards in WA and OR
   b. DNA based identification of the causal agent of cherry powdery mildew and multigene phylogenetic reconstruction of the evolutionary relationships among global cherry mildew entities
2. **Behavior:**
   a. Compare virulence structures of identified clades/ subgroups
   b. Identify niche (host tissue) preferences
   c. Identify reproductive strategies and, if heterothallic, mating type frequencies
3. **Control:**
   a. Evaluate response of powdery mildew spores to fungicide sprays before fruit infection is established (visible) using viability qPCR
   b. Identify critical spray periods in which fungicidal protection is most needed to suppress onset or minimize severity of fruit infection

Significant Findings

ADAPTATION

- 93 l isolates of *P. clandestina* were collected in Washington State and Oregon in 2017**.** An Additional 55 isolates were collected throughout WA and OR state in 2018. The focus of 2018 collection effort was to include isolates from distinct geographic locations throughout WA and OR. In 2019, the next-generation and Sanger sequencing were performed to evaluate genetic similarity of these *P. clandestina* isolates. Overall, one hundred and forty-eight isolates of *P. clandestina* were genetically evaluated during the project period.

- Representative *P. clandestina* isolates collected in 2018 are presented in Table 1. Phylogenetic analysis of partial ITS nucleotide sequence showed that all *P. clandestina* isolates (except one from Mexico) clustered together in a separate clade confirming their high level of genetic relatedness. The result indicated that only *P. clandestina* is the causal agent of powdery mildew in cherry orchards of the Pacific Northwest (PNW).

- A few representative isolates were used in cloning and sequencing of the *cytochrome b* gene, a target gene of QoI class (FRAC Group 11) fungicides. This served two purposes: 1) An additional gene for isolate characterization and 2) Fungicide resistance based on molecular target identification.

BEHAVIOR

- Two distinct foliar mildew growth habits were discovered. Fruit mildew growth patterns were different than those found on leaves. The two distinct growth habits of *P. clandestina* isolates were found to be genetically identical and did not differ in fungicide sensitivities. In the leaf-disc assays, growth habits of A and B type are interchangeable.
- We found evidence of mildew infection on leaves (most common), fruit (often seen), buds (green tissue) and, pedicels (fruit stem) but not on flowers.
- Infections were most severe on sucker shoots and young leaves in the upper canopy.
- Chasmothecia (the overwintering propagule) were found at all locations. Mating type genes were identified. Opposite mating-type genes, alpha box domain (MAT1-1) and HMG (MAT1-2) were found in all composite isolates collected in WA and OR. The presence of both mating-types in the orchard reiterates its significance in chasmothecia production.

CONTROL

- Foliar and fruit mildew disease pressure was monitored throughout the season. Two distinct peaks of increased conidia were identified in fruit infections. This information can be used to better time fungicide applications.
- Pilot-scale spray coverage was also studied to find out appropriate spray volumes using a water-soluble traceable dye. The current spray methods may not give adequate coverage on foliage higher in the canopies. The severe infestations in upper portions of the tree add significantly to the inoculum load, promote the spread of the pathogen, and provide a reservoir of chasmothecia to ensure available primary inoculum during the subsequent growing seasons.
- Although not a direct objective of this project, we used the opportunity to identify fungicide resistance using a pilot scale leaf-disc bioassay against different FRAC groups and molecular characterization of FRAC Group 11 (QoI) target gene, CytB. The results indicated presence of resistant isolates in Washington/ Oregon orchards. A separate project co-funded by Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission is currently investigating the nature, extent, and mitigation of fungicide resistance to different FRAC groups.

<u>Additional work accomplished:</u> Effects of postharvest treatments on harvested fruit in PNW

To aid in cherry export quarantine process to Australia and other countries requiring fumigation, we measured conidia viability on fruit after various applications of postharvest treatments. The results indicated the effectiveness of current PNW postharvest industry standards in removing all live and dead conidia. Specifically, hydrocooling removed more than 98% conidia and the remaining 2% were killed by fumigation and removed during packaging. The experiments were conducted in 2018 and results were published (Swamy, Probst, and Grove 2019). The final definitive results were further confirmed in 2019 using highly sensitive qPCR methods.

Methods, Results & Discussion

Adaptation and Behavior

Next-generation sequencing of P. clandestina isolates

High-throughput next generation sequencing of foliar powdery mildew isolates were performed in 2018. A minimum of 20 million reads were obtained from each sample indicating a greater depth in sequencing of *P. clandestina*. The reads were assembled in CLC Genomics Workbench platform (Qiagen, Redwood City, CA) and used in identification of full-length internal transcribed spacer (ITS) sequences. Additionally, the sequences were used to identify the full-length cytb gene, a target for QoI (FRAC group 11) fungicides. The sequence alignment and bioinformatics results indicated that most of the isolates used in the sequencing experiment harbored a mutation at position 143 of the cytb amino acid sequence, indicating a resistant isolate. The reads obtained in this study are also being used in 2019 co-funded project 'Fungicide Resistance: A Vital Need To Protect PNW Cherries From Mildew'

Phylogenetic analysis of P. clandestina isolates

Internal transcribed spacer (ITS) region analysis in 2017 and 2018 of all isolates exhibited high sequence identity confirming a single pathogen as the sole incitant of powdery mildew in Washington and Oregon. ITS sequences were cloned and sequenced in 2018 for fuller coverage spanning the entire ITS region. Sequence analysis indicated that all isolates were nearly identical (>99% identity) confirmed genetic uniformity of the pathogen across the PNW. This was confirmed by phylogenetic analysis of partial ITS nucleotide sequences from representative *P. clandestina* isolates collected in

2018. All *P. clandestina* (except one from Mexico) clustered together in a separate clade confirming their high level of genetic relatedness (Figure 1).

In addition to previous powdery mildew isolate DNA collections, several new orchard sites were identified and *P. clandestina* isolates were collected from these locations. During morphological analysis of mildew isolates, two distinct foliar mildew growth habits were apparent. They were often present in mixed populations. Morphological observations such as conidiophore branching, length and width of each conidial type were investigated. A-type mildew was identified as being highly branched conidiophore arrangement. The conidia were larger in size compared to B-type (Figure 2). B-type was characterized as most widely present mildew with many conidia in linear chains. Each conidiophore contained only one conidial chain. Each isolate collected in 2018 was morphologically characterized (Table 1). The role of both growth habits in the epidemiology of powdery mildew requires further study.

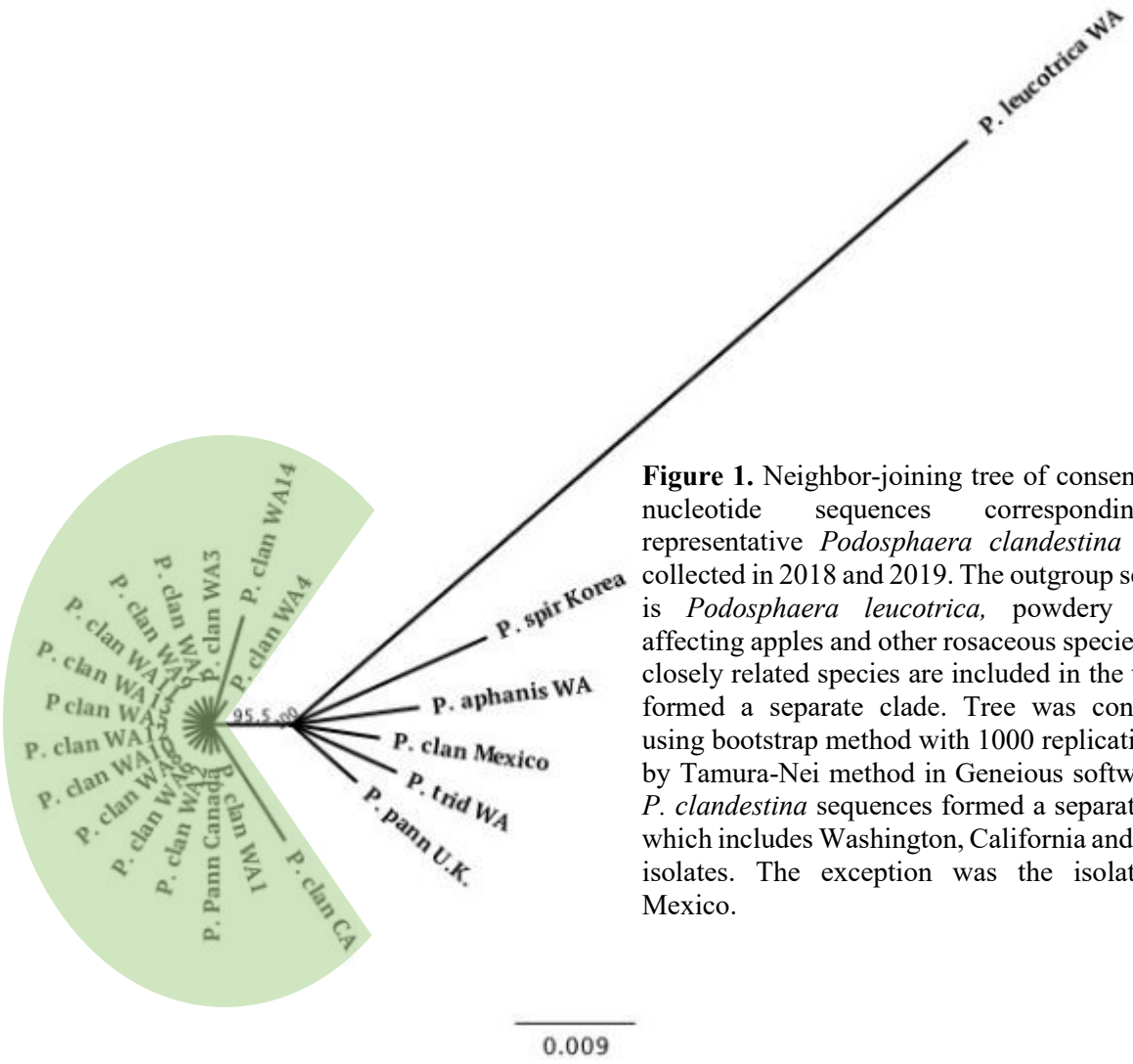

Figure 1. Neighbor-joining tree of consensus ITS nucleotide sequences corresponding to representative *Podosphaera clandestina* isolates collected in 2018 and 2019. The outgroup sequence is *Podosphaera leucotrica,* powdery mildew affecting apples and other rosaceous species. Other closely related species are included in the tree and formed a separate clade. Tree was constructed using bootstrap method with 1000 replications and by Tamura-Nei method in Geneious software. All *P. clandestina* sequences formed a separate clade, which includes Washington, California and Canada isolates. The exception was the isolate from Mexico.

CPM-A *Globular* CPM-B *Filamentous*

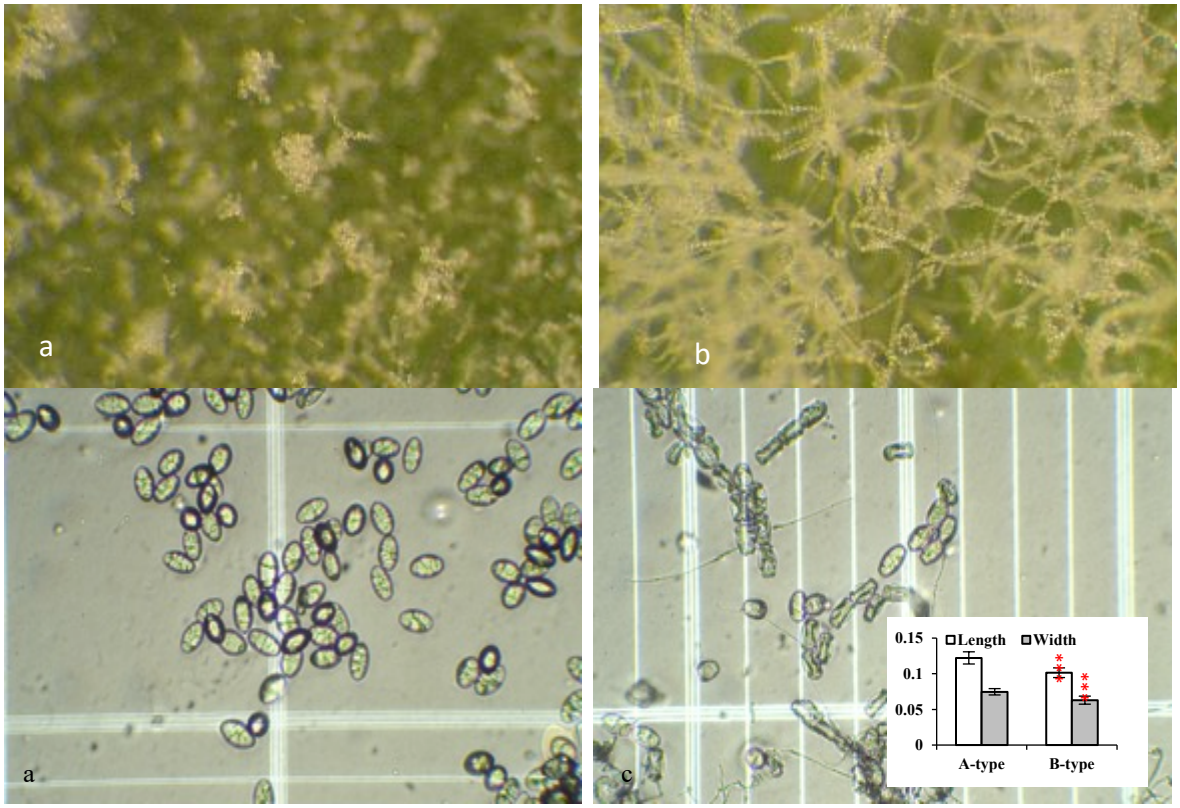

Figure 2. Note globular and highly branched CPM-A (a). Long linear chains observed in CPM-B (b). The sticky tape analysis exhibit slightly longer (and thicker) conidia of CPM-A (c) compared to CPM-B (d, e). Note the arrangement of conidia in both types. Significant differences (*asterisks) was determined using *t-test* ($p<0.001$).

Table 1. Cherry powdery mildew (*P. clandestina*) collections in 2018**.**

Region	Code	Host variety	Mildew type	ITS confirmation
Okanogan/ North Central Washington	BP-1	Lapins	A+B	Yes
	BP-2	Lapins	B	Yes
	OV-1	UN	B	No
	BR-1	UN	B	No
Chelan	MS-1	Bing	B	Yes
	MS-2	Bing	B	Yes
	MS-3	Bing	A+B	Yes
	MS-4	Bing	B	No
	MS-5	Bing	B	Yes
	MS-6	Skeena	B	Yes
	HF-1	UN	A+B	No
	MA-1	UN	B	No
Wenatchee	St1	Skeena	A+B	Yes
	St2	Skeena	A+B	Yes
IAREC Headquarters (Prosser, WA)	Roza-1	Bing	A	Yes
	Roza-2	Bing	B	No
	C-9	Bing	Fruit PM	No
Yakima Valley	MH-1	Lapins		Yes
	MH-2	Rainier	A+B	Yes
	MH-5	Bing	B	Yes
	MH-6	Bing	B	Yes
	MH-7	Lapins	B	No
	HT-1	Rainier	B	Yes
Columbia Basin	JP-1	Bing	A+B	Yes
	HL-1	Tieton	B	Yes
	HL-2	Santina	A+B	Yes
	HL-3	Santina	B	Yes
	HL-4	Bing	B	Yes
	HL-5	Rainier	B	Yes
	HL-6	Rainier	A+B	No
Lower Columbia Basin	DH-1	Bing	B	Yes
	DH-2	Bing	B	Yes
	DH-3	Bing	B	Yes
	DH-4	Bing	A+B	Yes
	DH-5	Bing	B	Yes
Dallesport area/ Oregon State	TP-1	Skeena	A+B	No
	SC-2	Skeena	B	Yes
	SC-4	Sweetheart	A+B	Yes
	SC-6	Rainier	A+B	Yes
	SC-7	Rainier	A+B	Yes
	AR-3	Bing	A+B	No

Mating-type and heterothallism

Chasmothecia, the overwintering propagules of *P. clandestina*, are an essential component in the epidemiology of powdery mildew (Grove and Boal 1991) in the PNW. Chasmothecia production depends on recognition of a mating type in the development of sexual structures, such as ascospores. This process is regulated by genes at mating-type loci in the fungal genome. In powdery mildews, the compatibility of mates is determined by mating-type locus, MAT-1 comprising of one of the two idiomorphs: MAT1-1 (alpha box domain) or MAT1-2 (HMG, high mobility group domain) but never both in each isolate. Identification of the reproductive strategy of *P. clandestina* is critical to the success of powdery mildew management in the PNW. At each location of isolate collection in 2018, late summer/ early winter visits were made to determine if the pathogen formed chasmothecia. We found chasmothecia in all sampling locations across PNW. These propagules serve as the source of primary inoculum for cherry mildew epidemics as reported earlier by Grove and Boal (1991). The presence of chasmothecia in each location implied that the pathogen is probably heterothallic and opposite mating types could be present throughout PNW. We used PCR-based approach to identify mating-types present in PNW to complement previous findings.

In order to identify the opposite mating-types, we used degenerate primers reported by (Brewer et al. 2011). The mating-type MAT1-1 (alpha box domain) was amplified as a 262 base pairs fragment, slightly longer than predicted. Other mating-type locus, MAT1-2 (HMG) was amplified as 282 base pairs fragment. The PCR amplicons were eluted from agarose gel and subsequently sequenced to design *P. clandestina* specific primers (Table 2) for analyzing the mating-type frequencies of isolates from Washington and Oregon cherry orchards. The multiplex PCR assay was performed on all single-colony or mixed cultures of *P. clandestina* isolates to measure the mating-type frequencies and to determine specificity of the primers that detect single or both mating-types in a single reaction. The results showed that all *P. clandestina* isolates (mixed cultures) amplified MAT1-1 and MAT1-2 in a single multiplex reaction (Figure 3). The populations of *P. clandestina* in the PNW contain both mating-types in equal frequencies.

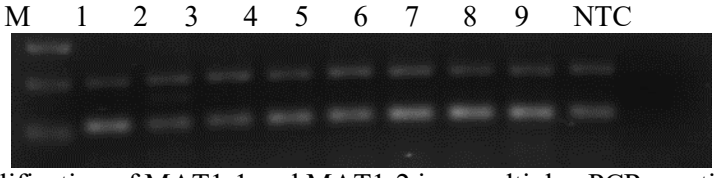

Figure 3. Amplification of MAT1-1 and MAT1-2 in a multiplex PCR reaction. Samples are *P. clandestina* composite isolates collected in Washington and Oregon orchards. M, marker; 1-9 DNA from *P. clandestina* isolates; NTC, no template control.

Table 2. *Podosphaera clandestina* specific PCR primers for identification of two mating-types in a multiplex reaction.

Primer type	Primer name	Primer sequence (5'→3')	Size of PCR product (bp)
P. clandestina specific primers	MAT1F	AGTCGGTGAATTCATGGATGGGA	108
	MAT109R	AGCGACACTGGGAAGACTAAAAA	
	HMG40F	TGAGGAAACTGTAGCCCGCA	207
	HMG247R	ACCAGGATTTTCAACAGCATGCT	

Control

Fungicide resistance

We investigated the occurrence of fungicide resistance of *P. clandestina* isolate collected at Washington State University's Roza farm in 2018. The isolate was subjected to leaf disc bioassays treated with fungicides from different FRAC groups, including a control that received no fungicide. These fungicides include myclobutanil (Rally, FRAC group 3), trifloxystrobin (Gem, FRAC group 11),

penthiopyrad (Fontelis, FRAC group 7), quinoxyfen (Quintec, FRAC group 13), a combination of trifloxystrobin and fluopyram (Luna Sensation, FRAC group 11 and 7, respectively), a combination of fluopyram and tebuconazole (Luna Experience, FRAC group 7 and 3, respectively), a combination of azoxystrobin and difenoconazole (Quadris Top, FRAC group 11 and 3, respectively) and, a combination of pyraclostrobin and boscalid (Pristine, FRAC group 11 and 7, respectively). After 14 days incubation (following inoculation), the inoculated leaf discs were microscopically examined for the presence of infection. Our results indicate that the fungicides quinoxyfen (Quintec) and penthiopyrad (Fontelis) containing fungicides were most effective.

In our bioassays, *P. clandestina* grew on leaf discs (of some isolates) treated with myclobutanil (Rally), trifloxystrobin (Gem) and a combination of pyraclostrobin and boscalid (Pristine) (Table 3). Although the colonies were smaller than those on the untreated controls, they produced abundant conidia. To confirm the resistant colonies, the colonies were re-inoculated onto fungicide-treated leaf discs and observations were made after 14 days of incubation.

Table 3. Summary of leaf disc fungicide resistance assay of Roza *P. clandestina* isolate.

Common name	Trade name	FRAC group	Rate of application*	Total leaf discs	Resistant colonies
Penthiopyrad	Fontelis	7	20 fl oz.	80	0
Myclobutanil#	Rally	3	6.0 oz.	80	5
				100	14
Trifloxystrobin#	Gem	11	3.8 fl oz.	80	22
				100	23
Quinoxyfen	Quintec	13	7 fl. oz.	80	0
Fluopyram/ Tebuconazole	Luna Experience	7/3	8.6 fl. oz.	80	0
Azoxystrobin/ Difenoconazole	Quadris Top	11/3	14 fl. oz.	80	0
Trifloxystrobin/ Fluopyram	Luna sensation	11/7	7.6 fl. oz.	80	0
Pyraclostrobin/ Boscalid#	Pristine	11/7	14.5 oz.	80	17
				100	19

*Rate of application per acre assuming 400 g spray material per acre.
#These fungicides were screened in two independent experiments.

Monitoring foliar and fruit mildew inoculum

Foliar and fruit disease pressure was monitored throughout the growing season by measuring live and total conidia. Ten leaves and 50 fruits were processed using laboratory protocols. Conidial suspensions were split into two aliquots and one of them received PMA-treatment. After DNA extractions, quantitative PCR (qPCR) were performed using PCR primers specifically developed for short fragment

of ITS gene (improvement to previous qPCR efforts). The results indicated that the live conidial density increased steadily in leaves while viable conidial incidence increased precipitously on fruit just one week prior to harvest (Figure 4) in cvs. 'Bing' and 'Sweetheart'.

Spray coverage

Preliminary studies on spray coverage on large trees were conducted in 2018. Pyranine dye was applied to mature trees at the WSU Roza orchard at 400 gallon per acre. Fruit samples were collected immediately after drying. The spray coverage was evaluated at different canopy levels (Figure 5). The results indicate that on large trees (traditional type with Mazzard rootstock) current industry application practices require further improvement. Spray coverage significantly decreased at higher canopy levels suggesting a need for improvement in current spray technologies and approaches. Results also explained the high number of infected fruits, the rapid increase of foliar powdery mildew following harvest, and number of in situ chasmothecia at higher levels in the canopy.

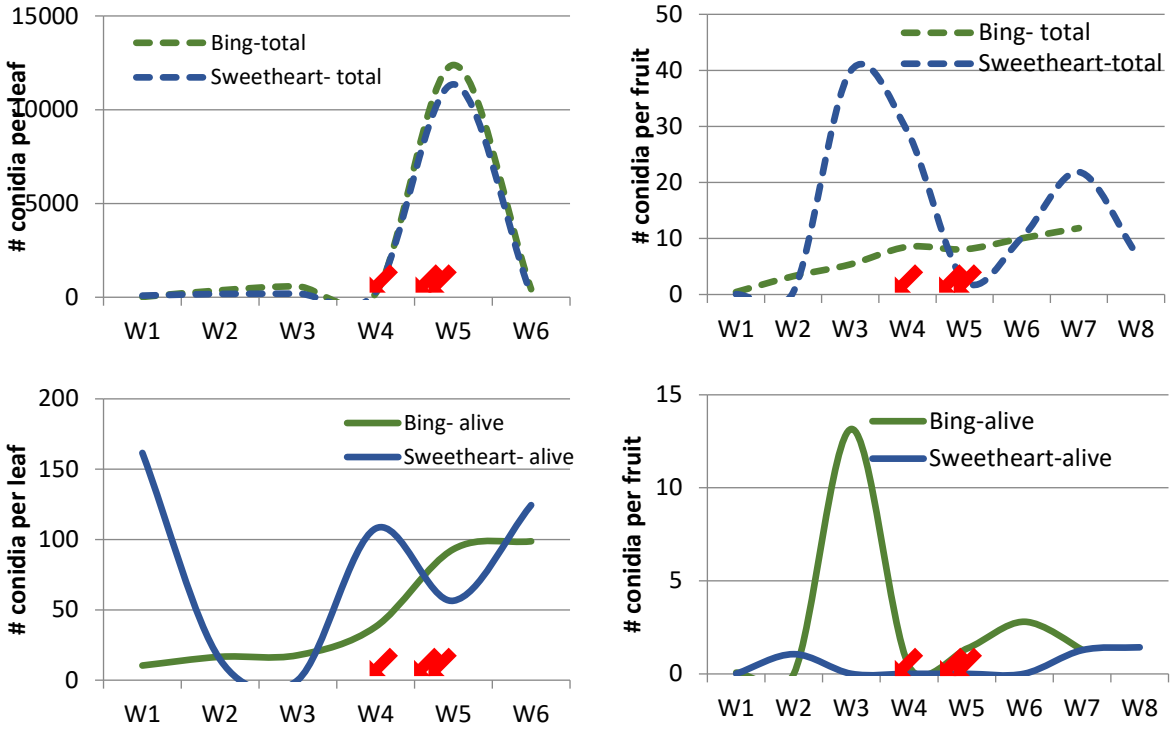

Figure 4. Conidial density of *Podosphaera clandestina* on cvs. Bing and Sweetheart trees. Leaves and fruits were analyzed separately from each tree. Red arrows indicate precipitation events. W, weeks after shuck fall.

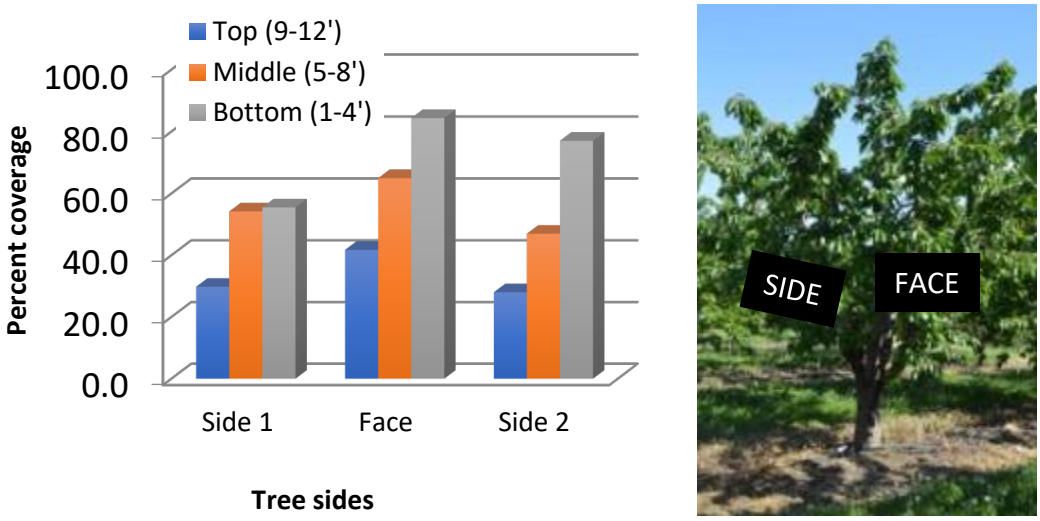

Figure 5. Spray coverage analysis at different canopy levels. Pyranine dye was quantified using images taken under UV-light and image processing was done using online tools.

EFFECTS OF POSTHARVEST TREATMENTS ON HARVESTED FRUIT

Mature Bing cherries were harvested in 2019 late June in commercial bins from the WSU's Roza experimental orchard. The cherry block was not treated with any fungicides in the past several years therefore powdery mildew infection on fruit was apparent and often exceeded 30% of the fruit surface. The cherries were immediately transported to Zirkle Fruit in Prosser for postharvest treatments. The fruit underwent hydrocooling (chilled chlorinated water) for about a minute and was later moved to a cold storage to achieve target fruit temperature of 48 °F for subsequent fumigation. Fumigation was carried out according to industry standards (for Australia as target market). The fruit on the following day received another round of hydrocooling on the packaging line and were packaged in 20 lbs. boxes. The boxes were then stored in cold storage to simulate shipping and arrival at the destination country. The samples (50 random fruit) were taken at each treatment stage in three replications for laboratory analysis. The fruit were processed according to established protocols (divided equally into two aliquots for further processing and PMA treatments) and DNA was extracted using a commercial kit. PMA was used to distinguish between membrane-compromised (dead) and total number of conidia (live and dead). A high sensitivity/specificity qPCR method was adopted to quantify conidia at each stage of postharvest treatment. The absolute number of conidia in each sample was deduced from a standard curve established on serial dilutions of known conidial concentrations. The fruit wash suspensions at each stage were used to inoculate foliage in the leaf-disc bioassay experiments. At the end of incubation period, none of the suspensions from fumigated or packaged cherries resulted in the establishment of mildew colonies on leaf discs. The results indicate that postharvest treatment of harvested fruit is extremely effective in removing all pathogenic conidia on the harvested fruit (Figure 6).

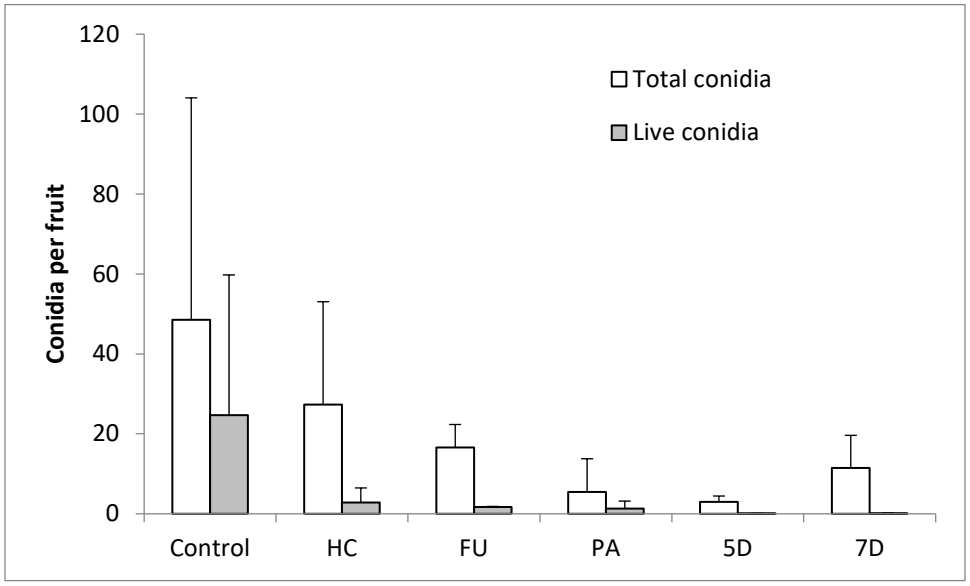

Figure 6. Number of total or pathogenic conidia at each stage of postharvest treatments of Bing cherries. Control: at harvest; HC: hydrocooling on receipt, FU: fumigation; PA: second hydrocooling at packaging; 5D: 5days post packaging; 7D: 7 days post packaging

Literature Cited

Brewer, Marin Talbot, Lance Cadle-Davidson, Paolo Cortesi, Pietro D. Spanu, and Michael G. Milgroom. 2011. 'Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi', *Fungal Genetics and Biology*, 48: 704-13.

Grove, G. G., and R. J. Boal. 1991. 'Overwinter survival of *Podosphaera clandestina* in eastern Washington', *Phytopathology*, 81: 385-91.

Swamy, Prashant, Claudia Probst, and Gary G. Grove. 2019. 'Incidence of *Podosphaera clandestina* on sweet cherries (*Prunus avium*) and the influence of postharvest handling practices on the survival of conidia on harvested fruit', *Postharvest Biology and Technology*, 156: 110929.

EXECUTIVE SUMMARY

Project Title: ABC of sweet cherry powdery mildew: adaption, behavior and control
Keywords: *P. clandestina,* fungicide resistance, heterothallism, ITS, spray coverage

In the Pacific Northwest (PNW), powdery mildew of cherry foliage and fruit is caused by *Podosphaera clandestina*. Analysis of internal transcribed spacer (ITS) sequence on many isolates of *P. clandestina* collected across Washington and Oregon states indicated a very high degree of genetic relatedness and were confirmed as the same species. Next-generation sequencing of representative *P. clandestina* isolates was completed and the data was used in identifying various *P. clandestina* full-length genes for the first time and the data is currently been used in other related projects. The fungus was identified as heterothallic in PNW due to the presence of two compatible mating types in all orchard locations studied. This is critical in the production of overwintering chasmothecia, the means of pathogen overwintering and a source of primary inoculum. In the PNW, *P. clandestina* occurs as two distinct growth habits on foliage while drastic differences were seen on pathogen colonies on infected fruit. Although genetically identical, different growth habits did not exhibit any variations in infectivity or fungicide sensitivities suggesting that the two growth habits could be due to physiological differences of their microclimate at sites of collection or unknown host factors. In the orchard conditions, the increase in the concentration of viable conidia on fruit and leaves largely depends on the precipitation events followed by appropriate temperature. A large increase in the viable conidial concentration just before the physiological maturity of fruit may be the cause of fruit infections in cvs. Bing and Sweetheart, as seen in the viability assays. Effective management of a disease is the function of uniform spray coverage of effective pesticides. The consistent observation of increased disease pressure at a higher canopy prompted us to examine the spray coverage in an established cherry orchard. The results of the preliminary study indicated that the spray coverage was <40 % in the higher canopy probably due to an inappropriate sprayer, improper spray calibration or a large tree canopy. Even when the spray coverage is optimal, the likelihood of fungicide resistance encounter in the PNW in cherry orchards was suspected due to a high frequency of synthetic spray applications. We investigated one of the collection sites for the presence of fungicide resistance of the *P. clandestina* isolate against four different FRAC groups either as a component of a premix formulation or single as a single compound. The pilot-scale study suggested that the isolate had developed insensitivities to at least two different FRAC groups. The problem is being investigated in a separate project co-funded by Washington Tree Fruit Research Commission and Oregon Sweet Cherry Commission. Furthermore, this project was used as an opportunity to investigate the presence of powdery mildew conidia in the fruit destined for commercial export. We found that the PNW postharvest treatments effectively removed all viable conidia before packaging and contain no disease inoculum that could infect the host tissue in the country of its destination. Overall, we investigated the biology and genetics of *P. clandestina* infection, establishment, and perennation on a cherry host. Additionally, the project was critical in unraveling problems in current disease management and identified a potential PNW-wide presence of fungicide resistance.