ABSTRACT

Title of Thesis: UNDERSTANDING LATE SEASON FRUIT ROT PATHOSYSTEMS AND INSECT INTERACTIONS IN MID-ATLANTIC VINEYARDS

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Fungal fruit rots and insect pests are among the most important problems negatively affecting the yield and quality of mid-Atlantic wine. In pathogenicity trials of fungi recovered from diseased Chardonnay and Vidal blanc grapes, *Alternaria alternata*, *Pestalotiopsis telopeae*, and *Aspergillus japonicus* were found to be unreported fruit rot pathogens in the region. Additionally, *P. telopeae* and *A. japonicus* had comparable virulence to the region's common fruit rot pathogens. Furthermore, a timed-exclusion field study was implemented to evaluate vineyard insect-fruit rot relationships. It was found that clusters exposed to early-season insect communities that included *Paralobesia viteana* had a significantly greater incidence of sour rot than clusters protected from insects all season. These results were contrary to the current assumption that fall insects are the primary drivers of sour rot in the region. This research provides diagnostic tools and information to develop management-strategies against fungal and insect pests for mid-Atlantic grape growers.

UNDERSTANDING LATE SEASON FRUIT ROT PATHOSYSTEMS AND INSECT INTERACTIONS IN MID-ATLANTIC VINEYARDS

by

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Chapter 1: Late season fruit rot pathogens of white grapes in mid-Atlantic vineyards

Introduction

The Maryland wine industry generates over $40 million annually and has been rapidly growing over the past decade, doubling vineyard acreage from 2006 to 2014, while almost tripling the tonnage of wine grapes produced during that same period (Anonymous, 2015a). This industry is based on a combination of traditional *Vitis vinifera* cultivars, such as cv. Chardonnay, Merlot and Cabernet Franc, together with hybrid varieties, including Vidal blanc (*Vitis vinifera* variety Ugni blanc x Rayon d'Or) and Chambourcin (Anonymous, 2010). *Vitis vinifera* cultivars are predominantly produced in the northwestern Piedmont, extending into the Appalachian Mountains, whereas hybrids dominate in the eastern and southern part of the state (Anonymous, 2010). As many of the hybrid varieties grown in the region, such as Vidal blanc, are relatively new to the US grape market, there is very little information on their susceptibility to pathogens—these cultivars may be susceptible

to diseases uncommon in *V. vinifera* cultivars, but this possibility has received relatively little attention.

Late season fruit rots pose a major challenge to wine grape production across all mid-Atlantic states (Delaware, Maryland, New Jersey, Pennsylvania, and West Virginia), compromising fruit quantity and quality (Barata et al. 2012a). Fruit rots are common due to the region's warm, humid summers and mild winters. *Vitis vinifera* cv. Chardonnay is one of the most economically important white wine grape cultivars in the region (Anonymous, 2010; Anonymous, 2013), and is highly susceptible to several fruit rot diseases, including bunch rot caused by *B. cinerea* (Baudoin, 2012; Carisse et al. 2006; Hed et al. 2014), ripe rot caused by *Colletotrichum* spp. (Greer et al. 2014) and sour rot, caused by diverse yeast and bacterial species (Zoecklein et al. 1992). The French-American hybrid Vidal blanc is the most widely grown white wine grape hybrid in the state (Anonymous, 2010), in part because it is considered to be less susceptible to *B. cinerea* than Chardonnay (Reisch et al. 1993); Vidal blanc susceptibility to ripe rot and sour rot are not known.

In addition to these, diverse other fungi such as *Alternaria* spp. and *Aspergillus* spp. have been associated with late season fruit rot symptoms in white wine grapes in the mid-Atlantic (Tony Wolf, personal communication), but the ability of these fungi to cause fruit rot has not been evaluated.

The objectives of this study were therefore to (i) evaluate pathogenicity of fungi collected from late season fruit rots in white wine grapes (cv. Chardonnay and Vidal blanc), focusing on species reported as fruit rot pathogens in other regions, but which had not been reported in the mid-Atlantic and (ii) assess the relative susceptibility of

the hybrid Vidal blanc to common and newly characterized fruit rot pathogens. This research provides a diagnostic resource for fruit rot disease identification in the region, as well as important information on appropriate cultivar section in new plantings in mid-Atlantic vineyards.

Methods

Isolation of fungi from fruit with late season fruit rot symptoms

Diseased Chardonnay clusters from Keedysville, MD (n = 40) and Vidal blanc clusters from two vineyards in southern Maryland (n = 6 and 9) were collected between August and September 2015. Disease symptoms included withering, discoloration, necrosis, loss of fruit integrity, and/or a vinegar-like fragrance. Symptomatic berries (3 to 5 per cluster) were surface disinfested by washing in 0.1% Tween 20, soaking 30 s in 70% ETOH and then 3 min in 1% NaClO. Berries were then incubated in Petri dishes containing sterile, pre-moistened filter paper at 17^0C 0:24 L:D. Emergent filamentous fungi were aseptically transferred to 100 mm Petri dishes containing potato dextrose agar (PDA) amended with antibiotics (agar 15 g/liter, dextrose 20 g/liter, potato extract 4 g/liter, streptomycin sulfate, 0.3 g/liter and tetracycline hydrochloride, 0.3 g/liter) and then a single germinating hyphal tip was excised and placed on sterile filter paper atop 10% PDA. Cultures on the filter paper were dried and stored at -20^0C until time of use.

Identification

Colonies were initially identified to genus based on culture characteristics and conidial morphology (Barnett and Hunter, 1998). A subset (20% to 100% (n = 3 to

10)) of isolates from each genus were then identified to species based on sequence analysis. DNA was extracted from cultures grown 3 to 4 days on PDA (as above) using PrepMan Ultra (Applied Biosystems). Amplification of the 5.8S rRNA gene, ITS (internal transcribed spacer) region, was carried out using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') on a Bio-Rad C1000 Touch thermal cycler (Bio-Rad Laboratories Inc.) using the following conditions: initial denaturation at 94^0C for 5 min, 40 cycles of denaturation at 94^0C for 30 s, annealing at 52^0C for 30 s, extension at 72^0C for 50 s, followed by a final extension at 72^0C for 7 min (White et al. 1990; Larena et al. 1999). Amplified products were visualized on a 1.5% w/v agarose gel after staining with EZ-Vision Three (Amresco) and then purified using UltraClean PCR Cleanup Kit (MoBio Laboratories Inc.), sequenced on a Sanger sequencing platform, and resulting sequences were BLAST analyzed using GenBank. The ITS region was uninformative for species identification of *Pestalotiopsis* and *Colletotrichum* isolates, so alternative gene regions were used. *Pestalotiopsis* isolates were identified based on amplification of the translation elongation factor (Maharachchikumbura et al. 2011) gene, using primers EF1-728 (5'-CATCGAGAAGTTCGAGAAGG-3') (Carbone and Kohn, 1999) and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (O'Donnell et al. 1998) under the following conditions: initial denaturation at 96^0C for 2 min, 35 cycles of denaturation at 94^0C for 30 s, annealing at 56^0C for 30 s, extension at 72^0C for 30 s, followed by a final extension at 72^0C for 7 min. (O'Donnell et al. 1998). *Colletotrichum* isolates were identified based on amplification of the Histone 3 gene, using primers HIS3-F

(5'-AAGAAGCCTCACCGCTACAA-3') and HIS3-R (5'-CTGAATGGTGACACGCTTGG-3') under the following conditions: initial denaturation at 95^0C for 1 min, 30 cycles of denaturation at 95^0C for 1 min, annealing at 60^0C for 1 min, extension at 72^0C for 1 min, followed by a final extension at 72^0C for 10 min (Kou et al. 2015).

Proof of pathogenicity

Two isolates each of *Alternaria alternata* (SL 215 and SL 227), *Aspergillus japonicus* (SL 230 and SL 231), and *Pestalotiopsis telopeae* (SL 752 and SL 754) were tested for pathogenicity on non-wounded and artificially wounded fruit (Table 2). Negative controls in each experiment consisted of intact and wounded berries, mock inoculated with 5% KCl + 0.1% Tween 20. Five incubators were used so that each isolate x inoculation method treatment combination was replicated five times, with four berries (subsamples) per incubator, and the experiment was conducted twice.

In preparation for inoculations, ripe Chardonnay and Vidal blanc clusters were collected from a vineyard in Western Maryland, and stored at -1^0C until the time of use (up to eight weeks after harvest). Asymptomatic, intact berries were cut from the rachis, leaving the cap stem attached, and then surface disinfested (as above) and dried in a laminar flow hood at ambient temperatures to remove excess moisture. Brix of fifteen berries, measured using a digital refractometer (PAL-1, ATAGO INC.) at the time of inoculation, was consistent across experiments (Chardonnay, $20.5^0 \pm 0.15^0$; Vidal, $23.8^0 \pm 0.33^0$).

Alternaria alternata isolates were grown for ten to fourteen days at 24°C under near ultraviolet light for 12:12 L:D (Carvalho et al. 2008). All other isolates were grown

on PDA for seven to ten days at 24°C 12:12 L:D. Inoculum was then prepared by suspending spores in 0.5% KCl + 0.1% Tween 20 solution and adjusting to 10^5 spores / ml. Inoculum was stored at 4^0C and used within 48 hours. Diluted subsamples of inoculum were spread over the surface of 2 to 5 PDA plates at a target dose of twenty-five spores per plate to evaluate spore viability at the time of inoculation (Aegerter and Gordon, 2006). The mean inoculum densities of *A. alternata* isolates SL 215 and SL 227 were 5.58 $x10^4 \pm 2.28x10^4$ and $4.86x10^4 \pm 1.4x10^4$ spores / ml respectively across both experiments; *A. japonicus* isolates SL 230 and SL 231 were $5x10^4 \pm 3x10^4$ and $9.06x10^4 \pm 0.66x10^4$ spores / ml respectively; *P. telopeae* isolates SL 752 were SL 754 were $6.15x10^4 \pm 0.45x10^4$ and $5.2x10^4 \pm 2.27x10^4$ spores / ml respectively.

Berries were placed in incubators (plastic 1.89 liter Gladware containers) containing a synthetic rubber ice cube tray on top of two-9 mm saturated filter papers, and a 5 mm circle of sterile Vaseline was made on the shoulder of the fruit. To inoculate intact (non-wounded) berries, a 10 µl droplet of inoculum was placed in the Vaseline circle. To wound inoculate, a 1 mm diameter incision was made within the Vaseline circle by penetrating just through the epidermis with a 24-gauge sterile hypodermic needle, and a 10 µl droplet of inoculum was placed on the wound. Incubators were maintained at 23^0C with 16:8 L:D.

Disease incidence was quantified as the percent of fruit that developed symptoms (out of four / incubator) within a four to six-day period, and disease severity was evaluated based on a ranking system (Table 1) at two, four, and six days post inoculation. Qualitative data on symptom expression was also collected based on visual

assessment and photo-documentation. Six days post inoculation, fungi recovered from 10% of berries cultured and identified to species as described above to confirm identity as the inoculated species. Each experiment was conducted twice.

Table 1 Ranking system scale for evaluating disease severity.

Rank	Description, based on visible berry surface[1]
0	No fungal growth and/or disease symptoms at inoculation site— 0% of the visible surface
1	Fungal growth and/or disease symptoms at inoculation site only—5% of the visible surface
2	Fungal growth and/or disease symptoms extending to the Vaseline ring—25% of the visible surface
3	Fungal growth and/or disease symptoms extend beyond the 5 mm Vaseline ring—50% of the visible surface
4	Fungal growth and/or disease symptoms on approximately 75% of the visible surface
5	Fungal growth and/or disease symptoms covering 100% of the visible berry surface

[1]Rating scale was based on the visible berry surface so as not to disturb the berry during the incubation period

Relative susceptibility of Vidal blanc to fungal fruit rot pathogens

Vidal blanc was evaluated for susceptibility to the most virulent *A. alternata* (SL 215), *A. japonicus* (SL 230), and *P. telopeae* (SL 752) isolates from pathogenicity tests describe above, together with *B. cinerea* (SL 179) and *Colletotrichum fioriniae* (SL 237), both originally recovered from symptomatic Chardonnay. Negative controls were mock inoculated with 5% KCl + 0.1% Tween 20. Three replicate incubators containing a three berry set of the subsamples were used for each treatment, and the experiment was conducted twice.

In preparation for inoculations, ripe Vidal blanc clusters were removed from cold storage, berries were surface disinfested and spore suspensions were prepared, as described above. Across both experiments, *A. alternata* was inoculated at $7.18 \times 10^4 \pm$

$1.88x10^4$ spores / ml; *A. japonicus* was inoculated at $11.67x10^4 \pm 2.74x10^4$ spores / ml; *P. telopeae* was inoculated at $8.53x10^4 \pm 0.27x10^4$ spores / ml; *B. cinerea* was inoculated at $4.93x10^4 \pm 0.67$ spores / ml; and *C. fioriniae* was inoculated at $12.56x10^4 \pm 1.15x10^4$ spores / ml. Brix of berries was $24.5^0 \pm 0.21^0$ across both experiments, based on evaluation of fifteen berries at the time of each inoculation. Inoculations were conducted in the absence of wounding and disease incidence was evaluated as described above at two, four, six, and eight days post-inoculation.

Analysis

Significance of main effects (isolate and inoculation method) on disease incidence was analyzed using ANOVA and Tukey's multiple means comparisons within R x64 2.15.2, following arcsine square root transformation of percentage data. Main effects of disease severity (rank values) were analyzed conditionally for those berries that became infected, based on the paired-samples Wilcoxon signed rank test with continuity correction or the Kruskal-Wallis rank sum test (if there were more than two treatment variables), using the Rcmdr plugin within R x 64 2.15.2. Conditional analysis was used because it provides a measure of extent of host colonization in those individuals that became infected (McRoberts et al. 2002; Swett and Gordon, 2015). Unless otherwise noted, experiments were combined for analysis based on the absence of significant interactions with the main effects in ANOVA ($P < 0.05$).

Results

Isolation and identification of fungi from fruit with late season fruit rot symptoms

2014 and 2015 field collections recovered *A. alternata, A. japonicus, B. cinerea, C. fioriniae, Epicoccum nigrum, Penicillium glabrum, P. telopeae,* and *Pestalotiopsis* sp. from diseased berries (Table 2). *Alternaria alternata, B. cinerea, C. fioriniae, E. nigrum, P. glabrum,* and *P. telopeae* were recovered from 55%, 2.5%, 44%, 12.5%, 5%, and 7.5% of diseased Chardonnay clusters respectively (n = 40). *Aspergillus japonicus, B. cinerea, C. fioriniae, P. glabrum,* and *P. telopeae* were recovered from 44.4%, 44.4%, 33.3%, 22%, and 22.2% of diseased Vidal blanc clusters, respectively (n = 15, data combined from both vineyards). As described above, *B. cinerea* and *C. fioriniae* have been previously reported in the region, and so were excluded from proof of pathogenicity trials. In preliminary analyses, neither *E. nigrum* nor *P. glabrum* were able to initiate disease (data not shown) and so were excluded from all downstream analyses.

Proof of pathogenicity

Alternaria alternata isolates were consistently able to initiate fruit rot on wounded Chardonnay berries; only one isolate was able to initiate symptoms on non-wounded berries. On wounded berries, disease symptoms were first detected two to four days post inoculation as white aerial hyphae emerging from the inoculation site, usually without discoloration of the berry skin (Figure 1A). As disease developed, the white to grey-green hyphae expanded, covering up to 100% of the visible berry surface, and colonized tissue turned light to dark brown. Symptoms also included areas of

extensive discoloration surrounding the inoculation site with little hyphal colonization (Figure 1B).

Effects of isolate treatment and inoculation method on fruit rot incidence at six days post inoculation were analyzed for both experiments combined. Background levels of *A. alternata* were detected in the negative controls, so this treatment was included in analyses.

The isolate x inoculation method interaction was significant in ANOVA ($P < 0.001$) so effects of inoculation method were analyzed separately for each isolate. The effect of inoculation method on incidence was highly significant for both isolates ($P < 0.001$), reflecting greater fruit rot incidence in the wound inoculation treatment (93% to 100% of berries infected, across the two isolates), compared to the intact inoculation treatment (0% to 5% of berries infected) (Table 3). The effect of isolate was not significant in the intact inoculation treatment ($P = 0.125$), reflecting low fruit rot incidence (0% to 5%) across all isolate treatments (Table 3). The effect of isolate was significant in the wound inoculation treatment—this reflected significantly lower disease incidence in the negative controls (17.5% ± 8.4% of berries infected), compared to both SL 215 (93% ± 3.8%) and SL 227 (100%) (Table 3).

The effect of isolate on conditional disease severity (severity in fruit that became diseased) was only analyzed for the wound inoculation treatment, since symptoms did not develop for both isolates on non-wounded berries. There was no significant difference between isolate treatments following wound inoculation ($P = 0.188$, Kruskal-Wallis rank sum test analysis) (Table 3). There was a trend wherein fruit rot severity was greater in the SL 215 and SL 227 treatments compared to the

Table 2. Species recovered from cv. Chardonnay and Vidal blanc in 2015 field surveys, based on sequence analysis

Species	Isolate	Primers	NRRL accession	Sequence length (bp)	Sequence homology (%)
Alternaria alternata	SL 215	ITS4, ITS1F	KP278204.1	505	100
	SL 220		KP278204.1	503	100
Aspergillus japonicus	SL 230	ITS4, ITS1F	KJ867624.1	483	100
	SL 231		KC128815.1	532	100
Botrytis cinerea	SL 179	ITS4, ITS1F	LN846783.1	481	100
Colletotrichum fioriniae	SL 237	HIS3F, HIS3R	JQ949283.1	292	100
Epicoccum nigrum	SL 241	ITS4, ITS1F	KF025954.1	487	100
	SL 242		KF025954.1	501	100
Penicillium glabrum	SL 234	ITS4, ITS1F	KP329741.1	510	100
Pestalotiopsis sp.	SL 749	EF1-728, EF2	KJ623227.1	489	100
Pestalotiopsis telopeae	SL 752	EF1-728, EF2	KM199498.1	567	98
	SL 754		KM199498.1	566	99

negative control, but these differences were not significant (Table 3). All fungi recovered from inoculated, symptomatic berries were identified as *A. alternata*.

Table 3. Disease incidence and severity on intact and wound inoculated cv. Chardonnay berries by two *Alternaria alternata* isolates.

	Incidence[a]		Severity[b]	
Isolate	Intact	Wound	Intact[c]	Wound
SL 215	5.0% ± 3.3% a	93.0% ± 3.8% a	1.0 ± 0.0	2.1 ± 0.2 a
SL 227	0.0% ± 0.0% a	100.0% ± 0% a	nd	2.3 ± 1.5 a
Control[d]	0.0% ± 0.0% a	17.5% ± 8.4% b	nd	1.9 ± 1.5 a

[a]Disease incidence six days post inoculation, quantified as the percent of berries infected per replicate, analyzed based on ANOVA for the two experiments combined. Means (± SE) within columns and rows separated by the same letter are not significantly different based on Tukey's means comparison ($P > 0.05$).
[b]Disease severity six days post inoculation, quantified on a rank scale (Table 1) and analyzed conditionally only for those berries which developed symptoms, based on the Kruskal-Wallis rank sum test. Means (± SE) within columns separated by the same letter are not significantly different ($P > 0.05$).
[c]The intact treatment was excluded from analyses due to absence of data in all but one isolate treatment.
[d]The negative control was included in analysis, due to background contamination by one or more *Alternaria* spp.

Pestalotiopsis telopeae was consistently able to initiate fruit rot in both intact and wounded Chardonnay berries. Disease symptoms differed between inoculation treatments. In wounded berries, white aerial hyphal growth was observed at the inoculation site as soon as two days post inoculation, but tissue was not noticeably discolored. By six days post inoculation, black acervuli had emerged within and around the area of hyphal colonization, and berry color remained normal (Figure 1C). In contrast, intact inoculated berries had no visible symptoms until six days post inoculation, at which point, large, irregularly shaped water soaked lesions formed on up to 100% of the visible berry surface, often accompanied by black acervuli rupturing through the epidermal layer inside of the lesion (Figure 1D).

There was a significant effect of isolate x inoculation method interaction on fruit rot incidence six days post inoculation ($P = 0.048$), so effects of inoculation method were

analyzed separately for each isolate. The effect of inoculation method was highly significant for both isolates ($P < 0.001$), reflecting greater fruit rot incidence in the wound inoculation treatment (85% to 95% of berries infected, across the two isolates), compared to the intact inoculation treatment (12.5% to 17.5% berries infected) (Table 4). The effect of isolate was not significant for either the intact ($P = 0.61$) or wound ($P = 0.13$) inoculation methods (Table 4).

Table 4. Disease incidence and severity on intact and wound inoculated cv. Chardonnay berries by two *Pestalotiopsis telopeae* isolates.

	Incidence[a]		Severity[b]	
Isolate	Intact	Wound	Intact	Wound
SL 752	17.5% ± 6.5% a	85.0% ± 5.5% b	3.1 ± 0.5 a	3.0 ± 0.2 a
SL 754	12.5% ± 5.6% a	95.0% ± 3.3% b	3.6 ± 0.5 a	2.6 ± 0.2 a

[a]Disease incidence six days post inoculation, quantified as the percent of berries infected per replicate and analyzed based on ANOVA for the two experiments combined. Means (± SE) within columns and rows separated by the same letter are not significantly different ($P > 0.05$).
[b]Disease severity six days post inoculation quantified on a rank scale (Table 1) and analyzed conditionally only for those berries which developed infection based on the Kruskal-Wallis rank sum test. Means (± SE) within columns separated by the same letter are not significantly different ($P > 0.05$).

In analysis of conditional disease severity, the isolate x inoculation method interaction was not significant ($P = 0.28$), so treatments were combined to evaluate main effects. The effect of inoculation method was not significant ($P = 0.15$, Kruskal-Wallis rank sum), reflecting similar severity for non-wounded (ranking of 3.1 to 3.6 across isolates) and wounded berries (ranking of 2.6 to 3 across isolates) (Table 4). There was not a significant effect of isolate on fruit rot severity ($P = 0.22$, Kruskal-Wallis rank sum) (Table 4). Disease did not develop in *P. telopeae* negative controls in any experiment and were excluded from analyses. All isolates recovered from symptomatic berries were identified as *P. telopeae*.

Aspergillus japonicus was able to initiate fruit rot in wounded and to a lesser extent, non-wounded Vidal blanc berries. Signs of *A. japonicus* infection usually developed in

advance of symptoms. As early as two days post inoculation, black conidiophores formed at the inoculation site. There were two different infection patterns—in the first, dense spore-bearing conidiophores developed at the inoculation site (Figure 1D); in the second, hyphal growth expanded radially from the inoculation site (Figure 1F). Both symptom types developed in both wounded and non-wounded berries. Epidermal tissue became bleached beyond the mycelium margin, covering 100% of the visible surface, and internal juices were commonly observed exuding from the infection site—leakage of juices may account for the second symptom type, since hyphae would be able to rapidly colonize those parts of the berry surface covered in juice. As disease progressed, white hyphae and dark conidiophores emerged from bleached areas (Figure 1E).

The effects of *A. japonicus* isolate and inoculation method on disease incidence were analyzed at four days post inoculation for both experiments combined. There was no significant effect of isolate ($P > 0.05$) on disease incidence, so data were pooled to analyze the effect of inoculation method (Table 5). Disease incidence was significantly greater ($P < 0.001$) following wound inoculation (67% to 90% of berries infected, across isolates) compared to intact inoculation (5% to 7.5% of berries infected) (Table 5).

The effect of isolate on conditional disease severity was analyzed for wound and intact inoculation treatments due to a significant experiment x isolate interaction ($P = 0.03$). For non-wound inoculation, data were analyzed for experiment one only, since symptoms did not develop in all isolate treatments in experiment two. The effect of isolate on conditional disease severity was not significant in the non-wound inoculation treatment ($P = 0.057$, paired-samples Wilcoxon signed rank test) in experiment one (Table 5). The effect of isolate was highly significant following wound inoculation ($P < 0.001$),

reflecting more severe disease in the SL 230 treatment compared to isolate SL 231 (Table 5). Disease did not develop in *A. japonicus* negative controls in any experiment, and so were excluded from analyses. All isolates recovered from symptomatic berries were identified as *A. japonicus*.

Table 5. Disease incidence and severity on intact and wound inoculated Vidal blanc berries by two *Aspergillus japonicus* isolates.

	Incidence[a]		Conditional severity		
	Intact	Wound	Intact[c]	Wound	
Isolate	Exp. Comb.	Exp. Comb.	Exp. 1	Exp. 1	Exp. 2
SL 230	7.5% ± 5.3% a	90.0% ± 5.5% a	2.0 ± 0.6 a	2.0 ± 0.2 a	1.2 ± 0.1 a
SL 231	5.0% ± 3.3% a	67.5% ± 6.5% a	1.0 ± 0.0 a	1.2 ± 0.1 b	1.0 ± 0.0 b

[a]Disease incidence four days post inoculation, quantified as the percent of berries infected per replicate, and analyzed based on ANOVA for the two experiments combined. Means (± SE) within columns separated by the same letter are not significantly different (P > 0.05).
[b]Disease severity four days post inoculation, quantified on a rank scale (Table 1) and analyzed conditionally only for those berries which developed symptoms using the paired-samples Wilcoxon signed rank test. Means (± SE) within columns separated by the same letter are not significantly different (P > 0.05).
[c]No data for experiment 2.

Relative susceptibility of Vidal blanc to fungal fruit rot pathogens

Relative virulence of species on Vidal blanc was assessed based on disease incidence (percent of berries infected), analyzed separately at two, four, and eight days post inoculation. At two days post inoculation, disease had only developed in the *A. japonicus* and *B. cinerea* treatments, with no significant species effect P = 0.14, ANOVA) (Table 6). By four days post inoculation, disease had also developed in the *C. fioriniae* treatment. The effect of species was significant at four days (*P* = 0.013), reflecting lower virulence of *A. alternata* and *P. telopeae* (0% of berries infected) compared to *A. japonicus* (33.3% ± 12.2%), with *C. fioriniae* and *B. cinerea* falling into an intermediate group (Table 6). At eight days post inoculation, disease had developed in all fungal treatments and there was a significant effect of species (*P* = 0.021), reflecting lower

incidence of *A. alternata* (22.3% ± 14.1% of berries infected) compared to both *A. japonicus* (72.3% ± 10.2%) and *C. fioriniae* (72.3% ± 10.2%), with *B. cinerea* and *P. telopeae* forming an intermediate group (Table 6). Since disease did not develop in the non-inoculated controls, they were excluded from analysis.

Table 3. Disease incidence of fruit rot pathogens on intact inoculated Vidal blanc, measured as the percent of berries infected per replicate (n = 6) at two, 4, and 8 days post inoculation (PI).

Species	2 Days PI	4 Days PI	8 Days PI
Alternaria alternata	0.0% ± 0.0% a	0.0% ± 0.0% a	22.3% ± 14.1% a
Aspergillus japonicus	11.0% ± 6.9% a	33.3% ± 12.2% b	72.3% ± 10.2% b
Botrytis cinerea	11.0% ± 6.9% a	11.0% ± 6.9% ab	50% ± 14.3% ab
Colletotrichum fioriniae	0.0% ± 0.0% a	5.5% ± 5.4% ab	72.3% ± 10.2% b
Pestalotiopsis telopeae	0.0% ± 0.0% a	0.0% ± 0.0% a	44.3% ± 14.1% ab

[a]Disease incidence quantified as the percent of berries infected per replicate, for the two experiments combined. Means (± SE) within columns separated by the same letter are not significantly different based on Tukey's means comparison (P > 0.05).

Discussion

In this study diverse fungal species were recovered from late season fruit rot symptoms and signs on white wine grapes. In addition to known late season fruit rot pathogens (*B. cinerea and C. fioriniae*), *A. alternata, A. japonicus*, and *P. telopeae* were all able to initiate fruit rot in the cultivar from which they were originally isolated. Sour rot yeast and bacterial species were also recovered (data not shown), but this study chose to focus on hyphal fungi (discussed further below). Disease symptoms and signs of *A. alternata* and *A. japonicus* were consistent with those described in previous studies in green table and white wine grape cultivars. *Pestalotiopsis telopeae* has not been previously reported as a fruit pathogen of grapes, but many symptoms and signs were consistent with those described for an uncharacterized *Pestalotiopsis* sp. in the white grape cultivar "Cheongsoo" (Deng et al. 2013). The high diversity of fruit rot pathogens now known to occur in this region is likely due to the ideal conditions (warm, humid summers and mild

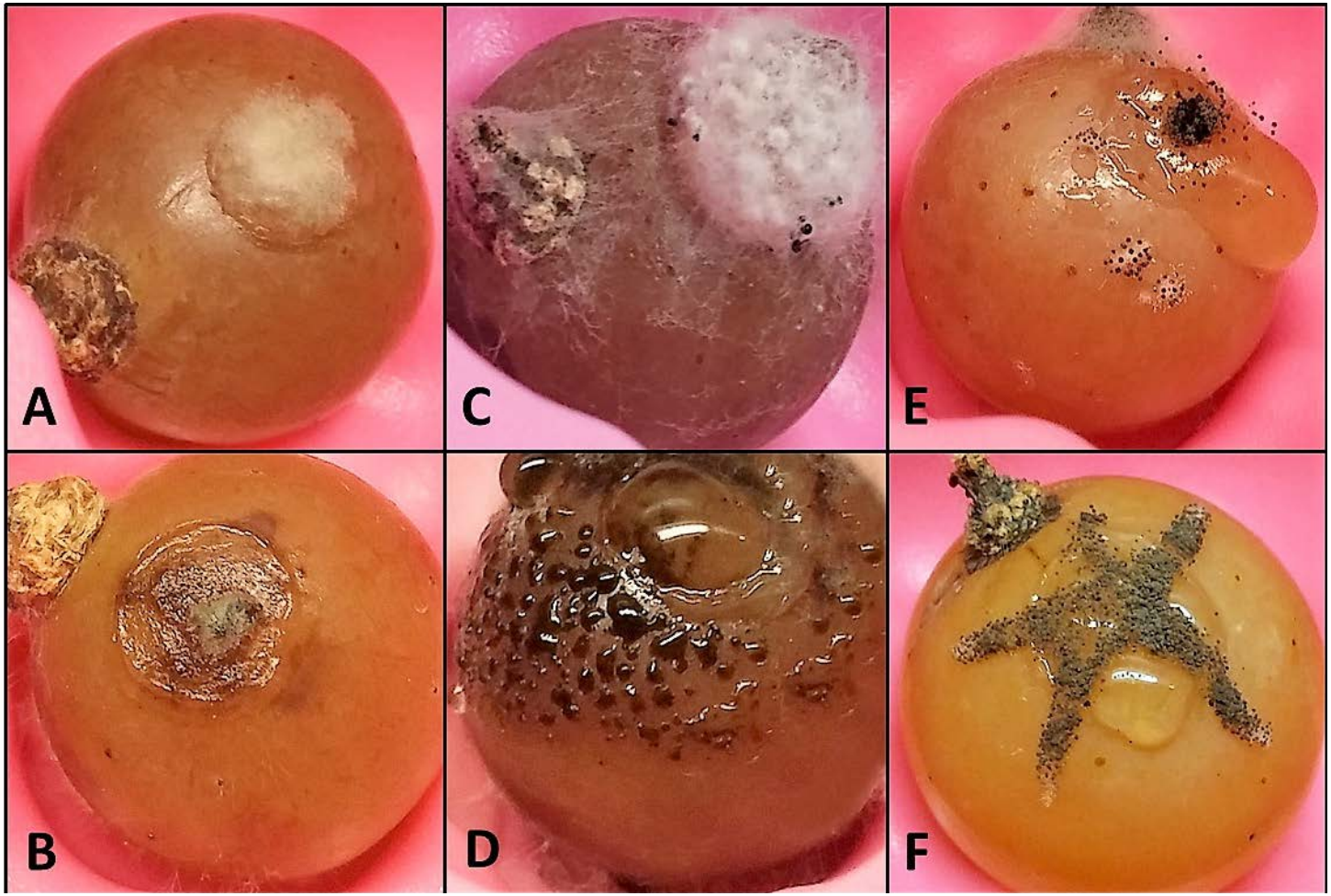

Figure 1. Fruit rot symptoms characteristic of *Alternaria alternata* (A, B), *Pestalotiopsis telopeae* (C, D), and *Aspergillus japonicus* (E, F). Some symptoms differed by type of wound treatment; Plates A, B, C, and E display symptoms of wound inoculated berries. Plates D and F show symptoms of intact inoculated berries.

winters) for fungal growth, reproduction and survival (Kakalíková et al. 2009; Urbez-Torres et al. 2009; Maharachchikumbura et al. 2011; Deng et al. 2013; Rousseaux et al. 2014; Jayawardena et al. 2015; Wilcox, 2015).

Wounding increased disease incidence of all fungi tested in pathogenicity trials, suggesting that wounds are epidemiologically important, but varied in their dependence on wounding for establishment. *Alternaria alternata* relied heavily on wounding for disease development, wherein up to 100% of wounded berries developed symptoms. This indicates that *A. alternata* is a wound-dependent opportunistic pathogen in grape (Keller et al. 2003). *Pestalotiopsis telopeae* and *A. japonicus* both were able to infect wounded berries with 85% and 90% incidence, but were also able to infect intact berries. This indicates that infection through a wound is not necessary for these pathogens, but will significantly increase the chances of development. Sources of wounds in mid-Atlantic vineyards include *Parabolesia viteana* (American grape berry moth) (Fermaud et al. 1992) which appears to facilitate late season fruit rot development in Maryland (Kepner and Swett, unpublished data), bird damage (Tracey and Saunders, 2003), and hail (Cozzi et al. 2008). In addition, studies in other regions indicate that *Aspergillus* spp. infect primarily during warm periods with high rainfall, when fruit swells, pulling the berry away from the cap stem, creating an entry point through which *Aspergillus* can colonize the fruit (Cozzi et al. 2009; Leong et al. 2006; Mikusova et al. 2010). Of note, high inoculum levels were applied in these studies, with a target dose of 1,000 spores / berry, in order to evaluate the infection capabilities of each organism. It is likely that lower inoculum levels would result in lower disease incidence on both intact and wounded berries.

With their capacity to initiate infection without wounding, *P. telopeae* and *A. japonicus* are likely to be the most economically significant of these newly described pathogens. Very little is known about the biology or management of *P. telopeae* or any other *Pestalotiopsis* species on grape berries. The delayed appearance of large sporulating lesions until the sixth day of evaluation, coupled with the extensive colonization of the berry rapidly thereafter suggests that this pathogen has a latent phase which could be similar to *Phomopsis viticola* or other early-season infecting pathogens (Mostert et al. 2000; Pscheidt and Pearson, 1989). *Pestalotiopsis telopeae* has not been reported on grapes in any other region in the US, but has been associated with leaf spot disease of *Telopea* sp. in Australia (Maharachchikumbura et al. 2011) and diseased kiwifruit vines in New Zealand (Anonymous, 2015b). There are other pathogenic *Pestalotiopsis* spp. that negatively impact grape production such as *P. uvicola* which causes grapevine trunk and fruit rot diseases worldwide (Rousseaux et al. 2014; Sergeeva et al. 2005; Urbez-Torres et al. 2009; Xu et al. 1999) and several other un-resolved putative *Pestalotiopsis* spp. which cause fruit rot. (Anonymous, 2015c; Deng et al. 2013; Jayawardena et al. 2015). It seems likely that this pathogen can establish latent infections, perhaps as early as the bloom period, similar to other coelomycetes that cause fruit rot in grapes (e.g. *Colletotrichum* spp.). Understanding the disease cycle will be necessary to develop effective chemical and cultural practices to manage *Pestalotiopsis* fruit rot.

The contribution of *Pestalotiopsis* to fruit rot losses in the region may have overlooked in part due to similarity with *Phomopsis viticola, Greenaria uvicola* (bitter rot), and *Colletotrichum* spp. (ripe rot). All species form black pycnidia or acervuli on the berry surface late in the season, giving the fruit a rough appearance and causing the epidermal

tissue to darken as disease progresses (Wilcox et al. 2015). In this study, *P. telopeae* spores oozing from the black acervuli were watery, light brown, and smeared easily when touched – these traits may be useful to differentiate from *Greenaria uvicola*, which produces abundant black sooty spores, and *Colletotrichum* spp., which produce salmon-colored spore masses. Further study of diagnostic symptoms will be critical to improve detection and control of *Pestalotiopsis telopeae*.

Aspergillus has been associated with late season fruit rots in the mid-Atlantic (Tony Wolf, personal communication) but its importance as a pathogen was previously not known. *Aspergillus* spp. are recognized as important fruit rot pathogens of grapes in California where, in addition to direct effects in reducing marketable yields, *Aspergillus* spp. are thought to play an important role in facilitating sour rot (Rooney-Lantham et al. 2008). In support of this association, sour rot was commonly found co-occurring on berries infected by *A. japonicus* in this study (data not shown). Sour rot is caused by a combination of yeasts and bacteria that together convert sugars into alcohol and acetic acid, negatively effecting wine quality (Barata et al. 2012b). If this is the case, then controlling *A. japonicus* may be critical to preventing sour rot development in susceptible cultivars. It also implies that *Aspergillus* infections may be masked in the field, if the pathogen is quickly displaced by sour rot organisms, making accurate diagnosis challenging (Rooney-Lantham et al. 2008). As another diagnosis challenge, *Aspergillus* fruit rot signs and symptoms may be mistaken for grey mold, which both have a fuzzy grey appearance on the berry. However, in the early stages of growth, *Aspergillus* conidiophores are greyish-green to black in color, whereas the conidiophores of *B. cinerea* are grey to dark brown, and this difference may be useful for field diagnosis.

Further investigation of pathogen distribution, infection biology, and sour rot associations of *A. japonicus* in this region will aid in developing control strategies to mitigate losses. *Alternaria alternata* is likely the least economically important of these newly described pathogens, due to its greater reliance on wounding for disease development. In other regions, *A. alternata* has been reported to cause summer bunch rot during the growing season (Kakalíková et al. 2009) it is often recovered as a common fungal endophyte (Hall and Emmett 2001; Rooney-Latham et al. 2008; Rosa et al. 2002; Rybárik et al 2014; Setati et al. 2015; Tao et al. 2014). Consistent with this, preliminary trials in this region recovered *Alternaria* from asymptomatic, unripe Chardonnay berries (Kepner and Swett, unpublished data). *Alternaria alternata* is likely acting as an opportunistic pathogen which primarily causes post-harvest losses (Karabulut et al. 2003; Steel et al. 2013). It is therefore unlikely that *A. alternata* poses a threat to wine grapes, which are rapidly processed, but may this pathogen be an issue for the expanding table grape industry in the region.

Vidal blanc is widely propagated in the mid-Atlantic as a replacement to Chardonnay for its similar enological properties and improved disease resistance traits. This study indicates that Vidal blanc is highly susceptible to *A. japonicus* and *C. fioriniae*. Both species initiated symptom development within two to four days, leading to degradation of 11 to 33% of berries by four days post inoculation and over 70% of berries by eight days post inoculation. Neither of these species are current management targets on Vidal blanc. *Botrytis cinerea* caused symptoms within two days post inoculation, with incidence reaching 50% by the end of the study, indicating that Vidal blanc is moderately susceptible to *B. cinerea*, consistent with Reisch et al. (1993). Although *P. telopeae*-

inoculated berries did not develop symptoms until six days post-inoculation (data not shown), disease incidence was similar to *B. cinerea* by the end of the study, indicating that Vidal blanc is also moderately susceptible to *P. telopeae*. Vidal blanc appears to be relatively resistant to *A. alternata*. This is the first study to characterize the relative susceptibility of Vidal blanc to a range of fruit rot pathogens and suggests that *A. japonicus* and *C. fioriniae* may have the greatest economic impacts on Vidal blanc production, especially as Vidal blanc acreage increases. This study was limited in the breadth of white cultivars examined. Further studies to screen for pathogen resistance will be critical to identifying cultivars with resistance to the diverse fruit rot diseases in the region.

These studies emphasized pathogenic hyphal fungi, since these are significant drivers of fruit degradation, both directly and by facilitating colonization of other pathogens (e.g. sour rot yeasts and bacteria). Several other fungal species isolated from symptomatic berries, including *Penicillium* spp. and *E. nigrum*, were not pathogenic in preliminary trials. Survey's that encompass a wider geographic and host range may reveal a greater diversity of fungal pathogens contributing to late season fruit rot in this region. For example, additional surveys in western Maryland indicate that *Cladosporium* spp. may also contribute to late season fruit rot (Swett and Hamby, unpublished data), as has been reported on table grapes in California (Swett et al. 2016, *in press*). Comprehensive evaluation of fruit rot pathogens will be critical to effective late season fruit rot management, which is necessary for an economically and environmentally sustainable wine grape industry in the mid-Atlantic.

Conclusions

The mid-Atlantic in general, and Maryland in particular, has the potential to be a productive and profitable grape growing region, but industry expansion is hindered by late season fruit rots, which are favored by the warm, humid summers and mild winters. Fruit rot disease epidemiology needs to be understood and losses mitigated to ensure profitability and high quality wine production. In this study, *A. alternata*, *A. japonicus,* and *P. telopeae* were isolated from and able to cause fruit rot symptoms in white wine grapes, representing the first report of all of these species as fruit rot pathogens of grapes in the region, the first report of *P. telopeae* as a pathogen of grapes, and the first report of *A. japonicus* as a pathogen of any hybrid grape variety. This study also represents the first comparative analysis of Vidal blanc susceptibility to multiple fruit rot pathogens, and indicates high susceptibility to *A. japonicus* and *C. fioriniae*. These results will provide foundational information for downstream studies to establish management strategies for late season fruit rots and to develop diagnostic resources to improve identification and management of diverse fruit rot diseases throughout the mid-Atlantic region.

Chapter 2: Insect-fruit rot interactions within the mid-Atlantic vineyard ecosystem at community and organismal scales

Introduction

Both table and wine grapes grown in the mid-Atlantic contribute over $1 billion to the region's economy (Anonymous, 2010; Anonymous 2013; Anonymous 2015). The region's long, warm summers and mild winters create an excellent environment for growing diverse grape cultivars. However, these same conditions are also conducive to the survival, dispersal and infection of many pathogens of grape. There are many important diseases affecting grapes but fruit rots are the number one reported grower concern (Poling 2007; Fiola 2008; Wilcox 2013). Fruit rots can cause severe damage, reducing crop yield by rendering both table grapes and wine grapes unmarketable. Wine grapes infected with fruit rot pathogens such as sour rot yeasts and bacteria generate off-flavors and render the wine undrinkable (Barata et al. 2012b; Steel et al. 2013). To avoid this, some wineries consider a grape cluster unacceptable if as little as five percent has

fruit rot. In addition to losses to individual growers, poor quality wines, when sold, can hurt this growing industry by diminishing the region's wine reputation.

Both summer and late season fruit rots are major contributors to yield loss. Summer fruit rot pathogens in the mid-Atlantic include the black rot pathogen *Guignardia bidwellii* (Travis, 1998; Luttrell, 1948), *Botrytis cinerea*, the cause of bunch rot (Wolf et al. 1997; Baudoin 2012; Hed et al. 2014), and ripe rot pathogens in the *Colletotrichum acutatum* and *C. gleosporioides* species complexes (Oliver and Nita, 2014, Kepner and Swett, in prep). Late season fruit rot pathogens include *B. cinerea* and *Colletotrichum* spp., as well as *Aspergillus japonicus*, *Pestalotiopsis telopeae* (Kepner and Swett, in prep), and sour rot yeasts (including *Pichia* spp., *Candida* spp., and *Hanseniaspora* spp.) and bacteria (including *Gluconobacter* spp. and *Gluconacetobacter* spp.). Sour rot pathogens produce compounds which negatively affect wine quality (Wolf et al. 1992; Barata et al. 2012b; Nally et al. 2013; Steel et al. 2013); in addition, volatile by-products can attract frugivorous (fruit-associated) insects which cause additional damage to grape clusters further reducing their quality (Witzgal et al. 2012; Davis et al. 2013)

Vineyard insects, including moths, fruit flies, sap beetles, wasps and ants, likely influence late season fruit rots in mid-Atlantic vineyards, by vectoring fungi and bacteria from diseased to healthy fruit, and also by creating wounds which allow pathogen entry (Poling 2007; Pfeiffer et al. 2013). Interactions between fruit rots and insects have not been documented in the mid-Atlantic, however, there are many documented interactions in other grape-growing regions.

In Europe, *Botrytis* bunch rot severity is markedly enhanced by *Lobesia botrana* (European grapevine moth; Lepidoptera: Tortricidae). Larvae carrying *B. cinerea* spores

both internally and externally can infect berries when tunneling through grape berries to feed, create wounds created by tunneling, and facilitate colonization by spores on the berry surface (Fermaud and LeMann, 1989; Fermaud et al. 1992; Isaacs et al. 2005; Ioriatti et al. 2015). Although *L. botrana* does not occur in the U.S., the closely related species *Paralobesia viteana* (American grape berry moth; Lepidoptera: Tortricidae) is a major pest in the mid-Atlantic (Wilcox, 2015). Despite its long history in the region (Hedrick, 1919), little is known about its relationship with fruit rot pathogens. Since both *P. viteana* and *L. botrana* are Tortricid moths, with larvae that share similar feeding habits (Botero-Garcés and Isaacs, 2003; Jordan et al. 2013), *P. viteana* is likely to have relations with fruit rot pathogens similar to *L. botrana*. In support of this hypothesis, *B. cinerea* is commonly associated with *P. viteana* infested berries in this region (Swett, personal communication).

Drosophila melanogaster (vinegar fly; Diptera: Drosophilidae), a common vineyard pest, can also vector *B. cinerea* spores (Louis et al. 1995). Diverse *Drosophila* spp. vector acidic acid bacteria, leading to sour rot development in wounded berries (Barata et al. 2012c). In addition, *Drosophila suzukii* (spotted wing drosophila; Diptera: Drosophilidae) has become an important agricultural pest causing significant losses in brambles (Biddinger et al. 2014), stone fruits (Poyet et al. 2014), blueberries (Kinjo et al. 2013), grapes, and other soft-skinned fruits (Walsh et al. 2011) following its introduction to North America in 2008 (Asplen et al. 2015). Like other drosophilids, *D. suzukii* feeds on yeast that naturally colonize fruits and requires a soft substrate, usually wounded or overly ripe fruit, for successful oviposition (Phaff et al. 1956; Kinjo et al. 2012;). However, *D. suzukii* is unique among other drosophilids in that the females have a

serrated ovipositor which allows for penetration and successful oviposition into intact fruit, even when unripe (Walsh et al. 2011). Ioriatti et al. (2015) examined the ability of *D. suzukii* to oviposit into different wine grape varieties and observed a range of results in both red and white grapes due to the penetrative resistance of the berry skin. Similar results were observed by Kinjo et al. (2013) in blueberries. The epidemiological interactions of *D. suzukii* with fruit rot pathogens in the mid-Atlantic vineyards are currently unknown, but the ability to infest intact berries and initiate wounds has potential to increase fruit rot severity (Barata et al. 2012c; Ioriatti et al. 2015)

In addition to the above, there are many other fruit-associated insects that may initiate fruit rots in mid-Atlantic vineyards, including sap beetles and stink bugs. Sap beetles (Coleoptera: Nitidulidae), common throughout the region, are drawn to damaged and diseased berries that have begun to ferment (Lindegren et al. 1992) and can externally acquire *B. cinerea* and other mold and yeast spores from infested berries (Considine, 2012). *Halyomorpha halys,* (brown marmorated stink bug), *Euschistus servus,* (native brown stink bug), and *Chinavia hilaris,* (native green stink bug; Pentatomidae*:* Hemiptera) have piercing mouth parts that cause wounds in berry tissue when feeding, and studies indicate abilities to vector pathogenic yeast species (Daugherty, 1967; Mizell, 2005; Brust and Rane 2011; Peiffer and Felton, 2014; Smith et al. 2014). The roles of these insects in facilitating fruit rot disease in grape berries are unknown.

Understanding the roles of insects in facilitating fruit rots is important to the continued success and economic growth of the mid-Atlantic grape industry. Consumer demands for sustainable production practices require more targeted disease management strategies than are currently being employed. For example, current fruit rot control practices do not

incorporate insect control strategies. To develop insect-fruit rot co-management methods requires an understanding of *whether insects can facilitate fruit rots*, and if so, *which fruit rots* can be better managed with insect control, *which insects* should be targets for management, and *when* insect management should occur.

The objectives of this study were to: (i) evaluate interactions between insect and fruit rot communities in a Maryland vineyard, to test the hypothesis that insects drive fruit rot development and to evaluate seasonal targets for insect management and (ii) develop a protocol to study the possible roles of *D. suzukii* in fruit rot epidemiology, using the fruit rot pathogen *C. fioriniae*, with a secondary aim of establishing preliminary data on *D. suzukii*-*C. fioriniae* interactions in grape berries.

Methods

Insect community-fruit rot interactions in the vineyard

Study site

Studies were conducted at the Western Maryland Research and Education Center in a mixed Chardonnay and Chambourcin vineyard in Keedysville, MD. This eighteen-year old, 0.31ha vineyard consisted of silty loam soil with 5m of grass between rows and 3m between vines. The vineyard was bordered by a row of mixed grape cultivars to the east, a mature Chardonnay-Chambourcin row followed by an apple orchard to the west, another experimental vineyard separated by a grass road to the south, and a native forest separated by another grass road to the north. Vines were treated weekly with pesticides from May 6 to August 12, 2015, with a rotation of carbaryl (Sevin), thiamethoxam (Actara), kaolin clay (Surround), and bifenthrin (Brigade). *Phomopsis*, black rot, *Botrytis* grey mold, downy mildew and powdery mildew were managed from May 5, 2015 to

August, 12, 2015 with a weekly rotation of ethylenebisdithiocarbamate (Manzate Pro-Stick), myclobutani, (Rally), n-trichloromethylthio-4-cyclohexene-1 2-dicarboximide (Captan 80 WDG), mandipropamid and difenoconazole (Revus Top), tebuconazole (Luna Experience), and phosphorus acid (Phostrol)[1].

Experimental design

The experimental vineyard was arranged in four blocks (rows) consisting of five Chardonnay vines per block, skipping the outermost vine in all blocks to minimize edge effects. One cluster per vine was allocated to each of four treatments. Treatments consisted of: (1) exposure to insects all season (no exclusion bag; positive control), (2) exposure to insects active from mid-summer (exclusion bags in place from June 23 to July 23, 2015) through harvest (September 23, 2015), (3) exposure to insects active from fall (bags in place from June 23 to September 2, 2015) until harvest, and (4) no exposure until harvest (bags in place from June 23 to September 23, 2015; negative control) (Table 7).[1]

To exclude insects, all treatment clusters (except positive control clusters) were placed into insect exclusion bags (five-gallon paint strainer bags, Home Depot) on June 23, 2015. Clusters with no visible damage at the time of bagging were selected randomly. Each cluster was shaken to dislodge insects (D'Alberto et al. 2010), then bagged, hand tied to the rachis, and secured with a twist-tie. Bags were adjusted to minimize contact with the cluster. This bagging method allowed air flow and sunlight exposure to the cluster, and preliminary studies indicate that fruit develops normally when bagged and there is no noticeable effect of bagging on fruit rot (Swett, unpublished data). The all season-exposed, positive control treatment clusters were inspected as above, and

[1] See Appendix Table A-1 for the complete spray schedule.

otherwise unaltered. Bags were then systemically removed to expose clusters to insects active during different periods within the season (Table 7).

Table 4. Experimental treatments of clusters and the associated stage of cluster development during exposure to insect communities.

Insect exposure treatment	Dates of exposure	Developmental stages of clusters during exposure
1. All insects	June 23 to September 23, 2015 (All season)	All stages
2. Insects active from mid-summer through harvest	July 23 to September 23, 2015 (Summer to harvest)	Cluster fill to ripe
3. Insects active from fall through harvest	September 2 to September 23, 2015 (Fall to harvest)	Veraison to ripe
4. No insects	Never	None

Fruit rot evaluation

Beginning on July 23, 2015, and at each sampling interval thereafter, clusters were evaluated for sour rot, ripe rot, and black rot. For each fruit rot, presence in each treatment cluster was quantified based on presence of diagnostic signs and symptoms on one or more berries in the cluster. Sour rot incidence was evaluated based on the presence of light brown, yeasty discharges, and the presence of acetic acid and/or ethyl acetate odors coming from one or more berries. Ripe rot was distinguished based on distinct marginalized areas of circular, reddish-brown necrotic spots on the berry epidermis. Also, berries may have had visible orange to salmon colored conidia on the surface. Black rot was identified based on the dark brown marbling of the epidermal tissue with or without visible acervuli on the surface (Wilcox, 2015). No other fruit rots were observed in this study. Incidence of each disease was quantified as the percentage of symptomatic clusters in each row, at each time point.

Monitoring Drosophila suzukii *populations*

Vinegar traps were used to monitor *D. suzukii* populations throughout the season. The traps consisted of plastic cups with plastic mesh holes (Trece SWD Trap, Trece INC.) on both sides containing about 200ml of a drowning mixture (4ml unscented dish soap per gal apple cider vinegar). A *D. suzukii* attractant (Trece 2015 SWD Lures RED item number TC/C0-5001-1R, Trece INC.) was adhered to the lid of the cup and hung in the center above the drowning mixture. One trap was placed within the center of each of four blocks. Traps were collected, emptied, and replaced weekly from July 8 to September 23, 2015. *Drosophila suzukii* flies were identified and enumerated weekly. Lures within the traps were replaced once, six weeks after initial deployment, on August 5, 2015.

Insect activity in monitor and treatment clusters

At each sampling interval (Table 7) in each block, three monitor vines in the center of the vineyard were surveyed for insects within clusters. Five random clusters per monitor vine were sampled using each of three methods: (1) aspiration of insects on the cluster into a glass vial, (2) beating of clusters over a plastic bag, and (3) collection and incubation of damaged berries at room temperature to observe emergence of any insects residing inside. Specimens were identified to at least the family level, and if possible the genus or species level. Insect activity was evaluated in treatment clusters by gentle examination of the cluster at each bag removal date, and berries were removed on the final sampling date to examine internal damage.

Paralobesia viteana *activity in indicator vines and treatment clusters*

Paralobesia viteana activity was monitored in two indicator vines at the vineyard edge, boarding a woodlot, where presence is most easily detected (Botero-Garcés and Issacs,

2003), and in treatment clusters starting on July 23, 2015, and monthly thereafter. Clusters were examined for signs of *P. viteana* based on the presence of entry wounds, larvae, larval frass, and silk webbing (Figure 2).

Developing a system to evaluate the role(s) of *D. suzukii* in fruit rot epidemiology

Since there have been few studies to examine *D. suzukii* in grapes, the primary goal of this project was to develop a system which could be used to conduct in-vitro studies of *D. suzukii*-fruit rot pathogen-grape berry interactions, with a secondary aim of establishing preliminary data on *D. suzukii-C. fioriniae* interactions in grape berries. *Colletotrichum fioriniae* was selected as a high priority pathogen in the region, amenable to in-vitro assays, which belongs to a genus reported to interact with *Drosophila* species (Peña et al. 1989).

Identification of grape cultivars supporting D. suzukii *oviposition*

The objective of this study was to identify table grape cultivars that permitted *D. suzukii* oviposition and F1 (first generation) development. This experiment was arranged in a split block design, wherein cultivar treatments consisted of Red Globe, Scarlett Royal, and Flame grapes, half of which received a wounding treatment, and half of which were left intact. Each treatment combination was replicated three times (incubators) with four sub-replicates (berries) per replicate.

All three cultivars were obtained from local supermarkets and stored at -1^0C until time of use. Asymptomatic, intact berries were aseptically cut from the rachis, in order to preserve the cap stem. Berries were then surface disinfested by washing in 0.1% Tween 20 for 5 sec, soaking in 70% ETOH for 30 sec, then 0.2% NaClO for 4 min, then rinsing in DiH_20 to remove excess NaClO, and surface dried for 30 min in a laminar flow hood.

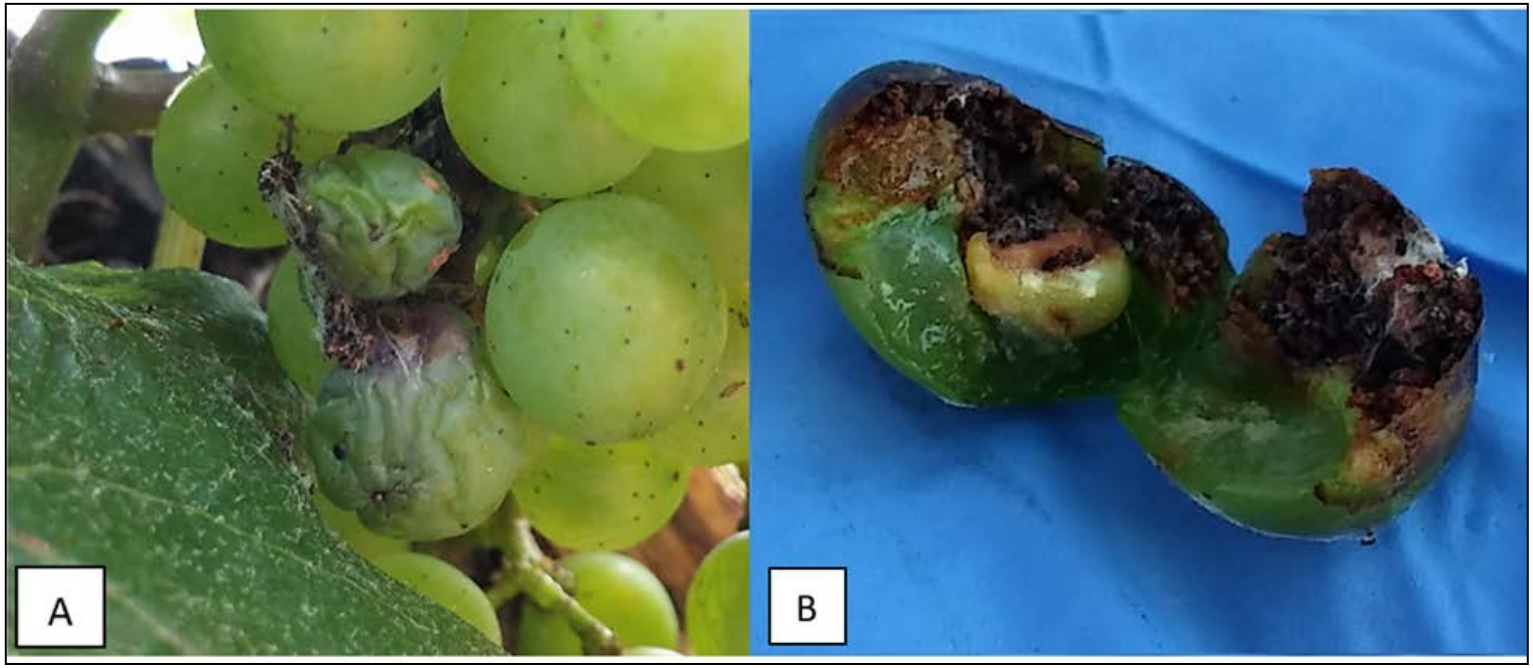

Figure 2. *Paralobesia viteana* damage on Chardonnay grapes during cluster fill. Typical damage includes tunneling between adjacent berries, necrosis at entry points, and the presence of webbing (A). When berries are opened up, internal decay, frass, and webbing were commonly observed (B).

In the wound treatment, 50% of the berry surface was wounded by puncturing through the epidermis with a sterile probe. Intact berries were not altered. Thereafter, berries were aseptically transferred into incubation chambers consisting of plastic 1.89 liter Gladware containers with a sanitized four-slotted synthetic rubber ice cube tray on top of two-7.5cm filter paper, saturated with 3 ml DiH_20. These incubation chambers were sealed with their factory made lids, retrofitted with a circular cutout mesh vent to allow air flow. *Drosophila suzukii* flies were reared in a semi-sterile laboratory environment on standard *Drosophila* rearing medium (27.5 liters deionized water, 15 liters molasses, 155 g agar, 1800 g yeast, 3.13 liter cornmeal, 375 ml Tegosept, and 135 ml propionic acid) and anesthetized with CO_2 before introduction into incubation chambers. Ten alert, healthy, and fully sclerotized 7-10 day-old mated males and females (five of each sex) were then placed into the incubation chambers immediately following berry placement (as described above). Chambers were incubated at 22^0C for 72h to allow for mating and oviposition. After 72h, the flies were anesthetized with CO_2 and removed. Emergence of the F1 generation was monitored for 21 days. Enumeration and removal of emergent flies were conducted at 18 and 21 days after the removal of mating adults. Results from this study were used to select a berry cultivar suitable for *D. suzukii* oviposition and F1 recovery which could be used in downstream studies examining *D. suzukii*-fruit rot interactions.

Developing a method to evaluate emergence and inoculum acquisition of D. suzukii *adults from* C. fioriniae *infested berries*

The objectives of this study were to develop a method[2] to (1) quantify F1 generation survivability in fruit rot infested berries and to (2) quantify external inoculum loads on

[2] See Appendix for the detailed protocol for in-vitro trials.

emergent adult flies. This study was conducted as a split plot design, wherein berries were either inoculated with *C. fioriniae* (isolate SL 237), or treated with sterile deionized water (non-inoculated control); half of the berries in each treatment were wounded and the other half were left intact at the time of inoculation (as described above). Berries in each treatment combination were exposed to adult *D. suzukii* flies 24 hours after treatment. Each treatment combination was replicated in triplicate with three sub-replicates (berries) per replicate (incubator).

Colletotrichum fioriniae cultures (originally recovered from diseased Chardonnay berries) were grown on potato dextrose agar (agar 15 g/liter, dextrose 20 g/liter, potato extract 4 g/liter) for six days (24^0C 12:12h L:D). Inoculum was prepared as a 10^5 spores / ml spore suspension in a 0.5% KCl + 0.1% Tween 20 solution, stored at 4^0C, and used within 48h. Spore viability, evaluated at the time of inoculation as described in Aegerter and Gordon (2006), was $2.6x10^4$ spores / ml. Wounded and intact berries were submerged in the spore suspension for 2 min (inoculated treatment) or in sterile deionized water for 2 min (non-inoculated control). Berries were then aseptically transferred to the incubation chambers (as described above). Incubation chambers were placed on top of a thick layer of moistened paper towels, inside an 8-liter plastic zip-lock bag[33]. Inoculated berries were incubated for 24h (24^0C 16:8h L:D) to allow berries to dry, and then ten *D. suzukii* adults were placed into the incubation chambers (as described above). After 24h, the mated adult flies were again anesthetized and removed. F1 emergence was monitored for 18 days, and F1 progeny were removed 13 days and 18 days after the removal of mating adults. F1 flies were anesthetized and aseptically transferred by

[3] See Appendix Figure A-1 for incubator design

replicate into glass vials containing a 1 ml solution of 0.5% KCl and 0.1% Tween-20. Vials were vortexed on a medium speed for 10 sec to dislodge spores. Spore viability was assessed by placing three-100mL subsamples of each suspension onto *Colletotrichum* selective medium (agar 20 g/liter, peptone 2 g/liter, magnesium sulfate heptahydrate 1.22 g/liter, potassium phosphate 2.72 g/liter, sucrose 2.8 g/liter, iprodione 0.025 g/liter, and tetracycline hydrochloride 0.3 g/liter; Correll et al. 1993). Cultures were maintained at 24^0C (16:8h L:D) and the number of colonies on each plate was enumerated two days after culturing.

Analysis

Effects of insect exposure treatment on fruit rot disease incidence at harvest (on September 23) was analyzed using ANOVA and Dunnett's test with the statistical software JMP 11 (SAS Institute, Cary, NC). Treatment effects were interpreted based on differences in insects active at each developmental stage of cluster development. Significance of main effects of pathogen and wounding treatment on *D. suzukii* F1 recovery and inoculum acquisition (fruit rot-*D. suzukii* interaction study only) were analyzed using ANOVA, and Tukey's multiple means comparisons (HSD) in JMP 11.

Results

Insect community-fruit rot interactions in the vineyard

Fruit rot evaluation

There was no significant effect of insect exposure treatment on incidence of either black rot or ripe rot ($P = 0.8662$ and 0.985, respectively) based on ANOVA (Figure 3). There was a significant effect of insect exposure treatment on sour rot incidence ($P = 0.045$,

ANOVA), reflecting 38.9% greater incidence of sour rot in clusters exposed from mid-summer to harvest compared to negative control clusters never exposed to insects (P = 0.0376, Dunnett's test) (Figure 3). There was no significant difference between negative controls and either clusters exposed all season or clusters exposed only in September (P = 0.18 and 0.97 respectively, Dunnett's test) (Figure 3).

Monitoring Drosophila suzukii *populations*

The *D. suzukii* population exhibited a bimodal growth pattern during the 2015 growing season. Population levels started off very low in late June, at the pea-size stage of cluster development[4]. The number of flies captured increased steadily until peaking on August 8, 2015, during cluster fill, with an average of 271 flies captured. Numbers then decreased to an average of 104 flies midway through veraison on September 16, 2015; at this point, the population again began to rise until harvest when sampling ended with an average of 189 flies captured. Across the season, there was a significant effect of month on population size ($P < 0.001$) based on ANOVA.

Insect activity in monitor and treatment clusters

Overall, there was an increase in the diversity of insects captured as the season progressed (Table 8). The abundance of these insects steadily increased throughout the season (Table 8). Some of the most active insects included *Drosophila* spp., ants (*Formicidae*), and sap beetles (*Nitidulidae*).

Paralobesia viteana *in indicator vines and treatment clusters*

Paralobesia viteana was active in both indicator vines and treatment clusters on June 23, where only berry damage was observed, and on July 23, 2015, where berry was damage observed and reared larvae were identified as *P. viteana*. Activity was not detected in the

[4] See Appendix Table A-2 for raw data of *Drosophila suzukii* populations.

vineyard on September 2 or September 23, 2015 (Table 8). When combining *P. viteana* incidence throughout the study, there was a significant effect of proximity to woodlot on incidence of *P. viteana* damage, with an 85% greater incidence of *P. viteana* damage in blocks closest to the woodlot ($P < 0.0001$, ANOVA).

Developing a system to evaluate the role of *D. suzukii* in fruit rot epidemiology

Identification of grape cultivars supporting D. suzukii *oviposition*

The F1 generation of *D. suzukii* was successfully recovered in each of the three red table grape cultivars examined (Table 9). There was a significant effect of wounding on F1 recovery ($P = 0.042$, ANOVA), reflecting greater F1 emergence in wounded berries (Table 9). There was no significant difference between cultivars receiving identical treatments ($P = 0.08$, and 0.31 for intact and wound treatments, respectively, ANOVA), but there was a trend wherein F1 recovery from intact berries was lowest in Scarlett Royal (0 *D. suzukii* F1 adults recovered).

Table 5. Recovery of F1 *D. suzukii* flies from three red table grape cultivars. Data combined from 18 and 21 days post oviposition results (n = 6).

	Treatment[a]	
Cultivar	Intact	Wound
Flame	1.0 ± 0.89 a	1.6 ± 1.6 b
Red Globe	1.5 ± 0.61 a	4.0 ± 1.9 b
Scarlet Royal	0.0 ± 0.0 a	1.7 ± 1.7 b

[a]F1 recovery from each variety per treatment based on Tukey's HSD means comparison. Means (± SE) within columns and rows separated by the same letter are not significantly different ($P > 0.05$).

Developing a method to evaluate emergence and inoculum acquisition of* D. suzukii *adults from* C. fioriniae *infested berries

Using the method developed, F1 progeny successfully emerged from *C. fioriniae*-infested

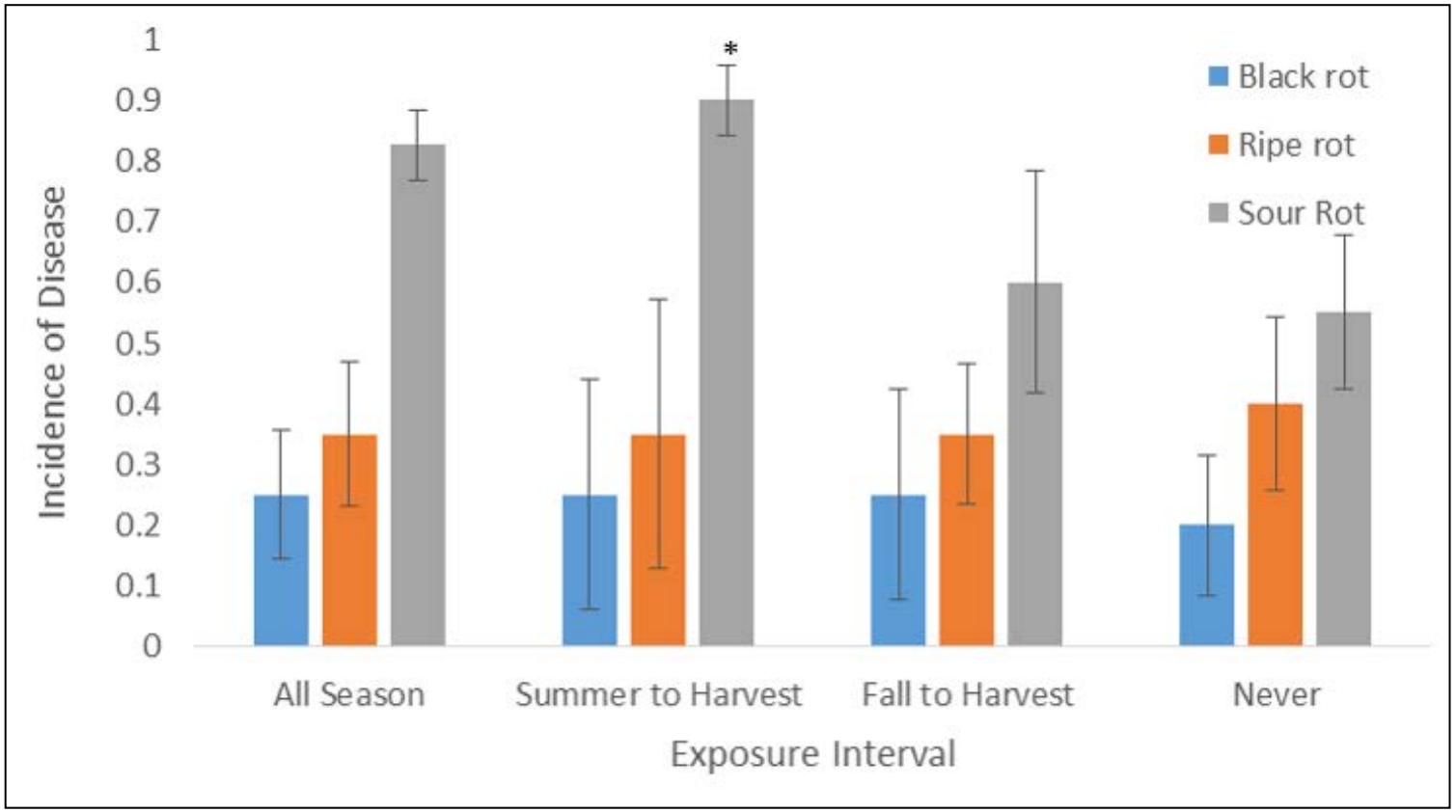

Figure 3. Incidence of black rot (blue), ripe rot (orange), and sour rot (gray) on treatment clusters (n=20) at harvest. Bars indicate standard error. (*) indicates a significant difference in disease incidence compared to the negative control (never exposed clusters) based on Dunnett's comparison with a control ($P < 0.05$).

Table 6. Insects recorded during field study and their respective abundance sampled by aspirating, beating, and recovered in damaged berries.

Sample date/ Cluster stage	Order	Family	Species	No. of specimens recovered	No. of vines recovered from
June 23, 2015/ Pea-size	*Coleoptera*	*Curculionidae*	Unknown sp.	1	1
	Diptera	*Cecidomyiidae*	Unknown sp.	2	2
		Simuliidae	Unknown sp.	3	3
		Unknown	Unknown sp.	1	1
	Hymenoptera	*Formicidae*	Unknown sp.	1	1
		Proctotrupidae	Unknown sp.	2	2
	Lepidoptera	*Tortricidae*	*Paralobesia viteana*	D[a]	
July 23, 2015/ Cluster fill	*Coleoptera*	*Scarabaeidae*	*Popillia japonica*	3	3
	Diptera	*Drosophilidae*	*Drosophila* sp.	1	1
			Zaprionus sp.	1	1
		Muscidae	Unknown sp.	1	1
	Hemiptera	*Pentatomidae*	*Euschistus servus*	1	1
	Lepidoptera	*Tortricidae*	*Paralobesia viteana*	1	1
September 2, 2015/ Veraison	*Coleoptera*	*Cleridae*	Unknown sp.	1	1
		Nitidulidae	Unknown sp.	2	2
		Unknown	Unknown sp.	1	1
	Diptera	*Drosophilidae*	*Drosophila* sp.	5	4
			Drosophila suzukii	1	1
			Zaprionus sp.	2	2
		Syrphidae	Unknown sp.	1	1
		Unknown	Unknown sp.	2	2
	Hemiptera	*Pentatomidae*	*Acrosternum hilare*	1	1
	Hymenoptera	*Formicidae*	Unknown sp.	6	5

Sample date/ Cluster stage	Order	Family	Species	No. of specimens recovered	No. of vines recovered from
September 23, 2015/ Harvest	*Coleoptera*	*Coccinellidae*	Unknown sp.	1	1
		Nitidulidae	Unknown sp.	5	2
		Unknown	Unknown sp.	1	1
	Diptera	*Drosophilidae*	*Drosophila* sp.	11	4
			Zaprionus sp.	5	2
		Unknown	Unknown sp.	2	2
	Hemiptera	*Pentatomidae*	*Euschistus servus*	1	1
	Hymenoptera	*Apidae*	*Apis mellifera*	3	3
		Formicidae	Unknown sp.	2	2
	Pscoptera	Unknown	Unknown sp.	1	1

[a]Indicates that typical *P. viteana* damage (D) was observed on one or more clusters in the field, but the larvae and / or moth were not present.

berries carrying fungal spores. The effect of pathogen treatment and wounding treatment on *D. suzukii* F1 recovery was not significant ($P = 0.38$ and $P = 0.66$, respectively) (Table 10). The effect of wound treatment on *C. fioriniae* infestation of F1 adults was not significant ($P = 0.47$, ANOVA), wherein F1 progeny were infested with an average of 198 +/-119 spores / fly across all treatments (Table 10). No *C. fioriniae* spores were recovered from F1 adults in the non-inoculated treatments (Table 10).

Table 7. Recovery of F1 *D. suzukii* and their acquired spore loads in intact and wound-inoculated Flame grapes. Data combined from 13 and 18 days post oviposition results

	Treatment[a]		Viable Spores per F1[b]	
Pathogen	Intact	Wound	Intact	Wound
C. fioriniae	6.33 ± 4.09 a	2.5 ± 1.0 a	215.44 ± 177.32 a	433.19 ± 323.20 a
Water	1.66 ± 0.88 a	7.66 ± 3.84 a	0	0

[a]F1 recovery from each variety per treatment based on ANOVA. Means (± SE) within columns and rows separated by the same letter are not significantly different ($P > 0.05$).
[b] Means (± SE) within the same rows separated by the same letter are not significantly different ($P > 0.05$).

Discussion

The results of the field study indicate that exposure to vineyard insects can influence sour rot epidemiology. When Chardonnay clusters were exposed to early to mid-summer insect communities, sour rot incidence nearly doubled compared to clusters protected during the same time period. Insects active during this period which have been implicated as drivers of fruit rot included *P. viteana*, several *Drosophila* spp., and *E. servus.* When clusters were exposed to insects only in the fall (after September 2), there was no difference in sour rot development compared to clusters that were never exposed, indicating that fall insect communities alone are not significant drivers of sour development. *Paralobesia viteana* was the only insect active in the summer that was not active in during the fall to harvest (September 2, to September 23, 2015) exposure time.

Based on this, we hypothesize that *P. viteana* is a primary early season insect driving fruit rot development.

These results suggest that early to mid-season insects active from bloom up until veraison should be the primary management targets for sour rot control, consistent with Pfeiffer et al. (2016). Improved control of *P. viteana* may be achieved by using effective chemistries starting earlier in the season, shortly before bloom (applications in this study began June 6, after bloom). According to our results, *P. viteana* was active early in the season, before exclusion treatments were implemented. An aggressive pesticide program was used in this study (applied primarily to protect against Japanese beetle), which included application of Sevin XLR, Actara, Surround, and Brigade, did not appear very effective in controlling *P. viteana*, based on observed persistent activity throughout the season despite chemical applications. There may be other insecticides that are more effective in controlling *P. viteana*, which were not used in this study. Further work to evaluate chemical efficacy and timing of applications may elucidate more effective early-season *P. viteana* control strategies.

These results run contrary to expectation that insects active in the fall are the sole drivers of late season fruit rots, including sour rot. This is a surprising finding, considering the greater diversity and overall abundance of insects known to feed on or cause direct damage to grape berries in the fall, compared to the early and mid-summer. This does not mean that fall insects, such as wasps (Hymenoptera: Vespidae) fruit flies, and ants (Hymenoptera: Formicidae), do not have significant roles in facilitating fruit rot development in the vineyard. Rather, these results imply that fall insects *alone* are not sufficient to enhance fruit rot incidence. One hypothesis for the greater significance of

early season insects is that they cause wounds which facilitate establishment of the late season insects, enhancing the epidemiological effects of late season insects on fruit rot development. Consistent with this hypothesis, in a parallel study, larvae of several *Drosophila* spp. and sap beetles were often observed in berries damaged by grape berry moth (Swett and Hamby, personal communication).

Although exposure to insects significantly increased incidence of insect damage and sour rot development, these results suggest that insect control alone is not sufficient to manage sour rot, which occurred at a high incidence even when clusters were never exposed to insects. Additional sour rot management control measures also need to be employed, including minimizing mechanical injury to berries during pruning, cordon training, leaf thinning, managing other pathogens which might facilitate fruit rot, and possibly fungicide applications (Wilcox, 2015).

Of note, *P. viteana* damage was observed in 25% of clusters at the time the bags were removed, indicating that *P. viteana* was present in some of clusters prior to bagging. This may account, in part for sour rot development in protected clusters, and likely reduced the effect of the exclusion treatments on fruit rot incidence. With an earlier exclusion date, effects of insect exclusion treatments on sour rot may be greater.

Unlike sour rot, black rot and ripe rot incidence were not affected by insect exposure treatments. Black rot may have also been unaffected by bagging because *Guignardia bidwellii* inoculum is mainly dispersed early in the season by rain splash to directly infect leaves and immature berries (Ferrin and Ramsdell, 1977). The majority of infections may therefore have already occurred by the time the bags were placed. Several of the *Colletotrichum* spp. that cause ripe rot have been shown to be vectored by insects in other

cropping systems including rice and soybean (Tebeest and Moore, 1992; Marcelino et al. 2008). This study did not support a role of insects in ripe rot, however, the incidence of ripe rot was lower (35 to 40% of treatment clusters) than sour rot (55 to 90% of clusters). Downstream studies at sites with higher ripe rot levels may provide additional insights into insect interactions.

The pesticide program used in this study might have prevented detection of treatment effects on *Colletotrichum* and other fruit rot pathogens, such as *Botrytis cinerea*, which was not observed during the course of the study. The fungicides that were applied during the study are effective in controlling hyphal fungi, but not most yeasts. Additionally, since copper or other bactericide was not used, bacteria were not actively managed. Therefore, the pesticide regime used in this study likely favored sour rot development, and indeed, sour rot occurred at higher levels than any other fruit rot, across all treatments. If so, treatment effects may have only been detected for sour rot because this was the only disease that was not suppressed by the pesticide treatments. The intensive fungicide applications throughout the season likely suppressed both black rot and ripe rot, which never reached an incidence greater than 40%. This could have prevented detection of a relationship between these diseases and insects. In follow-up studies, minimization of fungicide applications may allow detection of insect interactions with these and other fruit rot diseases.

As an alternative interpretation of the results, it is possible that the bagging treatment alone, and not insect interaction, altered cluster exposure to sour rot organisms. The pore size of the exclusion bags was large enough to permit fungal and bacterial entry (> 0.45 microns), but it is possible that the bag was a sufficient physical barrier to wind and water

dispersal that microbial exposure was significantly reduced. However, the bags were placed after fruit formation, providing opportunities for microbial establishment on fruit prior to bagging. In downstream studies, the effect of the bagging treatment on microbial exposure could be evaluated by comparing epiphyte abundance on berries in each treatment.

The increased incidence of sour rot during the mid-summer exposure treatment coincided with an increase in *D. suzukii* populations in the vineyard. These results, along with previous results from Ioriatti et al. (2015) and Kinjo et al. (2013), may implicate *D. suzukii* as a potential contributor to fruit rot development in the vineyard. To further evaluate the role of *D. suzukii* in facilitating fruit rots an in-vitro assay was developed. Initial trials focused on selecting an appropriate grape cultivar for downstream trials--Flame and Red Globe were selected as suitable cultivars, since both were able to support *D. suzukii* oviposition and development of F1 progeny. Significantly more *D. suzukii* F1 flies were recovered in wounded than intact berries, suggesting that berries with compromised epidermal integrity may be more susceptible to *D. suzukii* damage, consistent with previous studies (Kinjo et al. 2013; Biddinger 2014; van Timmeren and Isaacs, 2014; Ioriati et al. 2015). An assay was then developed (described above), wherein *D. suzukii* survival and inoculum acquisition could be examined for different fruit rot pathogens. Key to the development of this system was the placement of the moisture source (moisten paper towels) within a secondary chamber. With this design, high humidity was maintained (approximately 97% RH), but the flies were not directly exposed to moisture, which was important in preventing death of mating adults and

dilution of spores in emergent F1 flies. Additionally, this design provided greater *D. suzukii* F1 survival in wounded control treatments than in initial trials.

In preliminary evaluations, *C. fioriniae* did not affect F1 emergence, and F1 adults emerged carrying *C. fioriniae* spores with an average of 198 +/- 119 spores / fly in the inoculated berry treatments. These results indicate that *D. suzukii* may acquire and possibly transmit *C. fioriniae* spores to healthy berries in the vineyard. Based on observations during the study, disease severity (% of berries infected / incubator) was greatest in wound-inoculated berries, where F1 emergence was lowest – perhaps severely diseased berries are less supportive of instar development, due fungal alterations of fruit physiology and / or chemistry (Trienens et al. 2010). Including disease severity evaluations and increasing replications in downstream trials may help account for effects of severity on insect survival and acquisition of spores.

Conclusions

Field studies indicate that early and mid-summer vineyard insect communities influence sour rot development in the mid-Atlantic region. Based on this, sour rot management strategies should target insects active before veraison. These results expand on our understanding of sour rot-insect community dynamics in the mid-Atlantic, and run counter to the current assumption that sour rot is facilitated by late season insects, such as wasps and ants. An in-vitro assay was developed to examine interactions between fruit rot pathogens and the recently introduced vineyard insect, *D. suzukii*, which was among the insects active during the field study. Preliminary studies indicate that *D. suzukii* can reproduce in fruit rot infected berries, and emerge carrying pathogen spores. Further work is needed to identify insect targets for fruit rot management, narrow down the critical

time points of insect management, and establish mechanisms underlying relationships between fruit rot pathogens and insect pests.

Appendix

Table A- 1. Complete 2015 spray schedule used on the experimental vineyard during the insect community-fruit rot interaction study.

Date of Application	Material	Target Disease/Pest
May 5, 2015	Manzate	Phomopsis, Downy mildew, Anthracnose, Black rot
	Rally	Powdery mildew
June 1, 2015	Manzate	Phomopsis, Downy mildew, Anthracnose, Black rot
	Pristine	Powdery mildew, Black rot, Anthracnose
June 6, 2015	Manzate	Phomopsis, Downy mildew, Anthracnose, Black rot
	Luna Experience	Black rot, Botrytis, Powdery mildew
June 16, 2015	Manzate	Phomopsis, Downy mildew, Anthracnose, Black rot
	Revus Top	Powdery mildew, Black rot, Downy mildew
	Sevin XLR	American grape berry moth, Japanese beetle
June 22, 2015	Captan	Black rot, Downy mildew
	Revus Top	Powdery mildew, Black rot, Downy mildew
	Sevin XLR	American grape berry moth, Japanese beetle
July 1, 2015	Captan	Black rot, Downy mildew
	Luna Experience	Black rot, Botrytis, Powdery mildew
	Voliam Flexi	Japanese beetle
July 9, 2015	Captan	Black rot, Downy mildew
	Rally	Powdery mildew, Black rot
	Sevin XLR	Japanese beetle
July 16, 2015	Phostrol	Downy mildew
	Actara	Japanese beetle
July 21, 2015	Phostrol	Downy mildew
	Brigade	Japanese beetle
July 31, 2015	Phostrol	Downy mildew
	Sevin XLR	Japanese beetle
August 12, 2015	Phostrol	Downy mildew

Protocol for in-vitro insect-fruit rot interactions

Berry Preparation. Damage and blemish free grape berries were aseptically cut from the rachis leaving the cap stem intact on the berry. Then berries were then rinsed in 0.1% Tween-20, submerged in 70% EtOH for 30 sec, then soaked in a 0.2% NaClO solution for 4 min followed by a sterile deionized water rinse to remove any bleach from the surface. Berries were then air-dried in a laminar flow hood until the surfaces were completely dry. After drying, berries were then placed in a plastic zip lock bag with paper towels to absorb any extra moisture and stored in -1^{0}C incubator until use.

Pathogen Preparation. Colonies were initiated from stored filter paper and grown for 6 days on PDA (24^{0}C 12:12h L:D). A spore suspension was made by submerging the plates with a 0.5% KCl, 0.1% Tween-20 solution and spores were dislodged from the media using a sterile hockey stick. The resulting slurry was filtered through a sterile cotton cheese cloth into a sterile beaker. Spore solutions were enumerated by hemocytometer and diluted to the desired spores / ml using the previous KCl + Tween-20 solution. The resulting inoculum was stored in a 4^{0}C refrigerator until time of use (no more than 72h). Three 250 µl dilution platings of 10^2 spores / ml were performed on all inoculum immediately after inoculation to determine viability.

Inoculation. Incubation chambers were made from plastic, 1.89 liter Gladware containers that contained a sanitized four-slotted synthetic rubber ice cube tray. After being removed from cold storage and allowed to come to room temperature, berries were either wounded with a sterile probe to reach the desired amount of wounding, or left intact. The berries were then submerged by treatment type in the spore suspension for 2 min. The control berries were submerged in sterile deionized water for 2 min. Afterwards, berries were

transferred aseptically from the into the ice cube trays and sealed with their factory made lids that had a hole cut in the top which was covered with a fine mesh. The incubation chambers were then placed in a large plastic bag containing paper towels that were saturated in sterile deionized water in order to keep humidity high. The chambers incubated for 24h at 24^0C with a 16:8h L:D cycle in order for the inoculum to dry on the berry.

Insect introduction. Twenty-four hours after inoculation, *Drosophila suzukii* were anesthetized with CO_2 under a stereoscope. Five healthy and sclerotized males and females were selected and placed in glass vials together. After analgesia wore off, it was made sure that all flies awoke and were active. The vials were then emptied into the incubation chambers with berries yielding the desired treatments. The mesh lids were then closed and left to incubate (24^0C 16:8h L:D). After 24 hours, the mated adults were again anesthetized and removed from the chambers. The chambers were then left to incubate until offspring emerged.

Recovery of offspring and spores. Chambers were removed from their plastic bags and flies were anesthetization with CO_2. The emergent flies (offspring) were removed from the chambers with sterile tweezers, enumerated, and placed into vials filled with a 1 ml solution of 0.5% KCl and 0.1% Tween-20. The vials were vortexed on a medium speed for 10 sec to dislodge any spores. Then, three-100 ml of the resulting suspension was plated onto *Colletotrichum* selective media. Spore viability was assessed within 48 hours.

Analysis. Spore viability was averaged among the three plated suspension replicates per treatment replicate. Spore viability averages were then divided by the number of F1

offspring recovered from the treatment replicate and multiplied by 10, resulting in an average viable spore / fly count. Only treatment replicates that obtained an F1 generation were used in treatment calculations.

Notes for improvement. In order to better detect a treatment effect, replication should be increased to at least five per treatment combination (pathogen x wounding method). Observationally, disease severity among pathogen inoculated berries varied greatly both within replicates, and between wounding treatments. This phenomenon may be an important factor in F1 survival and a possible explanation for the high variances in the study. Therefore, quantifying disease severity by using the rating system described in Table 1 (pg. 7), could elucidate the relationship between extent colonization and F1 recovery. Additionally, as *D. suzukii* require between 2 to 3 weeks at 24^0C (16:8h L:D) in order to fully develop from egg to mature adult, adjusting the inoculum load may be helpful in managing disease severity issues that may be interfering with F1 recovery (this experiment used $2.4x10^4$ spores / ml). Furthermore, instead of F1 adults being placed into vials by replicate in bulk (as done in

Figure A- 1. Picture of incubation chamber design used to develop the method to evaluate emergence and inoculum acquisition of *D. suzukii* adults from *C. fioriniae* infested berries.

this experiment), each fly should be placed in its own vial to more accurately measure the spore / fly count.

Table A- 2. Raw data of Drosophila suzukii populations throughout the course of the field study.

Date	Block	Males	Females	Total	Stage of cluster development
7/8/15	1	0	0	0	Pea-size
7/8/15	2	0	1	1	Pea-size
7/8/15	3	0	0	0	Pea-size
7/8/15	4	0	0	0	Pea-size
7/15/15	1	0	0	0	Pea-size
7/15/15	2	0	0	0	Pea-size
7/15/15	3	1	0	1	Pea-size
7/15/15	4	1	0	1	Pea-size
7/22/15	1	1	3	4	Cluster fill
7/22/15	2	8	1	9	Cluster fill
7/22/15	3	5	1	6	Cluster fill
7/22/15	4	1	1	2	Cluster fill
7/29/15	1	17	11	28	Cluster fill
7/29/15	2	15	6	21	Cluster fill
7/29/15	3	11	9	20	Cluster fill
7/29/15	4	17	3	20	Cluster fill
8/5/15	1	41	50	91	Cluster fill
8/5/15	2	52	55	107	Cluster fill
8/5/15	3	44	79	123	Cluster fill
8/5/15	4	47	62	109	Cluster fill
8/12/15	1	58	43	101	Cluster fill
8/12/15	2	92	77	169	Cluster fill
8/12/15	3	151	90	241	Cluster fill
8/12/15	4	91	110	201	Cluster fill
8/19/15	1	96	59	155	Cluster fill
8/19/15	2	103	86	189	Cluster fill
8/19/15	3	338	134	472	Cluster fill
8/19/15	4	182	89	271	Cluster fill
8/26/15	1	143	97	240	Cluster fill
8/26/15	2	58	58	116	Cluster fill
8/26/15	3	85	98	183	Cluster fill
8/26/15	4	97	99	196	Cluster fill
9/2/15	3	96	115	211	Veraison
9/2/15	1	90	82	172	Veraison
9/2/15	2	59	52	111	Veraison
9/2/15	4	76	83	159	Veraison

Date	Block	Males	Females	Total	Stage of cluster development
9/9/15	1	99	57	156	Veraison
9/9/15	2	29	23	52	Veraison
9/9/15	3	82	77	159	Veraison
9/9/15	4	30	24	54	Veraison
9/16/15	1	93	56	149	Veraison
9/16/15	2	24	37	61	Veraison
9/16/15	3	65	60	125	Veraison
9/16/15	4	43	40	83	Veraison
9/23/15	1	67	139	206	Veraison
9/23/15	2	48	93	141	Veraison
9/23/15	3	108	157	265	Veraison
9/23/15	4	46	101	147	Veraison

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