

## ABSTRACT

Title of Thesis: THE EFFECTS OF SHOOT THINNING AND POST-BLOOM LEAF REMOVAL ON FRUIT ROTS AND DOWNY MILDEW FUNGICIDE RESISTANCE IN MID ATLANTIC VINEYARDS

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This thesis investigates integrated disease management strategies by evaluating essential canopy management practices and their effects on disease risk and development. Field trials were conducted across two vineyards in 2023 and 2024, involving three sites and five wine grape cultivars. Treatments included various combinations of high and low leaf removal and shoot density. Results demonstrated that low leaf removal significantly reduced ripe rot severity across trials and years, while low shoot density treatments lowered cluster powdery mildew and Botrytis severity. Disease severity varied by cultivar, with Merlot showing increased susceptibility to ripe rot and Vidal blanc to sour rot in 2024. Additionally, this research determined the fungicide resistance profiles of *Plasmopara viticola*, the causal agent of grapevine downy mildew. Between 2019 and 2023, 352 downy mildew samples were collected from 27 vineyards and 32 cultivars in Maryland and Pennsylvania. Resistance to common fungicides was assessed using whole-leaf bioassays, while amino acid mutations known for resistance development were confirmed via Sanger sequencing and PCR-RFLP. Overall, resistance was detected to azoxystrobin (69%), mandipropamid (39%), and phosphorous acids (33%) across all regions. Individual isolates, simultaneously displaying resistance to two or more chemical classes of fungicides, were frequently detected (20%). Notably, this study represents the first report of phosphorous acid resistance in North America, detected as early as 2020 and widespread across regions tested. These findings underscore the increasing threat posed by *P.*

*viticola* to grape production in the Northeast, as rising resistance to multiple fungicide classes significantly limits control options for growers. Further, canopy management practices such as leaf removal and shoot thinning can be tailored to specific diseases by forming less conducive microclimates.

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ON FRUIT ROTS AND DOWNY MILDEW FUNGICIDE RESISTANCE IN MID  
ATLANTIC VINEYARDS

by

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# Table of Contents

Acknowledgements.....	ii
Table of Contents.....	iii
List of Tables.....	v
List of Figures.....	vi
Chapter 1: Understanding the Effects of Shoot Thinning and Post-Bloom Leaf Removal on Late Season Rot Development in Maryland Vineyards.....	1
<i>Introduction</i> .....	1
<i>Materials and Methods</i> .....	7
Treatments and Experimental Design.....	7
Disease Surveys.....	10
Canopy Microclimate Sensors.....	10
Berry Measurements.....	11
Data Visualization and Statistical analysis.....	11
General Canopy Management.....	12
<i>Results</i> .....	12
Ripe Rot.....	12
Sour Rot.....	16
Cluster Powdery Mildew.....	17
Botrytis.....	20
RR risk model.....	25
Brix, TA, and pH.....	26
<i>Discussion</i> .....	28
Chapter 2: Characterization of Fungicide Resistance in <i>Plasmopara viticola</i> isolates from Maryland and Pennsylvania Vineyards.....	32
<i>Introduction</i> .....	32
<i>Materials and Methods</i> .....	35
Sample Collection.....	35
Bioassays.....	36
DNA extraction.....	37
G143A and G1105S verifications.....	37
Clade identification.....	38
<i>Results</i> .....	39
Bioassays.....	39
Geographic Distribution.....	41

Frequency of Resistance by Species .....	44
Frequency of Resistance by Cultivar .....	44
Chemical Class Resistance (CCRs) .....	45
PCR amplification.....	47
Clade Identification.....	47
<i>Discussion</i> .....	48
Summary.....	52
References.....	54

## List of Tables

Table 1. Table of Trials and Experimental Design .....	7
Table 2. Fungicide Treatments applied to WC trial 2024 season .....	9
Table 3. Analysis of split and split-split plot table for Ripe rot severity ES and WM trials . <b>Error! Bookmark not defined.</b>	
Table 4. Analysis of split-split plot table for sour rot severity ES trial .....	16
Table 5. Analysis of split- split plot table for cluster PM severity WC trial .....	18
Table 6. Nested ANOVA Table PM .....	19
Table 7 Analysis of split- split plot table for Botrytis severity WC trial .....	21
Table 8. Berry quality measurements of each trial .....	27
Table 9. Percentage of resistant isolates belonging to each respective vineyard and location. Highest levels of resistance belong to vineyards from the Lake Erie region.....	43

## List of Figures

- Figure 1.** Comparison of LR treatments to the raw ripe rot disease severity data for years 2023 and 2024 for the ES trial and the 2024 WM trial. Analyses were conducted on transformed data using Tukey's Honest Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05..... 14
- Figure 2.** Comparison of LR treatments of each cultivar in the ES trial to the raw Ripe rot disease severity data for years 2023 (top) and 2024 (bottom). Analyses were conducted on transformed data using Tukey's Honest Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05. .... 16
- Figure 3.** Post-hoc comparisons of treatment effects on log transformed sour rot severity, represented as mean  $\pm$  interquartile range (IQR), for (A) Spray, (B) Leaf Removal (LR), and (C) Shoot Density (SD). Analyses were conducted using Tukey's Honest Significant Difference (HSD) test, with treatments compared within each factor. Results illustrate significant differences among levels of Cultivar, while LR and SD showed no significant effects ( $p > 0.05$ ). ..... 17
- Figure 4.** Figure 4a compares the SD treatments to the raw Botrytis and Cluster PM disease severity data, with both diseases displaying lower disease severity among the low shoot density S treatments. Analyses were conducted on transformed data using Tukey's Honest Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05. Figure 4b compares the combined LR:SD treatments to the raw Botrytis and cluster PM disease severity data. Letters corresponding to each of the treatments display statistical differences and were performed using Tukey's HSD test. .... 19
- Figure 5.** Comparison of average morning (top) and afternoon (bottom) temperatures among LR:SD treatments. While morning temperatures appear to be consistent across all treatments, average afternoon temperatures vary with the high leaf removal (blue and yellow) displaying higher temperatures compare to low leaf removal (pink and red) treatments. .... 23
- Figure 6.** Comparison of average morning (top) and afternoon (bottom) leaf wetness counts among LR:SD treatments in the month of September. More variability among the treatments can be observed when considering the average LW for treatments. However, the LSS (blue) treatment consistently displays lower average LW both in the morning and afternoon. LLSS (red) and LS (yellow) treatments also consistently display higher LW counts throughout the day compared to LSS (blue) and LLS (pink) treatments. .... 24
- Figure 7.** Temperature and LW data from each of the LR:SD treatments was incorporated into the ripe rot risk model to identify which treatments observe the differences in infection risk. Results from the ripe rot risk model were consistent with the disease survey results with high leaf removal treatments (L) exceeding the disease risk threshold (0.47) more than the low leaf removal treatment (LL). N describes the number of times the model exceeded the risk threshold and M.S. displays the mean ripe rot severity for each respective LR:SD treatment according to 2024 disease surveys. .... 25
- Figure 8.** Overview of resistance profiles from bioassays. Figure 8a displays the total frequency of resistance among all isolates tested in bioassay. Figure 8b represents the percentage of isolates

resistant to 0, 1, 2, or 3 chemical classes with the most common group belonging to the 1CCR category and the least common belonging to 0CCR.....	39
<b>Figure 9.</b> Temporal distribution of isolate resistance profiles. Figure 9a displays the distribution of isolates associated with the collection year. Figure 9b displays the frequency of resistance of isolates by year of sample collection. ....	40
<b>Figure 10.</b> Regional distribution of fungicide resistance in DM isolates. Figure 10a compares the mean frequency of resistance to the regions from which isolates were collected from. Lake Erie displayed the highest levels of resistance for all three chemicals where resistance was found. Figure 10b displays the distribution of chemical class resistance (CCR) by vineyard. Each pie chart represents a different vineyard with the size of the pie chart representing the sample size. Shades within respective pie charts correspond with the different CCRs found within each vineyard.....	42
<b>Figure 11.</b> Distribution of resistance by Vitis species. Figure 11a displays the distribution Vitis species all isolates were collected from throughout the project. Figure 11b shows the distribution of resistant isolates compared to the Vitis species each DM isolate was collected from. Distribution of azoxystrobin and mandipropamid resistant isolates show similar distribution patterns to the overall species distribution, however, a higher proportion of phosphorus acid resistant isolates were collected for interspecific hybrids.....	44
<b>Figure 12.</b> Distribution of resistance by Cultivar. Figure 12a displays the distribution of all isolates associated with the cultivar they were isolated from and Figure 12b displays the distribution of resistant isolates by cultivar. Generally, the distribution of resistant isolates coincides with the overall distribution of cultivars except for a high number of phosphorus acid resistant isolates collected from ‘Vidal blanc’ leaves.....	45
<b>Figure 13.</b> Breakdown of resistance profiles using an Upset plot comparing the number of resistant isolates per chemical (Set size) to the varying combination of multi-resistant isolates. The highest number of isolates were 1CCR azoxystrobin resistant isolates, however, 241 isolates were resistant to azoxystrobin in total. The second most common resistance combination was isolates belonging to the 3CCR group. ....	46

# Chapter 1: Understanding the Effects of Shoot Thinning and Post-Bloom Leaf Removal on Late Season Rot Development in Maryland Vineyards

## Introduction

Mid-Atlantic wine grape production is a relatively new industry. Despite being in its formative stages, the Maryland wine industry generates around 3.1 billion dollars in total economic activity with approximately 13,500 direct employees (*Economic Impact Study of the American Wine Industry, 2022*). While wine is the source of profit for this industry, the locally grown grapes and land on which they are grown are the industry's foundation. Two key features of the Mid-Atlantic climate pose significant challenges to wine grape production: excessive precipitation during the growing season, and winter cold damage (Centinari et al., 2016). With excessive precipitation during the growing season comes favorable growing conditions for a diverse range of pathogens when compared to more common grape growing regions, making disease management a key concern among Maryland grape growers (Cosseboom & Hu, 2021).

High disease pressure within the region has led to reliance on chemical fungicides among Mid-Atlantic growers. Excessive use of fungicides exerts selection pressure on pathogen populations, increasing the likelihood of resistance development within vineyards (Brent & Hollomon, 1998). Therefore, it is important to implement integrated pest management (IPM) and reduce overall chemical input. IPM is a management strategy that incorporates biological, cultural, physical, and chemical strategies in a way that minimizes economic, health, and environment. Incorporation and optimizing efficacy of horticultural practices may help to reduce the number of required sprays and therefore, the rate of resistance development. This part of the research thesis focused on the potential of two specific viticultural practices, namely shoot thinning (SD) and post bloom leaf removal (LR), for improved disease management in wine grapes.

Canopy management is an essential practice in mid-Atlantic wine grape production. It involves several techniques that alter the position or number of shoots, leaves, and fruit on a vine to achieve a desired microclimate and resulting fruit yield and quality (R. E. Smart et al., 2017).

Common canopy management practices include pruning, shoot thinning, sucker and watersprout removal, shoot positioning, leaf and lateral removal, and hedging.

As one of the most widely adopted practices by growers, post-bloom leaf removal involves the removal of leaves surrounding grape clusters to alter the microclimate around clusters. Post-bloom LR has been reported to mitigate the impact of rainfall and high humidity on disease development in addition to improving the efficacy of pesticide application and penetration (English et al., 1989; R. Smart et al., 1990; Thomas et al., 1988; Zoecklein et al., 1992). The practice is generally performed between fruit set and bunch closure, however, the extent and intensity of leaf removal can vary significantly among vineyards.

Some vineyards adopt an aggressive approach, removing nearly all the leaves between the cane/cordon and the first catch wire, fully exposing grape clusters to sunlight and airflow. Many growers adopt this practice when their primary goal is disease management, as exposing fruits has been reported to reduce fungal diseases including Botrytis bunch rot (BBR) and sour rot (SR) in grape clusters (Blaauw et al., 2019; English et al., 1989; Hed & Centinari, 2024; Vogel et al., 2020; Zoecklein et al., 1992). Other growers take a more conservative approach, selectively removing fewer leaves to balance the benefits of improved cluster exposure with the potential risk of sunburn or other environmental stresses. The more conservative approach focuses more on fruit composition, as excessive temperatures have been reported to decrease anthocyanin levels in grapes in the Western US (Mori et al., 2007; Spayd et al., 2002; Tarara et al., 2008). This variability reflects differences in vineyard management goals, cultivar sensitivity, and regional growing conditions. Research in Virginia found that more intense and earlier leaf removal increased grape phenolics and maintained or improved anthocyanins, even in fully exposed clusters. Although exposed grapes were warmer, temperatures rarely exceeded thresholds that would harm berry quality, unlike in hotter, drier regions (Hickey & Wolf, 2019). Studies conducted in both the Northeastern and Southeastern United States have shown a decrease in BBR and SR severity on *Vitis vinifera* that were treated with post-bloom leaf removal (Hed & Centinari, 2018; Vogel et al., 2020). SR and BBR are among the most common diseases found in Mid-Atlantic vineyards in addition to *Colletotrichum*, *Aspergillus*, *Alternaria*, *Pestalotiopsis*, and *Neopestalotiopsis* (Cosseboom & Hu, 2021).

Shoot thinning is another important canopy management practice typically performed shortly after bud break when shoots are five to six inches in length. This practice aims to

improve canopy structure, fruit quality, and disease management by optimizing vine balance and enhancing light penetration and airflow. Shoot thinning enhances the balance between vegetative growth and reproductive output (source-sink balance). This adjustment helps allocate more resources to fruit development rather than excessive vegetative growth, improving vine health and longevity (Bernizzoni et al., 2011; Naor et al., 2002; Reynolds et al., 2005).

Shoot thinning reduces overall yield by decreasing the number of shoots and clusters per vine, but it can enhance fruit uniformity and quality. Studies have shown that shoot thinning results in fewer but larger clusters, as remaining shoots and clusters receive more resources (Naor et al., 2002). While overall yield is reduced, the increase in quality often offsets the economic impact of lower production. Shoot thinning improves canopy airflow and light penetration, reducing the humid microclimates that favor fungal diseases like powdery mildew (*Uncinula necator*) and BBR (*Botrytis cinerea*). Improved light exposure also promotes the synthesis of secondary metabolites such as anthocyanins and flavonoids, enhancing berry color and composition (R. Smart et al., 1990). By reducing canopy density, shoot thinning also helps regulate fruit-zone temperature and sunlight exposure, factors critical for berry ripening (Naor et al., 2002). This practice has been linked to increased soluble solids (Brix) and improved phenolic development, especially in red grape cultivars like ‘Pinot Noir’ and ‘Cabernet Franc’ (Reynolds et al., 2005). Wines produced from shoot-thinned vines often exhibit enhanced aroma, flavor complexity, and color intensity.

Common fruit rots in Mid-Atlantic vineyards include ripe rot, BBR, powdery mildew (PM), and SR. Ripe rot is caused by *Colletotrichum spp.*, particularly the species complexes *C. acutatum* and *C. gloeosporioides*. *Colletotrichum spp.* thrives in warm humid climates, making the Mid-Atlantic a favorable environment for ripe rot development. Symptoms of ripe rot manifest as dark concentric lesions on the berry with a leathery texture followed by mummification of the berry. Under humid conditions, the symptomatic lesions produce orange or salmon-colored acervili containing conidia. Conidia are then dispersed via rain droplets, thus spreading the disease to nearby berries and clusters (Hsieh et al., 2023). BBR is caused by *Botrytis cinerea*, a necrotrophic fungus and is characterized by berry discoloration and the presence of grey mycelia on infected berries. It is favored by cool and wet conditions and primarily dispersed by wind. Insects can act as vectors for the disease, spreading it between hosts (Latorre et al., 2015). Powdery mildew caused by *Uncinula necator* (syn. *Erysiphe necator*), is a

major biotrophic fungal disease in grapevine cultivation. It affects all green tissues of the plant, with cluster infections being particularly damaging to fruit quality and yield. Sour rot is a disease complex caused by a four-way interaction between the host, acetic acid bacteria, yeast, and insects. The disease is characterized by browning of berry skin and oozing of berry pulp accompanied by the smell of acetic acid. Sour rot is believed to be vectored by the insect *Drosophila spp.*, commonly known as vinegar flies (Hall et al., 2018). Unique epidemiology and etiology of the above-mentioned diseases may result in unique canopy strategies for effective disease management (Cosseboom & Hu, 2021).

A diverse range of wine grape cultivars are grown in the Mid-Atlantic, and with each cultivar comes varying susceptibility to the diverse pathogen population found in the region. A survey including 33 Maryland wineries performed by the Maryland Grape Growers Association (MGGA) reported in 2008 that 96 wine grape cultivars were being grown in the state. Of those, the most planted *vinifera* cultivars included ‘Cabernet Franc’, ‘Chardonnay’, ‘Cabernet Sauvignon’, ‘Merlot’, and ‘Pinot Gris’ and the most common hybrid cultivars comprised ‘Chambourcin’, ‘Vidal blanc’, and ‘Seyval blanc’ (Maryland Grape Growers Association, 2009).

The effects of grape species and their hybrids on ripe rot development have been previously documented (Jang et al., 2011; Shiraishi et al., 2007; C. C. Steel et al., 2012). Studies conducted in Japan and Australia (Shiraishi et al., 2007) identified cultivars belonging to *Vitis vinifera* as most susceptible to ripe rot infection. As a cultivar known for its susceptibility to a diverse range of pathogens, ‘Chardonnay’ is frequently used in laboratory studies in Australia to examine *Colletotrichum* growth requirements, histology, and tissue sensitivity (Greer et al., 2011; C. C. Steel et al., 2012).

BBR is a significant fungal disease affecting wine grapes worldwide, particularly in regions with humid conditions during the growing season. Cultivar susceptibility to this disease varies widely and is influenced by a combination of genetic, morphological, and environmental factors. Cultivars with compact clusters and thin-skinned berries are generally more prone to BBR due to reduced airflow and increased moisture retention within the cluster. For instance, ‘Gewürztraminer’ and Sauvignon blanc were identified as the most susceptible cultivars in field trials conducted in Chile and France between 2011 and 2015 (Pañitrur-De La Fuente et al., 2018). These results align with previous findings that compact-clustered cultivars like ‘Vignoles’

and ‘Cabernet Sauvignon’ are highly vulnerable to BBR (Fermaud et al., 2001; Hed et al., 2009; Vail et al., 1998). Cultivars with looser clusters and thicker berry skins show moderate resistance. ‘Chardonnay’, a cultivar characterized as having thin skin and tight clusters, has been noted for its intermediate susceptibility to BBR, depending on environmental conditions and management practices (C. Steel & Greer, 2006), however, the Chile and France study rated ‘Chardonnay’ as highly susceptible (Pañitrur-De La Fuente et al., 2018). Similarly, Grenache Noir exhibited moderate resistance to BBR across diverse growing conditions in the Chile and France trials, further highlighting the importance of cultivar-specific adaptability (Pañitrur-De La Fuente et al., 2018). Cultivars with naturally loose clusters, such as ‘Petit Manseng,’ or those bred for disease resistance tend to show greater resilience to BBR. In the Chile and France trials, ‘Cabernet Sauvignon,’ ‘Cabernet Franc,’ and ‘Petit Verdot’ were consistently identified as the most resistant cultivars, with similar classifications observed in both countries despite contrasting climatic conditions and cropping practices (Pañitrur-De La Fuente et al., 2018). Researchers in Chile and France reported a positive relationship between cultivar susceptibility and ripening indices, suggesting that later-maturing cultivars may have an increased risk of infection in BBR. This may be because *Botrytis cinerea* favors cooler, more humid conditions typical of late-season weather, meaning that late-ripening cultivars are accumulating sugars at a time when environmental conditions are especially conducive to pathogen development.

Susceptibility to PM varies significantly across grape cultivars and is largely influenced by genetic resistance, with cluster morphology and environmental conditions also affecting PM development. Numerous studies have reported that North American *Vitis* spp. are more resistant to powdery mildew than most European *V. vinifera* cultivars (Atak et al., 2017; Cadle-Davidson, 2008; Cadle-Davidson et al., 2010; Doster & Schnathorst, 1985a, 1985b; Eibach, 1994; Staudt, 2015). Cultivars like ‘Chardonnay’ and ‘Cabernet Sauvignon’ have been reported as highly susceptible to PM (Fuller et al., 2014; Peros et al., 2006). Cultivars like ‘Chardonnay’ and ‘Cabernet Sauvignon’ have been reported as highly susceptible to PM (Fuller et al., 2014; Peros et al., 2006). Cultivars developed through breeding programs that incorporate resistance genes from wild grape species show higher resilience. For example, hybrid cultivars such as ‘Chambourcin’ and ‘Vidal blanc’ display significant resistance to powdery mildew (Bouquet, 2011; Reisch et al., 2012). The presence of resistance loci such as *Ren1*, *Ren3*, and *Run1* in

certain cultivars has been linked to reduced fungal growth and spread (Coleman et al., 2009; Dalbó et al., 2001; Hoffmann et al., 2008; Welter et al., 2007).

When it comes to cultivar susceptibility to SR, studies have shown that those with larger berries displayed higher susceptibility to SR, due to larger berry size often resulting in tighter clusters (Bordelon, 2016; Wilcox et al., 2004). In addition to tight clusters, skin thickness has also been reported to increase susceptibility to SR. A study conducted by Lisek and Lisek in 2021 assessed SR susceptibility among 28 wine grape cultivars and found that cultivars including Riesling, Pinot Noir, and Seyval had the highest levels of susceptibility because of their thin skin and compact clusters (Lisek & Lisek, 2021).

Regional studies have highlighted that environmental conditions and vineyard management practices, such as canopy management, cluster thinning, and fungicide application, significantly influence the susceptibility of grape cultivars to both BBR and PM. For example, research conducted in Australia demonstrated that tailored management practices that create a more open canopy can mitigate disease pressure even in susceptible cultivars (C. Steel & Greer, 2006). Similarly, a study conducted in Geneva, New York, underscored the importance of proper canopy management and strategic interventions to reduce PM severity (C. N. Austin & Wilcox, 2011).

Due to the varying climatic conditions found in the Mid-Atlantic, the effects of canopy management practices widely accepted and commonly practiced in more common grape growing regions like California and New York are not fully understood when considering disease management. Testing the effects of different treatments of leaf removal and shoot thinning in Maryland vineyards will validate whether these universal viticultural practices are relevant to the unique growing conditions and diverse diseases found in Mid-Atlantic vineyards and how commonly grown cultivars respond to these practices.

## Materials and Methods

### Treatments and Experimental Design

**Table 1.** Table of Trials and Experimental Design

<b>Trial</b>	<b>Location</b>	<b>Year</b>	<b>Design</b>	<b>Main Plot</b>	<b>Sub Plot</b>	<b>Sub-Sub plot</b>
ES	Queen Anne Co. MD	2023-24	Split-split	Cultivar (5)	LR (2)	SD (2)
WC	Frederick Co. MD	2024	Split-split	Fungicide (2)	LR (2)	SD (2)
WM	Frederick Co. MD	2024	Split	LR (2)	SD (2)	n/a

### *Experimental Design*

In 2023 and 2024, an experiment involving three separate trials was initiated to investigate the effects of different treatments of leaf removal (LR) and shoot density (SD) on five wine grape cultivars commonly grown in Maryland (Table 1). The first trial (ES) was conducted in 2023 and 2024 at the University of Maryland Wye Research and Education Center vineyard located in Queenstown, MD (Queen Anne County on the Eastern Shore). The second (WM) and third trials (WC) were conducted in 2024 on Merlot and Chardonnay vines at a commercial vineyard in Jefferson, MD, near the foot of the Blue Ridge Mountains (Frederick County, Maryland).

The ES trial was conducted using a split-split plot (cultivar as the main plot factor) in a randomized complete block design with a 5×2×2 factorial treatment design with three vines per replication. The cultivars included in ES included three *Vitis vinifera*: ‘Chardonnay’, ‘Cabernet Franc’, and ‘Merlot’, and two interspecific hybrids: ‘Vidal blanc’ and ‘Chambourcin’. Similarly, the WC trial was performed in a plot of ‘Chardonnay’ vines using a split-split plot (fungicide application as the main plot factor, LR as the sub-plot factor, and SD as the sub-sub plot factor) in a randomized complete block design with a 2×2×2 factorial treatment design with six vines

per replicate. The main plot factor treatments included a sprayed and unsprayed treatment, where the sprayed trial was treated with fungicides to control late-season rots. The unsprayed treatment did not receive any fungicide applications specifically targeting late season rots. The WM trial was performed in a different part of the Frederick County vineyard in a plot of Merlot grapevines. This trial was the only split-plot factorial design with LR as the main plot factor and SD as the sub-plot factor.

All trials included in this study were factorial designs, including different levels of LR and SD treatments. Each trial included two high and low LR and SD treatments. LR treatments included the removal of two leaves per cluster for the low LR treatment (LL), and five per cluster for the high LR treatment (L). Shoot density treatments included a low-density treatment (S) in which vines were thinned to two shoots per linear foot, and four or five shoots per linear foot for the high SD (SS) treatment depending on the pruning system.

For the ES and WC trials, leaves were removed in early July, when berries reached pea-sized or stage 75 of the BBCH phenological development scale (Lorenz et al., 1995). Primary leaves adjacent to clusters were the primary target when performing leaf removal. For shoots lacking a sufficient number of leaves between the cane/cordon and the first catch wire in L treatments, the treatments were limited to the area below the first catch wire. Because the WM trial was subject to 2-4D damage from a nearby crop field that resulted in stunted growth of the vines in the trial, LR treatments were not performed until late July, when clusters were at berry touch or BBCH79.

The two SD treatments across all trials were made up of a high SD (SS) and a low SD (S) treatment. All S treatments were thinned to two shoots per linear foot, however, the SS varied depending on the pruning system. Cane pruned systems were thinned to four shoots per linear foot and included the ES and WC trials. Vines were thinned between early and mid-May when shoots reached a length of 5-6 inches. The WM trial received an SS treatment where shoots were thinned to five shoots per linear foot, as spur-pruned vines typically produce a denser canopy with more shoots, allowing for a greater contrast of SD treatments in the WM trial.

The Fungicide application treatments at the WC trial included two treatments where the sprayed treatment received fungicide applications targeting late season rots throughout the season, and the unsprayed treatment only received applications of fungicides targeting early season diseases such as black rot and Phomopsis. Downy mildew was also managed universally

with fungicides that have limited efficacy against fruit rots. In addition, the unsprayed treatment received one application of Captan pre-bloom to prevent botrytis infection at bloom. Table 2 provides a detailed description of all fungicide applications made to the WC trial in the 2024 season.

**Table 2.** Fungicide Treatments applied to WC trial 2024 season

Date	Product	Amount Per Acre	unsprayed	sprayed
19-Apr	Penncozeb	1.5 lb	yes	yes
	Sulfur	1 lb	yes	yes
29-Apr	Penncozeb	1.5 lb	yes	yes
	Sulfur	1 lb	yes	yes
13-May	Captan	1.5 lb	yes	yes
	Sulfur	1.5 lb	yes	yes
	Fungiphite	.75 qt	yes	yes
22-May	Penncozeb	2 lb	yes	yes
	Sulfur	2 lb	yes	yes
4-Jun	Captan	2 lb	no	yes
	Sulfur	2lb	yes	yes
	Rovral	1 qt	<b>no</b>	yes
	Carbaryl	1 qt	<b>no</b>	yes
	Fungiphite	1 qt	yes	yes
11-Jun	Phos acid	1.25 qt	yes	yes
	Nutrol	5 lb	yes	yes
24-Jun	Zilker	2.5 oz	yes	yes
10-Jul	Oxidate	3 qt	<b>no</b>	yes
	Belay	6 oz	yes	yes
	Captan	1.75 lb	<b>no</b>	yes
17-Jul	Oxidate	3 qt	yes	yes
22-Jul	Copper	1lb	yes	yes
	Captan	2 lb	<b>no</b>	yes

	Nutrol	5 lb	yes	yes
30-Jul	Miravus Prime	13 oz	<b>no</b>	yes
	Dipel	1 lb	yes	yes
	Carbaryl	1 qt	yes	yes
	Captan	2 lb	<b>no</b>	yes
13-Aug	oxidate	3 qt	<b>no</b>	yes
	Vanguard	5 oz	<b>no</b>	yes
	Mustang max	2 oz	yes	yes
15-Aug	Oxidate	3 qt	<b>no</b>	yes
22-Aug	Oxidate	2 qt	<b>no</b>	yes
	Elevate	1 lb	<b>no</b>	yes
	Mustang max	3 oz	yes	yes
12-Sep	Oxidate	2 qt	<b>no</b>	yes
15-Sep	Penncozeb	1.25 lb	yes	yes

#### Disease Surveys

Disease severity was a visual estimation of the percentage of berries per cluster exhibiting disease symptoms, including ripe rot, BBR, sour rot, cluster PM, aspergillus, bitter rot, and Alternaria. Briefly, three clusters per vine were randomly selected and visually assessed for disease. The percentage of berries displaying signs or symptoms on each cluster was determined and recorded in an Excel spreadsheet for further analysis. Harvest dates for Cultivars in the ES trial differed because each cultivar matured at different rates.

#### Canopy Microclimate Sensors

Temperature and leaf wetness sensors were placed in the cluster zone of the canopy of each of the four LR/SD ‘Cab Franc’ treatments. Leaf wetness (LW) was monitored with Phytos 31 leaf wetness sensors (Meter Group Inc., Pullman, WA), and temperature with Atmos 41 weather stations (Meter Group Inc.) in each vineyard. Data from these sensors was automatically transmitted to a cloud-based server every five-minutes. Ripe rot infection risk was calculated for

each treatment by implementing the ripe rot risk model designed by Cosseboom and Hu (Cosseboom & Hu, 2024).

#### Berry Measurements

When vines reached full ripeness or BBCH89, the number of clusters on each vine was counted and harvested. Each replicate was weighed at harvest to determine the yield and average cluster weight for each replicate. Average cluster weight was determined by dividing the total yield by the number of clusters in each replicate. One cluster per vine was collected at BBCH89, and transferred to the lab on ice. Clusters were combined with respective replicates, and juice was extracted and stored at -20 until further analysis. Measurements of berry pH and total tartaric acid content (TA) from each replicate were obtained using the Metrohm Titrando sample processor (Metrohm AG, Herisau Switzerland). Brix was measured using the Atago PAL-1 digital hand-held pocket refractometer (Atago CO., LTD., Tokyo Japan).

#### Data Visualization and Statistical analysis

Residuals were visualized in JMP to determine normality and transformed to meet the assumptions of normality and homogeneity of variances required for parametric statistical analyses. A square root transformation was implemented to normalize the 2023 ripe rot severity at the ES trial, ripe rot severity from the WM trial, and botrytis severity from the WC trial in 2024. A log transformation was utilized to normalize the ripe rot and sour rot severity response variables from the 2024 ES trial. Cluster PM severity residuals did not require any transformation.

Statistical analyses were performed on transformed data to assess the effects of experimental factors on disease severity. For the ES and WC trials, a split-split plot model was implemented using the function `ssp.plot` from the `agricolae` package in R, and the `sp.plot` function was utilized in the WM trial. The degrees of freedom and error terms for each factor were extracted from the model output to define the structure of the analysis. The coefficients of variation (CVs) were calculated to assess variability within the experimental design.

Post-hoc comparisons among treatment levels for all factors and levels were performed using Tukey's Honestly Significant Difference (HSD) test. These comparisons utilized the extracted degrees of freedom and mean square errors for the corresponding factors. Plots of the

HSD test results were generated with interquartile range (IQR) variation to visualize significant differences among treatment levels.

To further validate the findings, a separate analysis of variance (ANOVA) was conducted using the AOV function. The model included all factors, along with their interactions, with replicate as the error term for the nested structure. Factors were treated as categorical variables. The ANOVA results were summarized to determine the significance of main effects and interactions.

All analyses were conducted using R, and graphical outputs were adjusted for clarity and comparability.

### General Canopy Management

All vines were grown on vertical shoot positioning training systems and varied in age. Trials received cluster thinning treatments one week prior to LR treatments, where clusters were thinned to two clusters per shoot unless the shoot did not reach the third catch wire. In this instance, one cluster was left on the shoot. Due to the 24-D damage in the WM trial in 2024, many of the vines were stunted and shoots had not reached the third catch wire by late July. As a result, a large majority of shoots were thinned to one cluster per shoot to ensure consistent ripening and vine health in subsequent seasons.

### Results

#### Ripe Rot

Ripe rot was present in both vineyards, with the 2023 ES trial displaying the highest levels of ripe rot severity, followed by the WM and 2024 ES trials. Across all trials and years, leaf removal exhibited a significant effect (Table 3), with the higher density LL treatment outperforming the L treatment (Figure 1). In contrast, shoot density did not significantly affect ripe rot severity in any of the trials conducted, regardless of whether shoots were thinned to four or five shoots (Table 3). For the ES trial that involved multiple cultivars, the results from disease surveys revealed a highly significant effect of cultivars on ripe rot severity. This indicates substantial differences in performance among cultivars (Table 3). No significance in ripe rot was found among treatments in the WC trial.

In 2024, a marginal interaction effect, with a p-value of 0.05284, was observed between cultivar and leaf removal, suggesting that the effectiveness of leaf removal may depend on the cultivar (Table 3). However, interactions involving shoot density (SD x Cultivar, SD x LR, and SD x Cultivar x LR) were not significant ( $p > 0.05$ ). No interaction effects were observed at the ES trial in 2023 or the WM trial. Across all years and trials, the main plot, subplot, and split-split plot levels demonstrated high variability, reflective of environmental and biological noise inherent to field trials (Table 3).

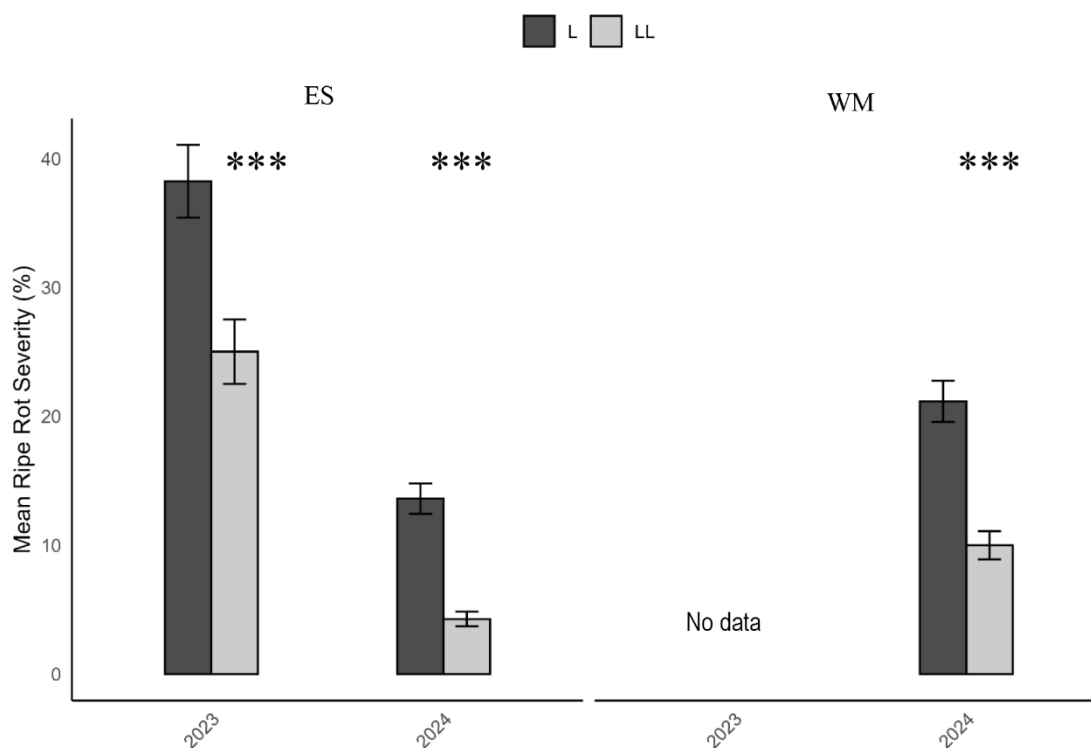
**Table 3.** Analysis of split and split-split plot table for Ripe rot severity ES and WM trials

Trial	Year	Factor	Df	Sum Sq	Mean Sq	F value	Pr (>F)	Signif.
ES	2023	Replicate	2	3.54	1.769	0.3269	0.7249	n/s
		Cultivar	4	595.04	148.76	24.3058	0.00016	***
		Ea	8	48.96	6.12			
		LR	1	61.8	61.803	22.9151	0.00074	***
		Cultivar:LR	4	17.22	4.306	1.5964	0.2496	n/s
		Eb	10	26.97	2.697			
		SD	1	9.63	9.628	1.7794	0.1972	n/s
		SD:Cultivar	4	4.54	1.134	0.2096	0.9301	n/s
		SD:LR	1	5.97	5.972	1.1037	0.306	n/s
		SD:Cultivar:L						
		R	4	16.65	4.162	0.7692	0.5578	n/s
		Ec	20	108.21	5.411			
		ES	2024	Replicate	2	5.5	2.752	2.0401
Cultivar	4			377.83	94.457	109.4438	5.11E-07	***
Ea	8			6.9	0.863			
LR	1			118.34	118.338	39.2259	9.35E-05	***
Cultivar:LR	4			41.08	10.271	3.4046	0.05284	.
Eb	10			30.17	3.017			
SD	1			0.9	0.903	0.6691	0.423	n/s
SD:Cultivar	4			2.99	0.747	0.554	0.69841	n/s
SD:LR	1			0.47	0.47	0.3485	0.5616	n/s
SD:Cultivar:L								
R	4			3.28	0.821	0.6086	0.66116	n/s
Ec	20			26.98	1.349			
WM				Replicate	5	88.585	17.717	4.8963
		LR	1	138.532	138.532	90.9646	0.000214	***
		Ea	5	7.615	1.523			

	SD	1	7.09	7.09	1.9593	0.19184	n/s
	LR:SD	1	1.103	1.103	0.3048	0.59299	n/s
	Eb	10	36.184	3.618			

Ea, Eb, and Ec represent error terms associated with the main plot, subplot, and sub-subplot levels, respectively. Replicate and Cultivar effects are tested against Ea. Leaf Removal (LR) and its interaction with Cultivar are tested against Eb. Shoot Density (SD) and all interactions involving SD are tested against Ec. For the WM trial. Replicate and LR effects are tested against Ea. Shoot density (SD) and its interaction with LR are tested against Eb. **Significance codes:**\*\*\*<0.001,\*\*<0.01,\*<0.05, n.s> 0.05.

### Ripe rot Severity by LR treatment, and Year ES and WM Trials

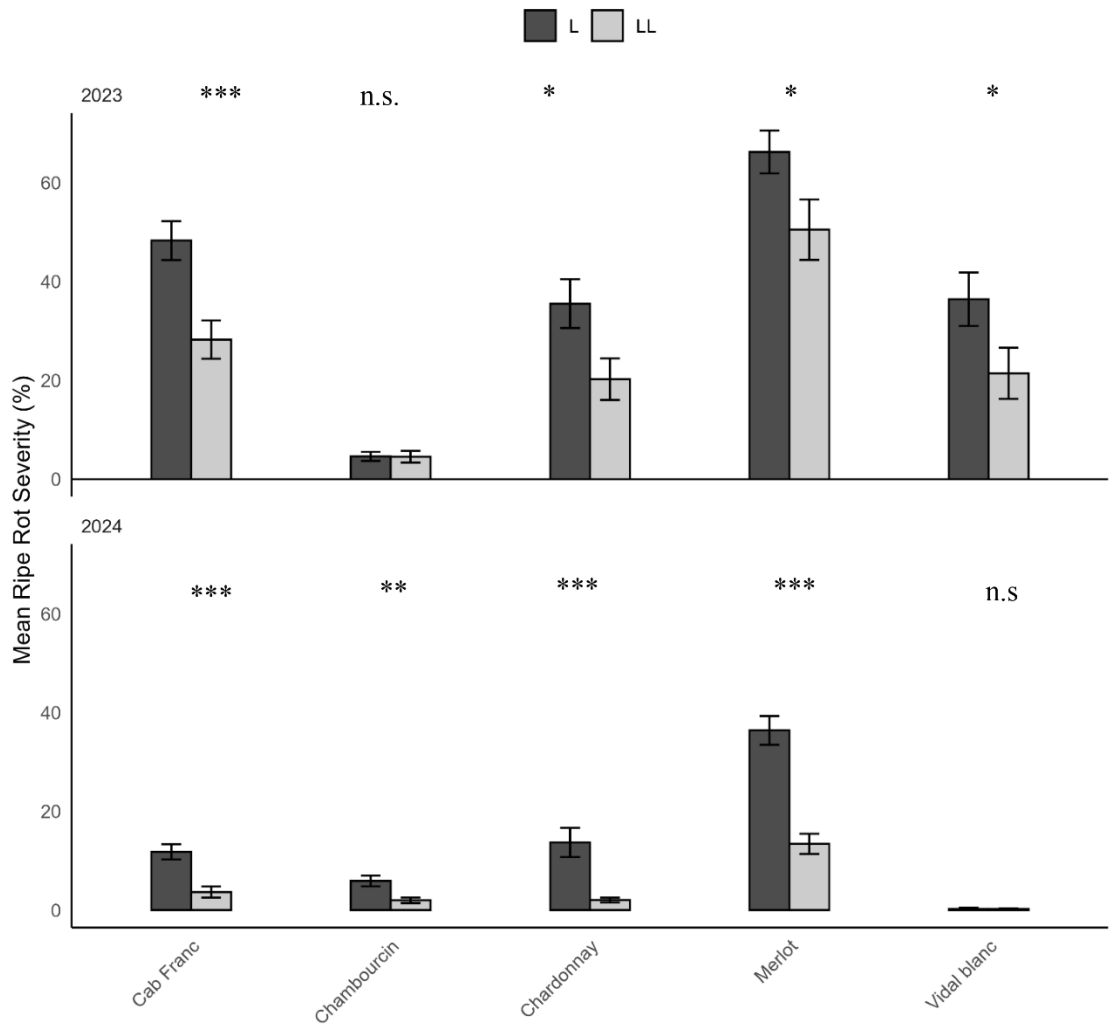


**Figure 1.** Comparison of LR treatments to the raw ripe rot disease severity data for years 2023 and 2024 for the ES trial and the 2024 WM trial. Analyses were conducted on transformed data

using Tukey's Honestly Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05.

Post-hoc comparisons using Tukey’s HSD test provided additional insights into treatment differences in ripe rot severity among cultivars. In both years at the ES trial, ‘Merlot’ exhibited the highest relative response in ripe rot severity and was significantly greater than all other cultivars (Figure 2). ‘Cab Franc’, ‘Chardonnay’, and ‘Chambourcin’ followed, while ‘Vidal blanc’ had the lowest response in 2024. However, the lowest levels of ripe rot were found in ‘Chambourcin’, with significantly lower RR severity than all other cultivars in 2023.

### Ripe rot Severity by LR treatment and Cultivar by year ES



**Figure 2.** Comparison of LR treatments of each cultivar in the ES trial to the raw Ripe rot disease severity data for years 2023 (top) and 2024 (bottom). Analyses were conducted on transformed data using Tukey's Honestly Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05.

Sour Rot

At the ES trial in 2024, an outbreak of sour rot was observed. A split-split plot analysis of variance (ANOVA) was performed to evaluate the effects of cultivar, leaf removal (LR), and shoot density (SD) on log-transformed sour rot severity (log\_SR), including their interactions. The model partitioned variability into appropriate error terms based on the experimental design: replicate, replicate × cultivar, and replicate × cultivar × LR, with residual error within plots. A highly significant effect of cultivar was observed on log-transformed sour rot severity (F = 3.933e-05, p < 0.001) (Table 4). Cultivar contributed substantially to the variability, as indicated by a high mean square (87.349) relative to residual mean square (Ea) (2.471). Post hoc Tukey HSD tests revealed that ‘Vidal blanc’ displayed significantly higher sour rot severity than all other cultivars included in the trial, followed by ‘Chardonnay’ and ‘Chambourcin’, and ‘Merlot’ and ‘Cabernet Franc’ displaying the lowest sour rot severity (Figure 3). In contrast, LR and SD did not display any significant effects on sour rot severity with p values greater than 0.05 for both effects. In addition, there were no significant interaction effects among treatments when considering sour rot severity. These results indicate that cultivar is the primary factor influencing sour rot severity, with highly significant differences among cultivars.

**Table 4.** Analysis of split-split plot table for sour rot severity ES trial

Trial	Year	Factor	Df	Sum Sq	Mean Sq	F value	Pr (>F)	Signif.
ES	2024	Replicate	2	0.64	0.319	0.2352	0.7926	n/s
		Cultivar	4	349.4	87.349	35.3544	3.933 e-05	***
		Ea	8	19.77	2.471			
		LR	1	0.22	0.217	0.1826	0.6782	n/s
		Cultivar:LR	4	1.82	0.455	0.3833	0.8158	n/s
		Eb	10	11.87	1.187			
		SD	1	0	0.003	0.0019	0.9654	n/s



at the WC trial in 2024. A split-split plot design was used to evaluate the effects of late season fungicide application (sprayed vs. unsprayed), leaf removal (L vs. LL), and shoot density (S vs. SS) on PM severity. Post-hoc comparisons using Tukey's HSD test further examined the main effects of the experimental factors. There was no significant difference in PM levels between sprayed and unsprayed treatments ( $p > 0.05$ ) or between L and LL treatments ( $p > 0.05$ ) (Table 5). A significant difference in PM levels was observed between S and SS treatments ( $p < 0.05$ ) (Table 5, Figure 4a). High variability was observed in the data, as indicated by coefficients of variation (CV) ranging from 99.9% to 190.3% across the experimental levels. The overall mean of PM severity was 47.34. The separate nested ANOVA revealed significant effects of shoot density ( $F = 112.828$ ,  $p = 2e-16$ ) on PM severity (Table 6). However, neither spray ( $F = 0.007$ ,  $p = 0.94$ ) nor leaf removal ( $F = 3.82$ ,  $p = 0.12$ ) showed significant main effects (Table 6). Interactions among the factors were generally nonsignificant, with the exception of a significant interaction between spray and shoot density ( $F = 7.504$ ,  $p = 0.00321$ ).

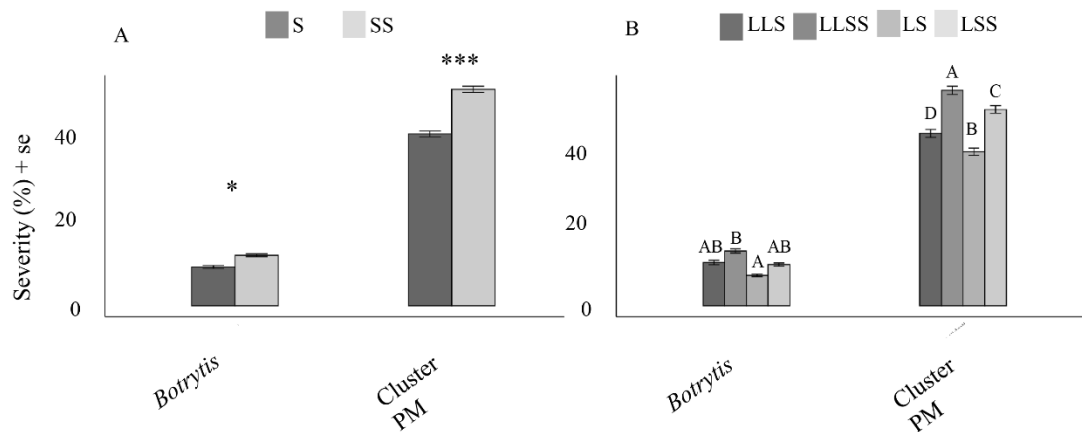
The results indicate that shoot density is a critical factor influencing PM levels, with higher densities (SS) associated with increased cluster PM severity. Neither fungicide application nor leaf removal had significant effects on PM levels, and the interactions among the factors were largely nonsignificant. However, the significant interaction between spray and shoot density suggests a potential synergistic relationship.

**Table 5.** Analysis of split- split plot table for cluster PM severity WC trial

Trial	Year	Factor	Df	Mean		F value	Pr (>F)	Signif.
				Sum Sq	Sq			
WC	2024	Replicate	2	156149	78075	34.3088	0.000112	***
		Spray	1	57	57	0.0071	0.940655	n/s
		Ea	2	16230	8115			
		LR	1	12365	12365	3.8167	0.122244	n/s
		Spray:LR	1	589	587	0.1812	0.692226	n/s
		Eb	4	12959	3240			
		SD	1	64245	64245	28.7251	0.000678	***
		SD:Spray	1	4273	4273	1.9105	0.204271	n/s
		SD:LR	1	4	4	0.0017	0.968341	n/s
		SD:Spray:LR	1	68	68	0.0305	0.835649	n/s
		Ec	8	17892	2237			

Ea, Eb, and Ec represent error terms associated with the main plot, subplot, and sub-subplot levels, respectively. Replicate and spray effects are tested against Ea. Leaf Removal (LR) and its interaction with Spray are tested against Eb. Shoot Density (SD) and all interactions involving SD are tested against Ec. **Significance codes:** \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05.

### Comparison of Botrytis and cluster PM Severity among treatments WC



**Figure 4. Comparisons of SD treatments to the raw Botrytis and Cluster PM disease severity data, with both diseases displaying lower disease severity among the low shoot density S treatments.** Analyses were conducted on transformed data using Tukey's Honest Significant Difference (HSD) test, with stars indicating the following values: \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05. Figure 4b compares the combined LR:SD treatments to the raw Botrytis and cluster PM disease severity data. Letters corresponding to each of the treatments display statistical differences and were performed using Tukey's HSD test.

**Table 6.** Nested ANOVA Table PM

<b>Error-Replicate</b>						
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>			
Replicate	2	156149	78075			
<b>Error-Replicate:Spray</b>						
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>	<b>F value</b>	<b>Pr (&gt;F)</b>	<b>Signif.</b>
Spray	1	57	57	0.007	0.941	n/s
Residuals	2	16230	8115			
<b>Error- Replicate:Spray:LR</b>						
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>	<b>F value</b>	<b>Pr (&gt;F)</b>	<b>Signif.</b>
LR	1	12365	12365	3.817	0.122	n/s
LR:Spray	1	587	587	0.181	0.692	n/s
Residuals	4	12959	3240			
<b>Error: Within</b>						
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>	<b>F value</b>	<b>Pr (&gt;F)</b>	<b>Signif.</b>
SD	1	64245	64245	112.828	< 2e-16	***
Spray:SD	1	4273	4273	7.504	0.00321	**
LR:SD	1	4	4	0.007	0.93533	n/s
Spray:LR:SD	1	68	68	0.12	0.72919	n/s
Residuals	2144	1220796	569			

Ea, Eb, and Ec represent error terms associated with the main plot, subplot, and sub-subplot levels, respectively. Replicate and spray effects are tested against Ea. Leaf Removal (LR) and its interaction with Spray are tested against Eb. Shoot Density (SD) and all interactions involving SD are tested against Ec. **Significance codes:** \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05.

#### Botrytis

Botrytis severity increased in the WC trial as clusters approached harvest. The results of the disease surveys conducted at harvest involved the analysis of transformed data with the same design as the cluster powdery mildew analysis. ANOVA showed significant effects of replicate ( $F = 8.04$ ,  $p = 0.012$ ) and SD ( $F = 9.39$ ,  $p = 0.015$ ). However, there were no significant effects for spray ( $F = 0.88$ ,  $p = 0.446$ ), LR ( $F = 0.87$ ,  $p = 0.403$ ), or any interaction effects (Table 7). Post-hoc HSD tests revealed the means for sprayed and unsprayed treatments were not significantly different, with a minimum significant difference of 2.90 ( $p = 0.446$ ). The means for

the two LR treatments, L (2.47) and LL (2.94), were also not significantly different, with a minimum significant difference of 1.39 ( $p = 0.403$ ). Shoot density levels did show significant differences, with the lower density S treatment displaying lower botrytis severity at 2.44 compared to the SS treatment with a mean of 2.96, with a p-value of 0.015 (Table 7, Figure 4). Results from the ANOVA confirmed that significant variability existed between replicate and SD, suggesting that SD significantly affected botrytis severity.

**Table 7.** Analysis of split- split plot table for Botrytis severity WC trial

Trial	Year	Factor	Df	Sum Sq	Mean Sq	F value	Pr (>F)	Signif.
WC	2024	Replicate	2	51.023	25.512	8.0356	0.0122	*
		Spray	1	43.488	43.488	0.8838	0.44641	n/s
		Ea	2	98.414	49.207			
		LR	1	23.648	23.648	0.8735	0.40289	n/s
		Spray:LR	1	5.61	5.61	0.2072	0.67255	n/s
		Eb	4	108.285	27.071			
		SD	1	29.821	29.821	9.3929	0.01547	*
		SD:Spray	1	3.454	3.454	1.088	0.32742	n/s
		SD:LR	1	0.147	0.147	0.063	0.83499	n/s
		SD:Spray:LR	1	3.414	3.414	1.0754	0.33006	n/s
		Ec	8	25.398	3.175			

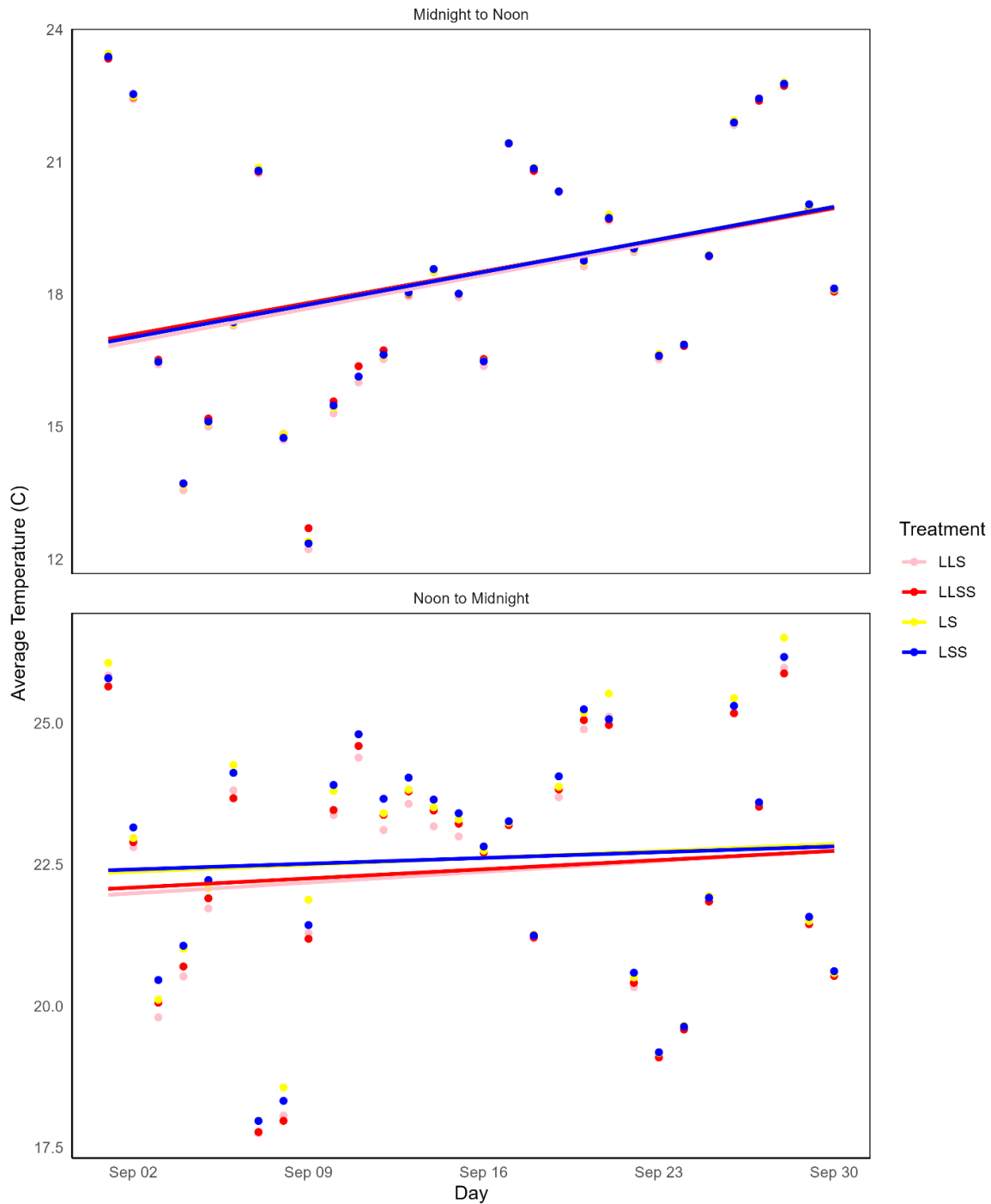
Ea, Eb, and Ec represent error terms associated with the main plot, subplot, and sub-subplot levels, respectively. Replicate and spray effects are tested against Ea. Leaf Removal (LR) and its interaction with Spray are tested against Eb. Shoot Density (SD) and all interactions involving SD are tested against Ec. **Significance codes:** \*\*\*<0.001, \*\*<0.01, \*<0.05, n.s> 0.05.

*Canopy microclimate data and ripe rot risk model*

Notable differences in temperature and leaf wetness (LW) among the four different leaf removal and shoot density treatments were observed when analyzing the weather sensor data for the 2024 ES trial in the month of September. While AM temperatures remained consistent between the hours of midnight and noon among treatments, L treatments displayed consistently

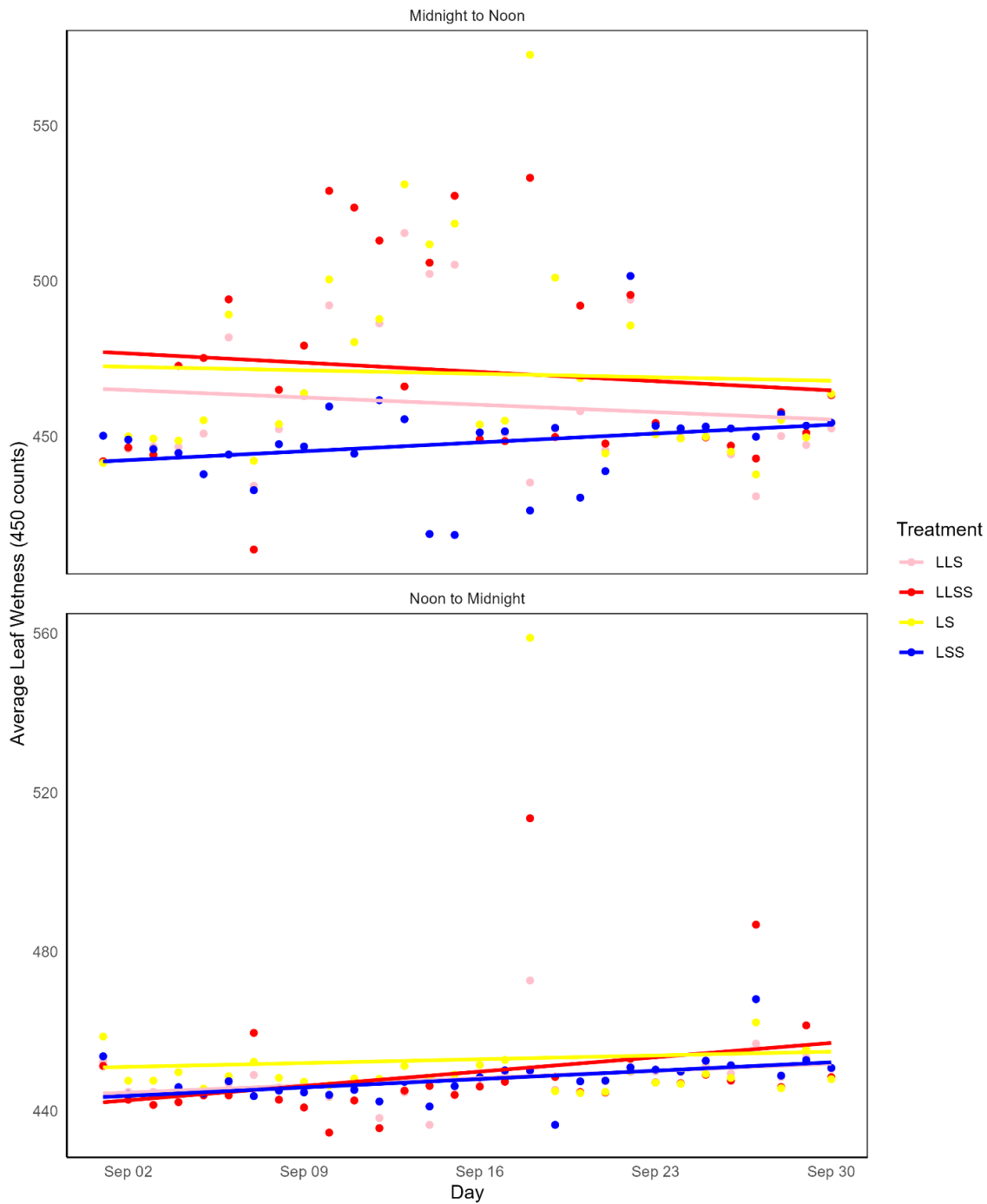
higher temperatures between the hours of noon and midnight compared to the low leaf removal treatments (Figure 5). Leaf wetness also varied considerably by treatment, with LS and LLSS treatments displaying high AM LW counts, and the LSS treatment showing the lowest morning LW counts. LW counts in the afternoon were more variable among treatments, with the LS treatment displaying higher LW counts compared to other treatments in early and mid-September, with the LLSS treatment superseding the LS treatment later in the month (Figure 6).

### Average AM and PM temperatures among LR:SD Treatments ES Trial



**Figure 5.** Comparison of average morning (top) and afternoon (bottom) temperatures among LR:SD treatments. While morning temperatures appear to be consistent across all treatments, average afternoon temperatures vary with the high leaf removal (blue and yellow) displaying higher temperatures compare to low leaf removal (pink and red) treatments.

### Average AM and PM LW counts for LR:SD Treatments ES Trial



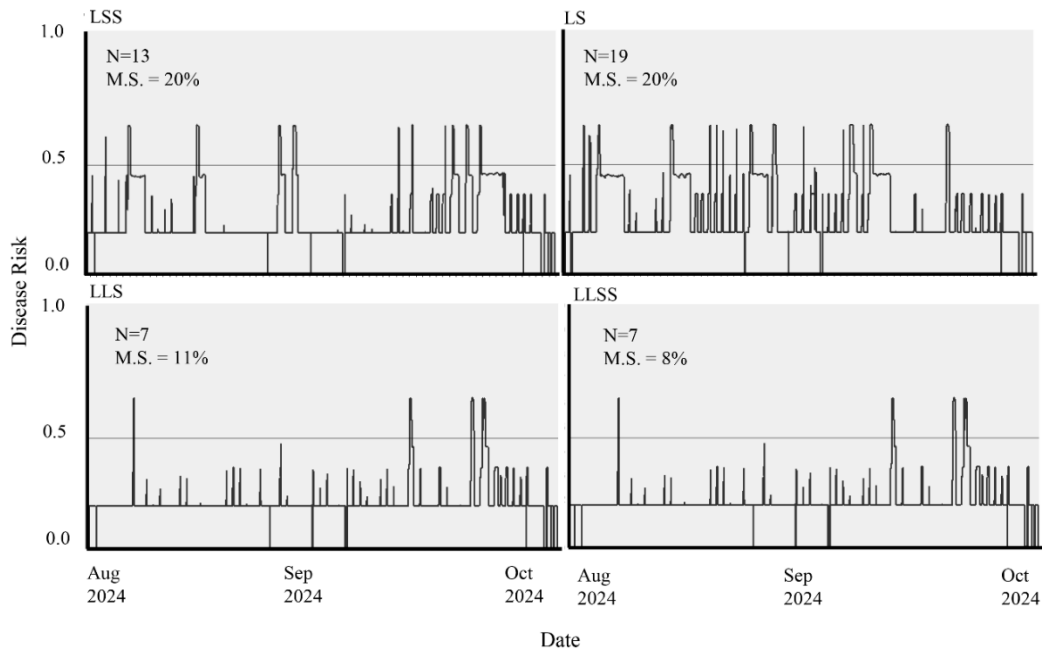
**Figure 6.** Comparison of average morning (top) and afternoon (bottom) leaf wetness counts among LR:SD treatments in the month of September. More variability among the treatments can be observed when considering the average LW for treatments. However, the LSS (blue) treatment consistently displays lower average LW both in the morning and afternoon. LLSS

(red) and LS (yellow) treatments also consistently display higher LW counts throughout the day compared to LSS (blue) and LLS (pink) treatments.

RR risk model

To further understand the role of canopy microclimate on ripe rot development, the data from temperature and LW sensors was integrated into the ripe rot risk neural network model designed by Cosseboom and Hu (Cosseboom & Hu, 2024), which combines leaf wetness duration, temperature, and grape cluster phenological stages to predict infection risk. The model revealed clear differences among the LR:SD treatments. Sensors placed in the high leaf removal (LSS, LS) treatments exceeded the disease risk threshold more frequently than those in the low leaf removal (LLSS, LLS) treatments (Figure 7). This finding aligns with the disease survey data previously reported in this study, suggesting that lower leaf removal creates a microclimate more conducive to ripe rot infection and development.

**Comparison of Ripe Rot Risk Neural Network Model and Severity among Treatments ES**



2024

**Figure 7.** Temperature and LW data from each of the LR:SD treatments was incorporated into the ripe rot risk model to identify which treatments observe the differences in infection risk. Results from the ripe rot risk model were consistent with the disease survey results with high leaf

removal treatments (L) exceeding the disease risk threshold (0.47) more than the low leaf removal treatment (LL). N describes the number of times the model exceeded the risk threshold and M.S. displays the mean ripe rot severity for each respective LR:SD treatment according to 2024 disease surveys.

#### Brix, TA, and pH

Berry measurements including brix, TA, pH, and average cluster weights were recorded to ensure that the LR and SD treatments did not compromise overall crop quality. The *Wine Grape Production Guide for Eastern North America* states that optimal ranges for brix, berry pH and TA range from 19 -23 °, 3.0-3.3, and 0.6-0.75 g/mL in white wines respectively. Optimal ranges for red wines are 20-24° brix, a pH between 3.2 and 3.4, and a TA between 0.6 and 0.75. When comparing these optimal ranges to the data collected in this experiment, berry pH was above average across all trials and years (Table 8). Comparatively, TA levels were consistently below average ranges across all cultivars with the exceptions of the ‘Vidal blanc’ treatment, L and SS ‘Chardonnay’ treatments and the S treatment in ‘Chambourcin’ in 2023 as well as the ‘Vidal blanc’ L treatment in 2024. Brix levels, in contrast, steadily fell into acceptable ranges for all treatments except the ‘Chardonnay’ treatments, the ‘Chambourcin’ SS treatment and the ‘Merlot’ LL treatment in 2023. The 2023 ES trial displayed higher than average Brix levels as clusters were harvested and juiced later than the typical dates of harvest to allow disease development for further study. The most notable difference in juice quality was observed among the fungicide application treatment at the WC trial where sprayed treatments had consistently higher brix levels compared to the unsprayed applications with a mean difference of 1.65°.

**Table 8.** Berry quality measurements of each trial

Year	Trial	Main Factor	Treatment	TA (g/100ml)	pH	brix	avg. cluster wt (oz.)
2023	ES	Cab franc		0.47	4.04	25.45	4.16
			L	0.46	4.07	25.53	4.18
			LL	0.47	4.02	25.37	4.15
			S	0.48	4.00	25.45	4.25
			SS	0.45	4.09	25.45	4.07
		Chambourcin		0.78	3.68	24.51	5.92
			L	0.76	3.76	24.35	6.16
			LL	0.80	3.60	24.67	5.68
			S	0.75	3.73	25.08	6.27
			SS	0.82	3.63	23.93	5.56
		Chardonnay		0.60	3.76	22.81	4.43
			L	0.75	3.76	22.65	4.36
			LL	0.44	3.76	22.97	4.51
			S	0.52	3.78	23.12	4.78
			SS	0.68	3.74	22.50	4.08
		Merlot		0.57	3.85	24.54	3.85
			L	0.55	3.90	25.15	3.74
			LL	0.58	3.79	23.93	3.97
			S	0.56	3.81	25.57	3.91
			SS	0.58	3.88	23.52	3.80
Vidal blanc		0.67	3.54	24.62	7.07		
	L	0.68	3.59	24.33	6.90		
	LL	0.66	3.50	24.90	7.24		
	S	0.66	3.55	24.95	7.64		
	SS	0.68	3.54	24.28	6.50		
2024	ES	Cab franc		0.42	3.89	20.77	3.45
			L	0.42	3.89	20.87	3.47
			LL	0.43	3.89	20.67	3.42
			S	0.43	3.88	20.75	3.23
			SS	0.42	3.90	20.78	3.66
		Chambourcin		0.66	3.54	22.63	6.74
			L	0.65	3.60	23.38	6.80
			LL	0.67	3.47	21.87	6.68
			S	0.66	3.61	23.70	6.77
			SS	0.66	3.47	21.55	6.72
		Chardonnay		0.50	3.74	20.61	3.73
			L	0.50	3.76	20.35	3.87

		LL	0.50	3.71	20.87	3.59
		S	0.50	3.71	20.17	3.77
		SS	0.50	3.77	21.05	3.68
		Merlot	0.48	3.82	20.24	3.57
		L	0.47	3.87	20.30	3.48
		LL	0.49	3.78	20.18	3.65
		S	0.49	3.80	20.60	3.56
		SS	0.47	3.85	19.88	3.58
		Vidal blanc	0.59	3.50	20.87	6.49
		L	0.60	3.50	20.80	6.59
		LL	0.58	3.50	20.93	6.38
		S	0.59	3.54	21.85	6.71
		SS	0.59	3.46	19.88	6.27
		no spray	0.58	3.48	20.53	5.17
		L	0.58	3.52	20.07	5.18
		LL	0.58	3.45	21.00	5.17
		S	0.58	3.52	20.60	5.27
		SS	0.58	3.45	20.47	5.08
2024	WC	spray	0.58	3.50	22.18	4.54
		L	0.57	3.50	22.03	4.38
		LL	0.59	3.50	22.33	4.70
		S	0.58	3.52	22.32	4.66
		SS	0.58	3.48	22.05	4.42
		n/a				
2024	WM	L	0.50	3.72	21.74	4.82
		LL	0.53	3.66	21.56	4.80
		S	0.53	3.70	21.68	4.66
		SS	0.50	3.68	21.63	4.97

### Discussion

In this study, cultivar appeared to be the driving force for pathogen development in all trials, as shown by the significant differences among cultivars in both sour rot and ripe rot severity found in the ES trial as well as the limited range of diseases observed in the WC and WM trials. ‘Merlot’ was found to be the most susceptible cultivar to ripe rot, with other *vinifera* cultivars also displaying higher levels of RR severity compared to interspecific hybrids. However in 2023, ‘Vidal blanc’ also displayed comparable RR severity but not in 2024. This is

likely because of the sour rot outbreak in the ‘Vidal blanc’ in 2024 that likely outcompeted ripe rot that season. Studies have shown that SR development impedes the development of other late season rots, which likely explains the low incidence of ripe rot found in ‘Vidal blanc’ in 2024 (Bisiach et al., 1982) In addition to ‘Merlot’ having the highest levels of ripe rot severity at the ES trial, it was almost exclusively found in the WM trial which was comprised of ‘Merlot’ grape vines in Western Maryland. Considering the differences in climate, soil, and vineyard management practices between the two vineyards, it can be inferred that ‘Merlot’ is highly susceptible to ripe rot, supporting previous research conducted in Japan (Shiraishi et al., 2007). While ripe rot was the major pathogen found at the WM trial, the WC comprising ‘Chardonnay’ vines displayed a completely different pathogen population within the trial. *BBR* and cluster powdery mildew were the two most devastating pathogens found in the WC trial, which is consistent with previous reporting of Chardonnay’s high susceptibility to the two diseases (Gadoury et al., 2012; Naegele, 2018; C. C. Steel et al., 2012). Understanding what diseases are most likely to infect cultivars planted within a vineyard can potentially aid growers in the development of canopy management plans that target the greatest threats to successful wine grape production depending on what cultivars are being grown.

The canopy management strategies imposed in this experiment may contribute to hindering the unique dispersal mechanisms of each pathogen, effectively excluding them from the environment required for successful development and reproduction. *Colletotrichum* spp. are mainly a water-dispersed pathogen that relies on rain or water splash for infection (Dowling et al., 2020). It is possible that the denser canopies in the cluster zone of the LL treatment may have created a protective barrier around the clusters, preventing *Colletotrichum* spores from reaching and colonizing the berries.

Sunlight is another major factor that influences pathogen development. *Erysiphe necator*, the causal organism of powdery mildew, is highly sensitive to UV radiation. Exposure to UV radiation has been reported to reduce conidium germination and appressorium formation, effectively deterring colonization of host tissue (C. Austin, 2010) . Open canopies provide more light penetration to the clusters, which may explain why the low shoot density treatments displayed the lowest levels of cluster PM severity in the WC trial (C. N. Austin & Wilcox, 2011; R. Smart et al., 1990).

*BBR* is a notoriously versatile and problematic pathogen for a multitude of crops worldwide. It has numerous dispersal mechanisms, with the primary mode being airborne dispersal and can penetrate host tissue through appressoria. However, wounding from hail, insects, or other pathogens can significantly increase host susceptibility to *Botrytis*, as the wounds create an easy mode of entry for the disease (Williamson et al., 2007). The early outbreak of Cluster PM in the WC trial likely provided numerous entry points for *Botrytis*, which may in turn have affected the effects of the different treatments on the pathogen. Sour rot is a unique disease, due to the complex interactions of yeast, acetic acid, and insects give rise to its development in vineyards (Hall et al., 2018). As SR is theorized to be vectored by *Drosophila spp.* insects, the canopy management strategies in this study may not have affected the insect's ability to reach clusters and spread the disease. These findings underscore the importance of tailoring canopy management strategies to disrupt the specific dispersal and development mechanisms of individual pathogens, highlighting the nuanced relationship between environmental conditions, canopy structure, and disease pressure in vineyards.

In addition to cultivar susceptibility, it is important for growers to consider environmental factors like topography, proximity to water, nearby vegetation and soil type that may influence pathogen populations within a vineyard. For example, the trials included in the Western Maryland vineyard differed dramatically in both topography and vegetation. The WC trial was located at the highest elevation point in the vineyard, next to a large forest with wild *Vitis* species growing nearby. In general, higher elevations result in increased airflow and cooler temperatures. Both *Botrytis cinerea* and *Erysiphe necator* are wind-dispersed pathogens that prefer cooler temperatures than *Colletotrichum spp.* These environmental conditions may have contributed to the higher frequency of cluster PM and Botrytis in the WC trial. In addition, nearby wild grapes may have provided a native host to the biotrophic and host-specific *Erysiphe necator*, increasing the PM population near the WC trial. In contrast, the WM trial was located near the lowest elevation point of the vineyard, with decreased airflow and warmer temperatures, which displayed high ripe rot severity. These warm temperatures and long periods of leaf wetness critical for ripe rot development likely contributed to increased disease development in this trial.

Considering the effects of environmental factors is critical for vineyard managers when developing effective disease management strategies. Understanding how topography, vegetation,

and microclimatic conditions influence pathogen populations can help optimize control measures and minimize disease risk. As shown in the Western Maryland vineyard trials, variations in elevation, airflow, temperature, and proximity to wild grape hosts created distinct disease pressures for each trial. By tailoring management practices like leaf removal and shoot thinning to these environmental conditions, growers can more effectively address specific disease challenges and improve overall vineyard health and productivity.

This study highlights the effects cultivar, leaf removal and shoot density practices have on disease management in vineyards, and how they interact with disease development. Cultivar susceptibility appears to be the main factor driving disease presence in vineyards, however different shoot thinning and leaf removal practices displayed potential improvements for the management of diseases even on susceptible cultivars. Denser canopies around the cluster zone like those found in the LL treatment of this study were found to reduce ripe rot severity, whereas thinner canopies displayed improved management of cluster powdery mildew and *BBR*. Understanding the relationship between host, pathogen and environment and implementing canopy management practices on a site-by-site basis within a vineyard is essential for sustainable disease management in Mid-Atlantic vineyards.

## Chapter 2: Characterization of Fungicide Resistance in *Plasmopara viticola* isolates from Maryland and Pennsylvania Vineyards

### Introduction

*Plasmopara viticola*, the causal agent responsible for Downy mildew (DM) in grapevines, is a biotrophic heterothallic diploid oomycete indigenous to eastern North America (Koledenkova et al., 2022). The pathogen undergoes multiple generations of asexual reproduction throughout the growing season and one sexual reproductive cycle before senescence in Autumn. It then overwinters as oospores until spring brings forth favorable conditions for sporangia production, thus repeating the organism's life cycle (Allègre et al., 2006; Chen et al., 2019; Rossi et al., 2009). The disease was first reported in Europe in 1878 in the Bourdeaux region of France and remained a devastating epidemic in European vineyards until Pierre-Marie Alexis Millardet discovered copper sulfate's effectiveness against the disease and released the Bourdeaux mixture in 1885 (Koledenkova et al., 2022; Millardet, 1885). *P. viticola* has been documented on numerous members of the Vitaceae family with evidence of five distinct clades that display host specificity and specific geographic distribution (Rouxel et al., 2013). Among these clades, four are responsible for infection of domesticated grapes including clades *aestivalis*, *vinifera*, *riparia* and most recently, *vulpina* (Hong et al., 2019; Rouxel et al., 2013, 2014). Clade *aestivalis* dominates global DM infections, rendering it the most pivotal clade in the context of downy mildew management strategies (Camargo et al., 2019; Fontaine et al., 2021; Taylor et al., 2019; Yin et al., 2014).

While Downy Mildew has been documented on multiple hosts belonging to the Vitaceae family, the disease includes five distinct species complexes or clades that display host specificity and specific geographic distribution. Rouxel et. al 2013 first observed complete specialization of host plants on *Parthenocissus quinquefolia* and *Vitis riparia*, while the cryptic species associated with *V. aestivalis*, *V. labrusca*, and *V. vinifera* showed less specificity. Clades belonging to the *Plasmopara* genera include *P. viticola* cl. *riparia* which can infect *V. riparia* and interspecific hybrids, clade *aestivalis* which infects interspecific hybrids, *V. vinifera*, and *V. aestivalis*, clade *vulpina*, which infects *V. vulpina*, clade *vinifera*, which infects *V. vinifera*, and *Plasmopara muralis* cl. *quinquefolia*, which infects *P. quinquefolia* (Rouxel et al., 2013, 2014).

In addition, reports claim that within these clades, clade *vinifera* had the largest geographic distribution in North America, spanning from northern Michigan to central Florida, but clade *aestivalis* made up 83% of samples collected on vineyards comprised of European grapes (*V. vinifera*) (Rouxel et al., 2014). It has since been reported that clades *aestivalis*, *vulpina* and *vinifera* are present in vineyards in Georgia and Florida, with clade *vulpina* broadening its host range to *V. vinifera* species (Hong et al., 2019).

In East Canada, temporal distribution of clades has been documented in vineyards with clade *riparia* being dominant during the early season and clade *aestivalis* progressively taking over infection rates as the season progressed (Carisse et al., 2021). It was later reported that clade *aestivalis* is more aggressive than clade *riparia*, displaying higher levels of competitive ability (Mouafo-Tchinda et al., 2022). Aside from *P. viticola*'s native region of the Eastern North America, where there is evidence of four clades infecting domesticated grapes, clade *aestivalis* overwhelmingly prevails in global downy mildew infections, rendering it the most pivotal clade in the context of downy mildew management strategies (Camargo et al., 2019; Fontaine et al., 2021; Taylor et al., 2019; Yin et al., 2014).

Because of the high disease pressure in the Eastern US, the vineyards in this region rely heavily on fungicides for DM management. This high frequency of spray applications expedites the resistance development of *P. viticola* to fungicides being used to prevent and treat the pathogen. Growers currently rely on multi-mode of action fungicides including mancozeb and Bourdeaux mixtures, in addition to single-site inhibitors like quinone outside inhibitors (QoIs) (FRAC11), phenylamides (FRAC4), carboxylic acid amides (CAAs) (FRAC40), and phosphorous acids (FRACP07). Resistance to QoIs such as azoxystrobin and pyraclostrobin in *P. viticola* emerged within a mere four years following their introduction to the market in France and Italy and spread globally within a few years (Collina et al., 2005; Corio-Costet et al., 2011; Gisi et al., 2002). Furthermore, resistance to carboxylic acid amide (CAA) fungicides was documented in Europe and the US in 2005 (Baudoin et al., 2008; Feng and Baudoin, 2018; Gisi et al., 2007).

QoI fungicides exert their antifungal activity through a specific mode of action targeting the cytochrome bc1 complex (*cyt b*) in the mitochondria of fungal and oomycete cells. By binding to the Qo site of the *cyt b* protein, QoIs disrupt the electron transfer chain, thereby inhibiting respiration and ultimately leading to the death of the fungal cell (Gisi et al., 2002).

One well-documented mutation associated with QoI resistance in *P. viticola* involves a substitution of the amino acid glycine with alanine at position 143 in *cyt b*. The G143A mutation has been extensively studied and is known to confer high levels of resistance to QoIs in *P. viticola* populations (Gisi et al., 2007). Another mutation occurring at position 129 of the amino acid sequence in *cyt b* leads to a substitution from phenylalanine to leucine (F129L), which has been identified as conferring resistance to QoI fungicides in various species such as *Pythium aphanidermatum*, *Magnaporthe oryzae*, and *Alternaria solani*. However, it is noteworthy that the degree of resistance associated with the F129L substitution is comparatively lower than that conferred by the G143A mutation (Gisi et al., 2007; Grasso et al., 2006; Kim et al., 2003). These mutations alter the structure of *cyt b*, disrupting the binding affinity of QoIs to the target site and effectively reducing the efficacy of fungicides involved in mitochondrial respiration inhibition, allowing strains to survive and proliferate in the presence of QoIs.

In addition to QoIs, resistance to CAAs in *P. viticola* has been characterized previously, with evidence of CAA resistance in the Mid-Atlantic previously documented (Feng and Baudoin, 2018). CAAs target oomycetes in the Peronosporales family by inhibiting cellulose synthesis, a vital process in oomycete cell wall formation. Resistance to CAAs in *P. viticola* is a result of a mutation of an amino acid substitution from Glycine to Serine at location 1105 in the cellulose synthase 3 (CeSA 3) gene (Blum, Waldner, et al., 2010). Resistance as a result of the G1105S mutation is a recessive trait, and will only confer resistance to CAAs if both alleles of the diploid pathogen contain the single nucleotide polymorphism (Blum et al., 2010b; Gisi and Sierotzki, 2008).

Both phosphonates and phenylamides have been effective against DM, with little evidence of resistance emergence in the eastern US thus far. Phenylamides inhibit ribosomal RNA synthesis through obstruction of RNA polymerization, however, the molecular target gene is currently unknown. Resistance to phenylamides has long been documented in *P. viticola* in European vineyards (Gisi and Sierotzki, 2008). Although the likelihood of resistance emergence to phenylamides is deemed high, it seems to exert a stabilizing influence over time, leading to the resurgence of sensitivity in untreated populations following sexual recombination (Gisi and Sierotzki, 2008). Like phenylamides, the resistance mechanism of phosphonates remains largely unknown and has been debated for many years. Early studies suggested an "indirect" mechanism, proposing that phosphites may interfere with the biosynthesis of certain phytoalexins related to

plant innate resistance, despite showing no direct effect on pathogen growth *in vitro* (Nemestothy and Guest, 1990; Saindrenan et al., 1988; Sanders et al., 1983; Vo-Thi\_Hai et al., 1979). However, subsequent research challenged this hypothesis, indicating that the efficacy of phosphonates on pathogens depends on the phosphorous status of the growth medium. Moreover, the "direct" effect hypothesis gained support from the emergence of resistant mutants of *Phytophthora capsici* and *P. palmivora* both *in vivo* and *in vitro* (Bower and Coffey, 1985; Sanders et al., 1990). Studies also revealed that inhibitors of phenylalanine ammonia-lyase, involved in phenylpropanoid biosynthesis, reduced the uptake of phosphonate by *P. capsici in vitro*, supporting the notion of a direct effect (Fenn and Coffey, 1985). Further investigations demonstrated that phosphonate fungicides induce the accumulation of polyphosphate and pyrophosphate in *Phytophthora* species, affecting phosphorous metabolism and inhibiting various enzymes involved in metabolic pathways such as glycolysis and the hexose monophosphate bypass (Andreu et al., 2006; Niere et al., 1994; Stehmann and Grant, 2000). Currently, there is only one documented incidence of phosphonate resistance in *P. viticola* reported in India (Khilare et al., 2003).

Although resistance to some commonly used fungicides has been investigated in the southeastern US including Virginia (Baudoin et al., 2008; Feng and Baudoin, 2018) and Georgia (Campbell et al., 2020) more recently, the situation is largely unknown in the northeastern region. Thus, the objectives of this study were to 1) determine the frequency of resistance to commonly used fungicides in Maryland and Pennsylvania, and 2) confirm their mechanisms of resistance. In addition, we aim to identify which *P. viticola* clades are present and investigate the roles of clade, season, and geographic distributions when managing DM in regional vineyards.

## Materials and Methods

### Sample Collection

A total of 352 leaves containing DM infections were collected from 14 Maryland and 13 Pennsylvania vineyards involving 32 *Vitis* cultivars between the years 2019 and 2023. The cultivars were classified into 3 species: *Vitis vinifera*, *labrusca*, and Interspecific hybrids. To ensure the coverage and representation of resistance issues, samples were collected from

vineyards located in various geographic regions, including Western Maryland, the Eastern Shore Maryland, Southern Maryland, Central Maryland, Southeastern PA, and Lake Erie. In each vineyard, at least two cultivars were selected for sample collection. Ten symptomatic leaves were collected from each cultivar across various sections of the vineyard. Leaves were individually stored in Ziploc bags and carefully placed on ice to preserve their integrity during transportation to the laboratory for further analysis.

## Bioassays

For the bioassays, four commercially available fungicide products, including Abound (a.i. azoxystrobin), Revus (ai. mandipropamid), Fungi-phite (ai. Phosphorous acid), Ridomil Gold (a.i. Mefenoxam), and one technical grade (i.e. oxathiapiprolin) were used for the resistance detection. Oxathiapiprolin is the active ingredient in commercially available products such as Orondis or Orondis Gold. Neither of the products is currently registered for grapes. Resistance was tested at the lowest dose of labelled rates for all three formulations. For oxathiapiprolin, the doses registered to target oomycete diseases in horticultural crops ranging from 1.2 to 2.4 fl oz per acre, and 1.2 fl oz/acre was chosen for the bioassays.

*Leaf preparation.* Greenhouse grown Cabernet Sauvignon and ‘Chardonnay’ leaves were used to incubate the isolates tested. Leaves between the third and fifth fully expanded from the apical meristem of each shoot were collected, placed in a Ziploc bag and transported to the laboratory. Leaves were disinfected with a solution of 10% sodium hypochlorite and distilled water for two minutes and rinsed three times with distilled water then placed on a baking sheet with sterile paper towels in a laminar flow hood to dry. Once dry, leaves were dipped in a mixture containing respective fungicides for one minute and placed on a baking sheet lined with sterile paper towels under a laminar flow hood to dry. A positive control of sterile distilled water was also included along the five fungicide treatments. Dried treated leaves were transferred to petri dishes containing 0.75% water agar and half strength MS media.

*Inoculation.* Fresh spores were generated by rinsing field leaf samples with distilled water followed by dark incubation on a wet paper towel in a Ziploc bag at 25 °C overnight. Fresh spores were then used to make spore suspensions at a concentration of  $1 \times 10^5$ /ml. Briefly, a single lesion from an infected leaf was rinsed with 500  $\mu$ l of sterile water by repeatedly adding and

removing water from the leaf surface using a pipettor. Inoculation was carried out by applying spore suspensions at a concentration of  $1 \times 10^5$ /ml to six separate leaves, each containing one of the six treatments. Five 10  $\mu$ l drops of each individual spore suspension was added to a partitioned section of each treated leaf, allowing five isolates to be tested per treated leaf. Following inoculation, dishes were parafilm and incubated in the dark for 24 h at 25 °C. After incubation, lids were removed to allow droplets to dry. Once dried, lids were replaced, and isolates were placed in open gallon Ziploc bags and incubated at 25 °C in a 12 h photoperiod for 14 d. Lesion growth was observed starting from 5 d post-inoculation until 12 d post-inoculation. Growth on treated leaves was monitored, and any growth that resulted in the presence of sporangiophores on treated leaves was considered resistant. Isolates that failed to produce any mycelial growth on the abaxial surface of treated leaves were considered sensitive. Isolates that produced oil spots at the site of inoculation but failed to sporulate were also considered sensitive.

#### DNA extraction

DNA extraction was largely performed using a combination of methods described previously (Chi et al., 2011; Hong et al., 2019). Briefly, 500ul of DNA extraction buffer containing 50x EDTA, KCl, and water and 300 ul of 2-propanol were dispensed into 1.5 ml centrifuge tubes and labelled. 100ul of DNA extraction buffer from corresponding tube was used to rinse spores from infected leaf, microwaved on medium-low for twenty seconds three times and centrifuged at 5000 rpm for ten minutes. Supernatant was then decanted into 300 ul of 2-propanol, mixed, then centrifuged at 10,000 rpm for ten minutes. Supernatant was discarded without disturbing pellet and dried. Once dry, 30 ul of nuclease free water was added to tube and DNA was stored at -20 °C until further analysis.

#### G143A and G1105S verifications

For QoI resistance, the G143A mutation in the *cyt b* gene from resistant *P. viticola* isolates was verified. This was achieved through PCR amplification followed by Sanger sequencing of the amplified region using specific forward and reverse primers: 5' CGATGCATTACACCCCGCATA-3' and 5'-TACAGCCCCTACAATTACAAATGGA -3'.

These primers were designed in this study using the Geneious primer tool (version 2024.0.5, Biomatters Ltd., Auckland, New Zealand). PCR amplification of the cytochrome b (*cyt b*) region containing the G143A mutation was achieved using 10 µl Apex Taq RED Master Mix, 1 µl forward primer, 1 µl reverse primer, 6 µl nuclease free water, and 2 µl DNA template. Amplification was performed using a PCR Thermal Cycler with initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 30 seconds. The final extension step was conducted at 72°C for 5 minutes. PCR products were visualized on 1.5% agarose gel and then purified for sanger sequencing through Genewiz by Azenta (Burlington, MA, USA) using ExoSap IT (Thermo Fisher Scientific, Waltham, MA, USA). For CAA resistance, the G1105S mutation of the CeSA3 gene was confirmed using the PCR-RFLP method, described by (Aoki et al., 2011).

#### Clade identification

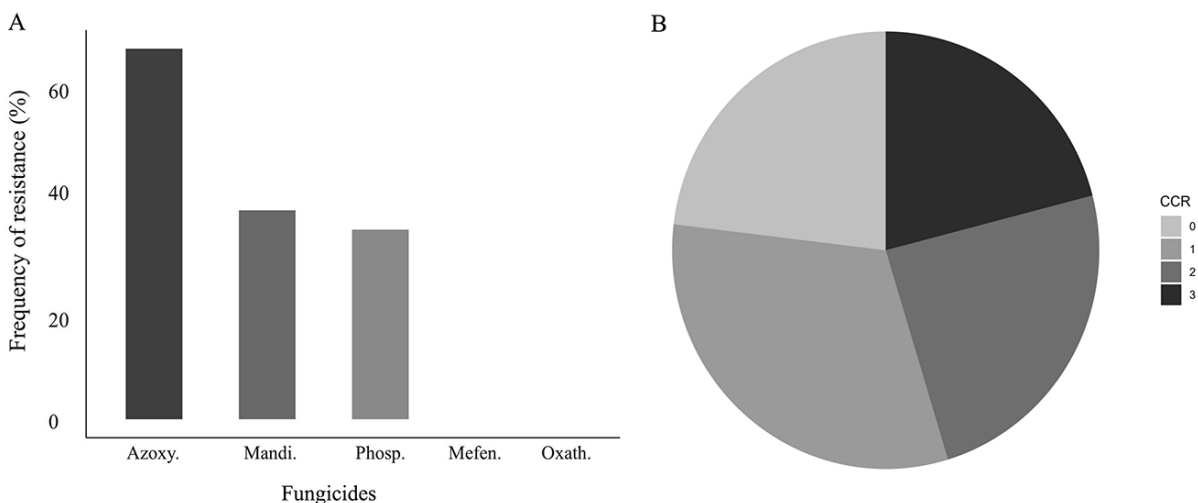
Partial sequences of the internal transcribed spacer (ITS) and *cyt b* gene sequences obtained for G143A confirmation were used to identify *P. viticola* clades. Specific forward and reverse primers: 5' CGGAAGGATCATTACC-3' and 5'- GCTGCGTTCTTCATCGATGC-3', designed by Rouxel et al. (2013), were used for ITS amplification and Sanger sequencing, following the protocol described above. Phylogenetic trees were generated through the alignment of sequenced samples with referenced genes belonging to each clade obtained on NCBI (<https://www.ncbi.nlm.nih.gov/>) followed by concatenation of *cyt b* and ITS sequences using UPGMA algorithm in Geneious software (version 2024.0.5, Biomatters Ltd., Auckland, New Zealand), facilitating the identification and classification of different clades within the studied isolates. Sequences representing each genotype described in this study were deposited in the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under the accession numbers PQ4726324 for ITS gene, PQ508260 for *cyt b* and PQ625492 and PQ625493 for CeSA3 gene, respectively.

## Results

### Bioassays

Among the 352 samples tested from 27 respective vineyards, a total of 69%, 39%, and 33% displayed resistant phenotypes to azoxystrobin, mandipropamid, and phosphorous acid, respectively. In contrast, no resistance was detected to mefenoxam or oxathiapiprolin (Figure 8a). Resistance to the three fungicides was widely distributed, with resistance detected in all distinct regions sampled in our study. Similarly, chemical class resistance (CCR) among isolates tested varied, with 22%, 34%, 24%, and 20% of samples displaying resistant phenotypes of 0, 1, 2, and 3 CCR, respectively (Figure 8b). For example: 0 CCR isolates are sensitive to all fungicides tested, whereas 3 CCR are simultaneously resistant to three chemical classes of fungicides.

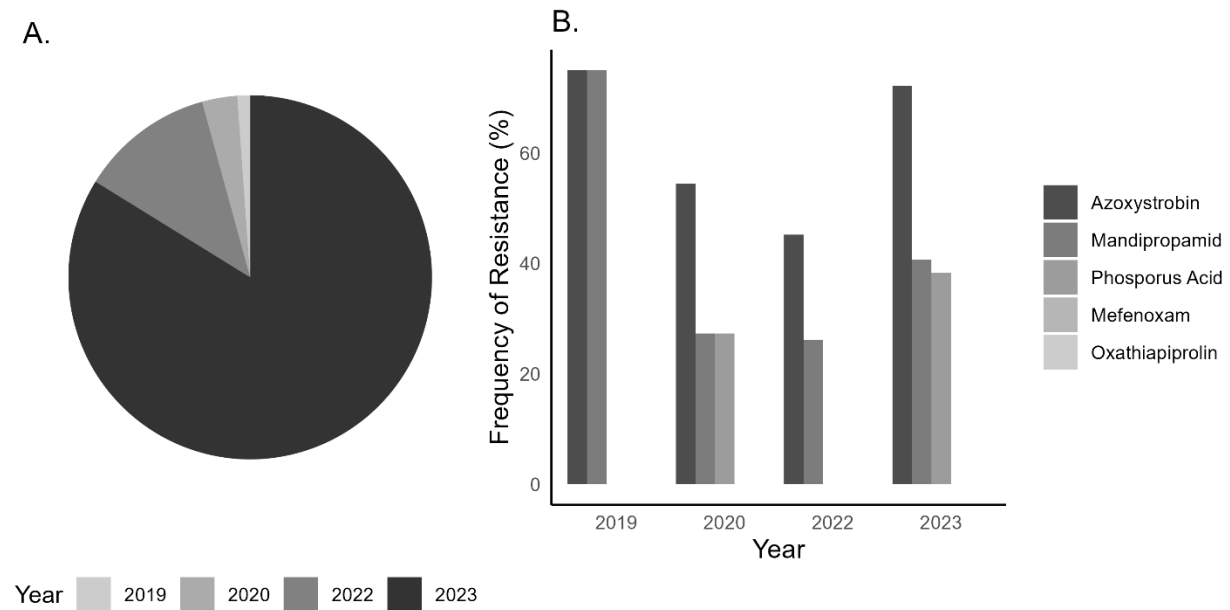
### Overview of resistance profiles from bioassays



**Figure 8.** Overview of resistance profiles from bioassays. Figure 8a displays the total frequency of resistance among all isolates tested in bioassay. Figure 8b represents the percentage of isolates resistant to 0, 1, 2, or 3 chemical classes with the most common group belonging to the 1CCR category and the least common belonging to 0CCR.

While testing was conducted in the years 2019, 2020, 2022, and 2023, the majority of isolates (84%) came from 2023 followed by 2022 at 12% of total isolates, then 3 and 1% belonging to years 2020 and 2019 respectively (Figure 9a). The highest frequencies of resistance to azoxystrobin and mandipropamid were found in 2019, with 75% of the isolates resistant to both fungicides. In 2023, azoxystrobin and mandipropamid resistance were the second highest, at 72% and 41% respectively. Resistance levels to azoxystrobin and mandipropamid between 2020 and 2022 remained relatively consistent, with azoxystrobin resistance ranging between 45 and 55%, and mandipropamid resistance remaining between 26 and 27%. Notably, phosphorous acid resistance was first detected in 2020. It was undetected in 2022, however, 38% of isolates collected in 2023 displayed resistance to this fungicide (Figure 9b). As noted above, isolates collected in 2023 represent the most extensive and geographically diverse vineyards and cultivars.

### Temporal distribution of isolate resistance profiles

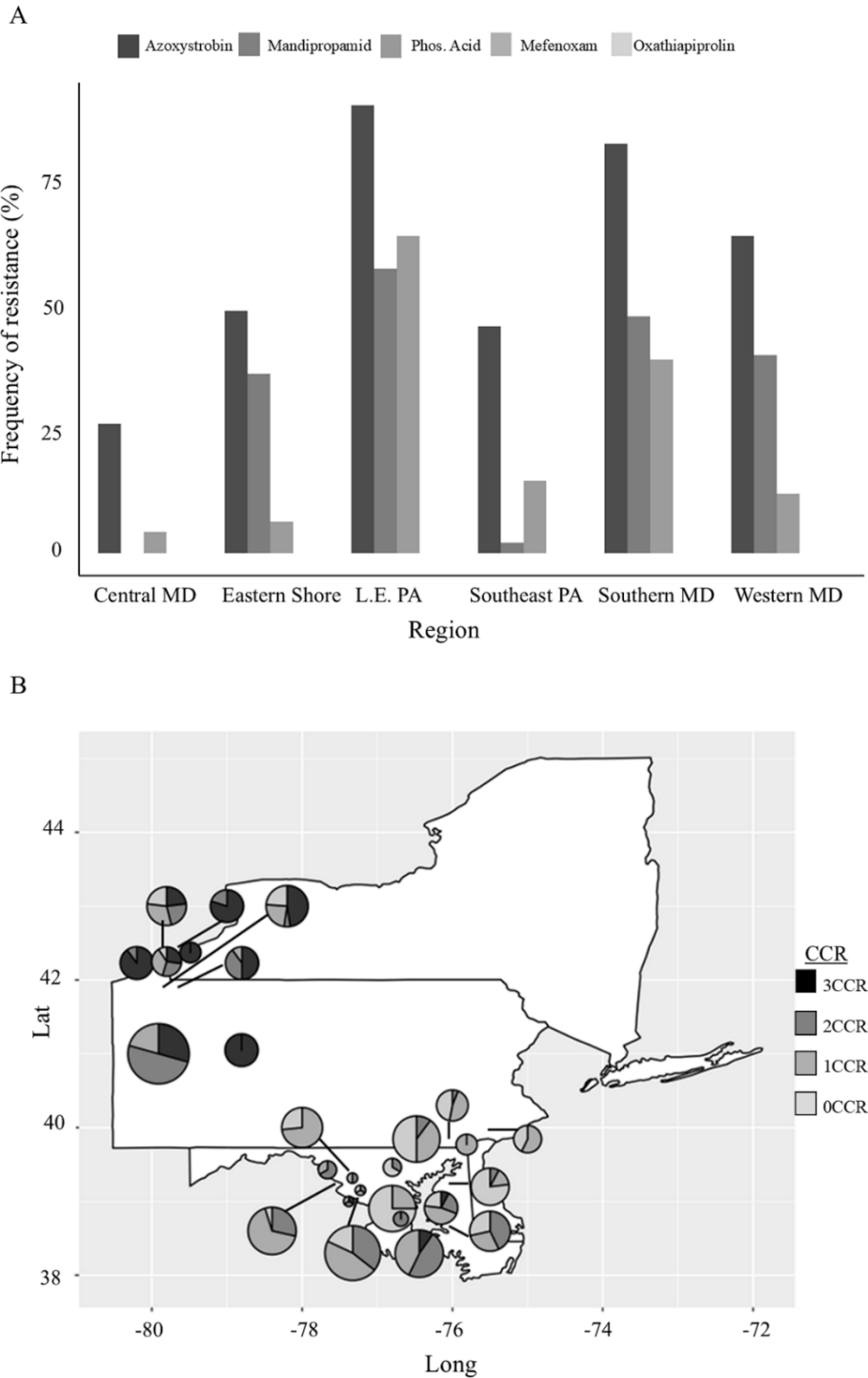


**Figure 9.** Temporal distribution of isolate resistance profiles. Figure 9a displays the distribution of isolates associated with the collection year. Figure 9b displays the frequency of resistance of isolates by year of sample collection.

## Geographic Distribution

When considering the geographic distribution of resistance in DM, frequency of resistance varied among regions, with the highest levels of resistance to all three chemical classes occurring in the Lake Erie region. This region included vineyards in Pennsylvania and New York surrounding Lake Erie (Figure 10a). The Lake Erie region displayed 90%, 57%, and 64% resistance to azoxystrobin, mandipropamid, and phosphorous acid, respectively. Southern Maryland displayed the second highest level of resistance to all three chemicals. Western Maryland displayed the third highest resistance to azoxystrobin, at 64%, and 40% and 12% to mandipropamid and phosphorous acid. The Eastern shore displayed 49%, 36%, and 6% resistance to azoxystrobin, mandipropamid and phosphorous acid. Southeastern PA displayed the second lowest resistance levels to azoxystrobin and mandipropamid, at 46 and 2% respectively. Central MD displayed the lowest levels of resistance overall and was the only region where no mandipropamid resistant isolates were found. Nevertheless, resistance was found to be widespread across those regions. In addition, the frequency of resistance varied by vineyard (Table 9), showing no discernible trend across different geographical regions.

## Regional distribution of fungicide resistance



**Figure 10.** Regional distribution of fungicide resistance in DM isolates. Figure 10a compares the mean frequency of resistance to the regions from which isolates were collected from. Lake Erie displayed the highest levels of resistance for all three chemicals where resistance was found. Figure 10b displays the distribution of chemical class resistance (CCR) by vineyard. Each pie

chart represents a different vineyard with the size of the pie chart representing the sample size. Shades within respective pie charts correspond with the different CCRs found within each vineyard.

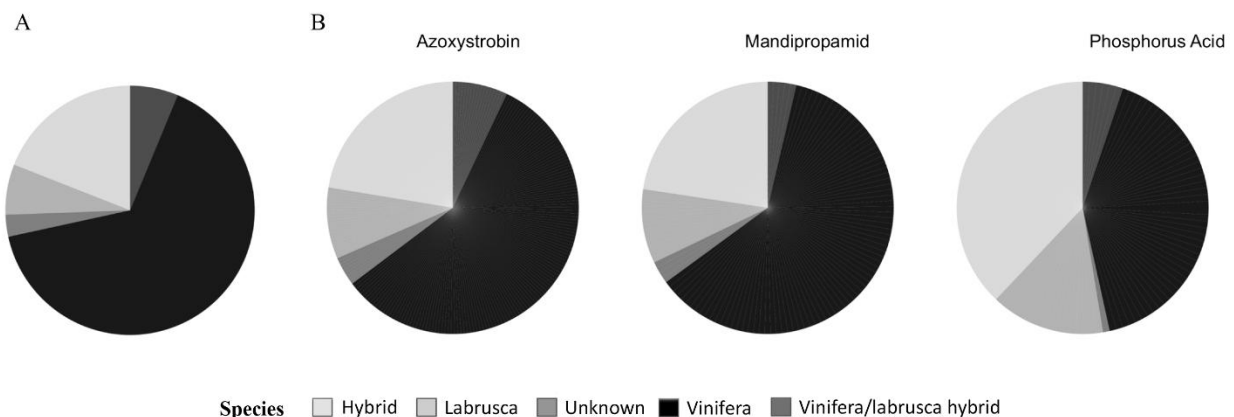
**Table 9.** Percentage of resistant isolates belonging to each respective vineyard and location. Highest levels of resistance belong to vineyards from the Lake Erie region

Region	Vineyard #	# Isolates	Frequency of Resistance (%)				
			Azoxy.	Mandi.	Phos Ac.	Mefen.	Oxath.
C MD	Vineyard 10	20	25	0	0	N/A	0
	Vineyard 14	3	33	0	33	N/A	0
ES MD	Vineyard 11	21	62	48	5	0	0
	Vineyard 12	13	69	31	15	0	0
	Vineyard 13	13	8	23	0	0	0
LE PA	Vineyard 15	10	100	100	100	0	N/A
	Vineyard 16	34	100	68	41	0	N/A
	Vineyard 17	26	77	23	46	0	N/A
	Vineyard 18	10	90	50	100	0	N/A
	Vineyard 19	10	100	80	100	0	N/A
	Vineyard 20	10	100	90	100	0	N/A
	Vineyard 21	21	76	48	52	0	N/A
	Vineyard 22	11	91	27	55	0	N/A
	Vineyard 23	4	100	100	100	0	N/A
	SE PA	Vineyard 24	4	25	0	75	0
Vineyard 25		20	45	0	15	0	0
Vineyard 26		7	43	14	0	0	0
Vineyard 27		17	53	0	6	0	0
S MD	Vineyard 8	21	81	48	38	0	0
	Vineyard 9	2	100	50	50	0	0
	Vineyard 1	28	39	54	25	0	0
	Vineyard 2	3	33	67	0	0	0
W MD	Vineyard 3	15	73	0	0	0	0
	Vineyard 4	21	86	38	0	N/A	0
	Vineyard 5	3	100	33	67	N/A	0
	Vineyard 6	2	100	50	0	0	0
	Vineyard 7	3	67	100	0	N/A	0
<b>Total</b>	<b>27</b>	<b>352</b>	<b>69</b>	<b>39</b>	<b>33</b>	<b>0</b>	<b>0</b>

### Frequency of Resistance by Species

When comparing the frequency of resistance to the *Vitis* species DM samples were collected from, samples collected from *Vitis vinifera* constituted 65% of isolates and made up for 58%, 59%, and 41% of azoxystrobin, mandipropamid, and phosphorous acid resistant isolates respectively (Figure 11a). DM samples collected from *Vitis labrusca* hosts comprised of 7% of total isolates and accounted for 10% of both azoxystrobin and mandipropamid resistant isolates, and 15% of phosphorous acid resistant isolates. Despite making up only 24% of the total isolates, interspecific hybrids constituted 43% of phosphorous acid resistant isolates. Azoxystrobin and mandipropamid resistance was more consistent, with interspecific hybrids making up 28% of the original hosts for both respective chemicals (Figure 11b).

### Distribution of resistance by *Vitis* species



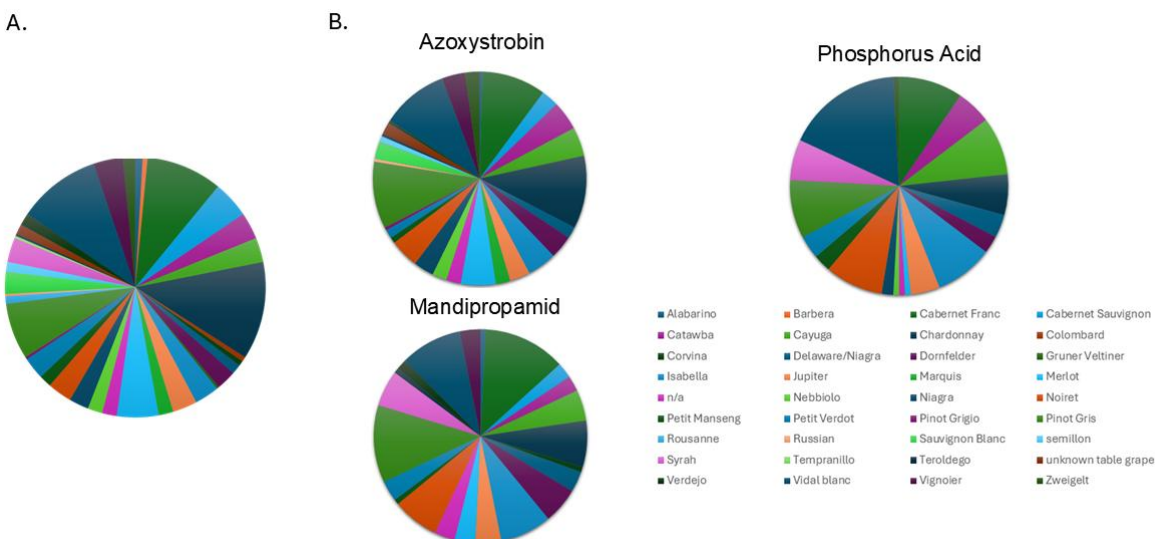
**Figure 11.** Distribution of resistance by *Vitis* species. Figure 11a displays the distribution *Vitis* species all isolates were collected from throughout the project. Figure 11b shows the distribution of resistant isolates compared to the *Vitis* species each DM isolate was collected from. Distribution of azoxystrobin and mandipropamid resistant isolates show similar distribution patterns to the overall species distribution, however, a higher proportion of phosphorus acid resistant isolates were collected for interspecific hybrids.

### Frequency of Resistance by Cultivar

The host range of DM samples used in this study included 32 different cultivars. Among these cultivars, the three collected most frequently for bioassays included ‘Chardonnay’, ‘Cabernet Franc’, and ‘Vidal blanc’, making up 11, 9, and 10% of original isolate hosts respectively

(Figure 12a). The resistant phenotypes of the isolates collected from these cultivars showed similar results, with ‘Chardonnay’ accounting for 10%, 7% and 6% of isolates resistant to azoxystrobin, mandipropamid, and phosphorous acid respectively (Figure 12b). ‘Cabernet Franc’, which accounted for 8% of total DM isolates made up 9%, 12%, and 9% of azoxystrobin, mandipropamid and phosphorous acid resistant isolates. Finally, isolates that were originally collected from ‘Vidal blanc’, making up 10% of total isolates accounted for the highest number of phosphorous acid resistant isolates, at 17%. Azoxystrobin and mandipropamid resistant isolates obtained from ‘Vidal blanc’ leaves comprised of 10% and 9% of total resistant isolates respectively (Figure 12b).

### Distribution of resistance by Cultivar



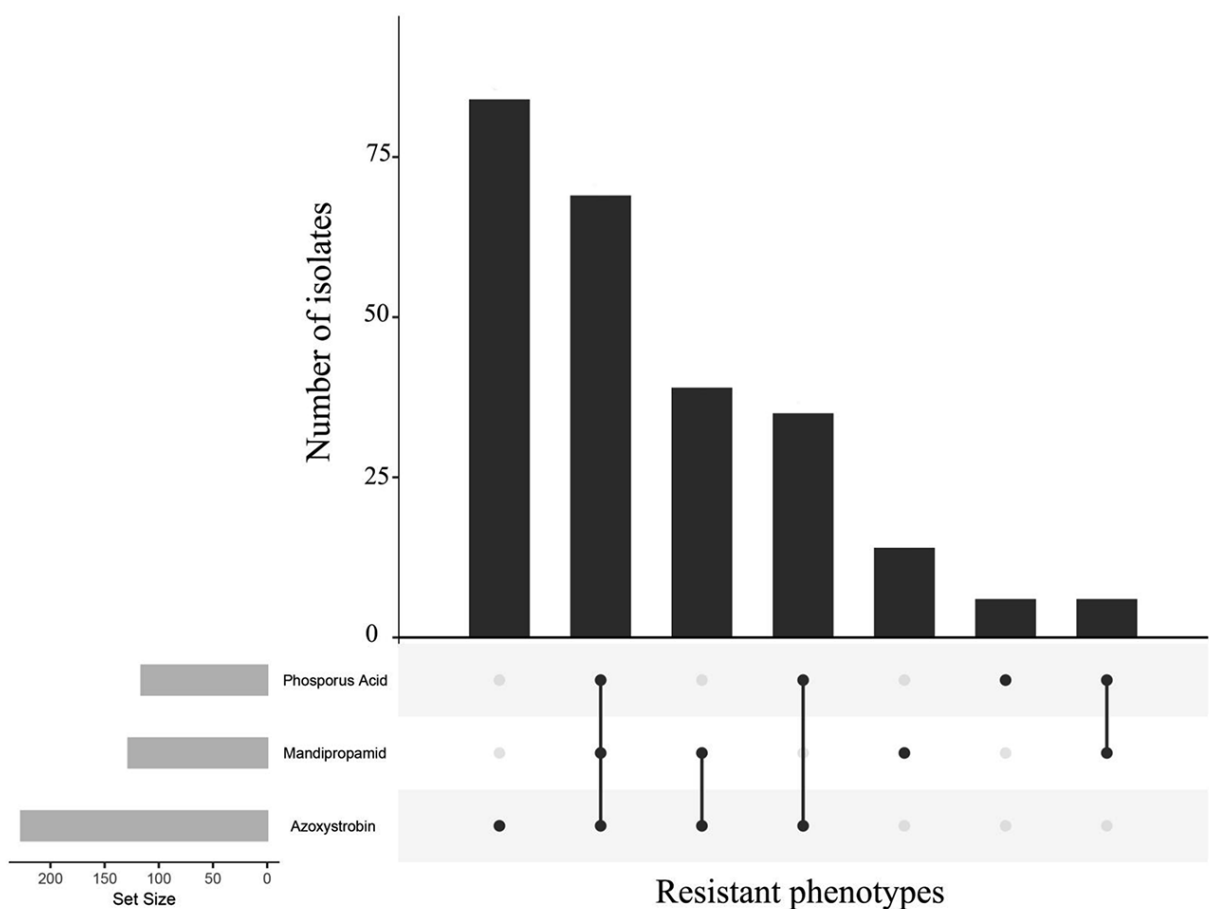
**Figure 12.** Distribution of resistance by Cultivar. Figure 12a displays the distribution of all isolates associated with the cultivar they were isolated from and Figure 12b displays the distribution of resistant isolates by cultivar. Generally, the distribution of resistant isolates coincides with the overall distribution of cultivars except for a high number of phosphorus acid resistant isolates collected from ‘Vidal blanc’ leaves

### Chemical Class Resistance (CCRs)

Among the 27 vineyards sampled for resistance testing, twelve (43%) displayed at least one isolate with 3CCR, nine of which belonged to the Lake Erie region (Figure 10b). 3CCR isolates were discovered in a vineyard in Western Maryland as early as 2020, however in only one

instance. No more 3CCR phenotypes were detected until 2023. Eleven vineyards tested had a maximum of 2CCR, and four with 1CCR. There were no vineyards tested that resulted in no resistance across all isolates (Table 9). The majority of 1CCR isolates displayed resistance to azoxystrobin, followed by mandipropanid and phosphorous acid (Figure 13). The second most common combination of resistance in isolates was those belonging to the 3CCR category. The least common combinations of resistance were those with a 2CCR, displaying resistance to phosphorous acid and mandipropanid, and 1CCR isolates displaying only phosphorous acid resistance, with six isolates belonging to each category, respectively.

### Breakdown of resistance profile chemical combinations



**Figure 13.** Breakdown of resistance profiles using an Upset plot comparing the number of resistant isolates per chemical (Set size) to the varying combination of multi-resistant isolates. The highest number of isolates were 1CCR azoxystrobin resistant isolates, however, 241 isolates were resistant to azoxystrobin in total. The second most common resistance combination was isolates belonging to the 3CCR group.

PCR amplification.

Confirmation of known mechanisms of azoxystrobin resistance was carried out by sequencing a segment of the *cyt b* gene containing the location of G143A mutation. DNA of 52 samples were selected for sequencing, 39 of which displayed resistant phenotypes, five displaying a sensitive phenotype, and eight of which failed to be cultured during initial bioassays. DNA selected for sequencing varied by region, vineyard and cultivar. Despite variation in DNA selection, all 52 sequences displayed the G143A mutation, indicating that azoxystrobin resistance in Mid-Atlantic vineyards may be more prevalent than results from bioassays indicate.

DNA from 51 DM isolates were selected for PCR-RFLP, five of which displayed sensitive phenotypes of mandipropamid during initial resistance screening bioassays. Of the 51 DNA isolates that underwent PCR-RFLP, 34 displayed genotypes consistent with observed phenotypes from previous bioassays. Results from PCR-RFLP of 17 isolates did not match those of bioassays, two of which displayed a sensitive phenotype and homozygous recessive resistant genotype, 15 of which had resistant phenotypes based on bioassays but displayed mandipropamid sensitive genotypes. To confirm PCR-RFLP results, the 14 phenotypically resistant isolates displaying sensitive genotypes were sequenced. After sequencing, six of the fifteen isolates were confirmed as resistant homozygous recessive, two displayed double peaks, with an “A” peak much larger than the “G” peak, two displayed both A and G peaks similar in size, one displayed heterozygosity with a larger G peak, and three displayed sensitive homozygosity. All sequences displaying either heterozygosity with dominant G peaks or homozygous sensitive genotypes were from the same vineyard in Lake Erie, Pennsylvania, except for one isolate from the Eastern Shore of Maryland.

#### Clade Identification

All isolates sequenced for clade identification were found to belong to clade *aestivalis*. The ITS 1 and 2 region of 63 isolates varying in phenotype, region, vineyard, cultivar and *Vitis* species were amplified and sequenced. Among them, the *cyt b* region of 38 of the isolates was also sequenced. Twelve isolates underwent sequencing of the *cyt b* region alone, yet all isolates sequenced matched clade *aestivalis* when aligned with reference genes belonging to clades *aestivalis*, *riparia*, *vulpina*, *vinifera*, and *quinquefolia*.

## Discussion

This study has shown that *P. viticola* populations resistant to multiple chemical classes are increasingly prevalent, reducing the available fungicide arsenal for growers. Vineyards sampled exhibited diversity in terms of their age, approaches to canopy management, and fungicide applications. This variability is expected to contribute to fluctuations in disease prevalence throughout the region, influenced by distinct management practices and environmental factors. Resistance levels varied by vineyard, indicating that a grower's spray program significantly affects resistance in populations on a site-to-site basis. High spray frequency is associated with the selection of resistant populations, and the Mid-Atlantic's standard spray program for downy mildew involves applications on a 7–14-day interval from bud break to senescence. This intensive spray program likely accelerated the development of resistant populations in the region similarly to what has been observed in other pathogens (Hahn, 2014).

Sequencing and PCR-RFLP data resulted in higher levels of resistance to azoxystrobin, and similar resistance frequency to mandipropamid previously reported in the Eastern US (Baudoin et al., 2008; Campbell et al., 2020; Feng and Baudoin, 2018). Notably, this study reported the first incidences of phosphorous acid resistance in *P. viticola* in North America. When considering bioassays, results displayed azoxystrobin resistance levels consistent with previous findings with the exception of the Lake Erie region, which displayed the highest levels of resistance to all three chemical classes where resistance was found. Higher resistance levels in the Lake Erie region are possibly explained by the use of 'Chardonnay' leaves, a more susceptible cultivar, as the growth medium in the bioassays performed. As a more susceptible cultivar, the leaves may have facilitated the growth of less fit *P. viticola* strains when compared to the moderately susceptible Cabernet Sauvignon. With this in consideration, using highly susceptible cultivars like 'Chardonnay' may improve the accuracy of bioassays in future studies. The sequencing results of cyt b also seemed to be more aligned with the bioassay results using 'Chardonnay' leaves than Cabernet Sauvignon leaves. The susceptibility of 'Chardonnay' can also be observed when considering the primary host cultivar of DM samples collected

throughout this study. DM samples in this study were most frequently collected from infected ‘Chardonnay’ leaves and the cultivar was often the first cultivar to display signs of DM infection in the field at the beginning of the growing season. Limiting the production of highly susceptible cultivars like ‘Chardonnay’ and ‘Vidal blanc’ could potentially help eliminate less fit resistant strains and even shorten the infection season of DM in a vineyard (Hawkins and Fraaije, 2018; Mikaberidze and McDonald, 2015).

The frequency of resistance did not display a steady increase over the five years in which this study was conducted, but rather a spike in the first and last years with the lowest resistance levels during the middle two years (no collection occurred in 2021). However, as noted above, the sample size is small in all years except 2023, which may not completely capture the temporal changes in frequency of resistance. When comparing the average monthly rainfall, the highest rainfall occurred in 2020 with 25.58 inches between the months of June and October and the lowest occurred in 2023 with 17.3 inches of rain. It is possible that more fungicide applications of phosphorous acids were made to combat the high disease pressure leading to the detection of resistant populations in 2020. A field study conducted in Virginia tested the efficacy of Prophyt, a fungicide in the same chemical class (i.e. FRAC P07), and found that the chemical’s ability to control DM reduced over time when sprayed on a 14-day interval. However, the same study did not find any loss of sensitivity to Prophyt when conducting bioassays or potted plant experiments (Feng, 2018). Considering the majority of phosphorous acid resistant acid isolates came from the year 2023, further investigation into the stability of resistant phenotypes may be warranted to better understand the future of phosphonates in Northeastern viticulture.

It was reported in the Southeastern US that unique multi-locus genotypes of *P. viticola* continue to appear throughout the growing season. This suggests the possibility of oospores from the previous year germinating throughout the growing season, increasing population diversity within a vineyard (Hong et al., 2020). Limiting the application of single mode of action fungicides later in the season may allow new sensitive populations to increase. An increase in sensitive populations could potentially reduce the frequency of mandipropamid and phosphorous acid resistance the following season through a combination of reduced selection pressure and sexual recombination. Mandipropamid resistance may particularly benefit as sensitive strains recombine with resistant populations during sexual reproduction in the fall, effectively reducing

the homozygous recessive population containing both alleles that confer the G1105S mutation (Blum et al., 2010b).

Isolates belonging to interspecific hybrids displayed higher levels of phosphorous acid resistance when comparing the distribution of phosphorous acid resistant isolates belonging to respective *Vitis sp.* to the overall distribution of host sample species. Most grapevines cultivated in Maryland and Southeastern Pennsylvania belong to *Vitis vinifera*, whereas the Lake Erie region primarily grows more cold tolerant grape cultivars, many of which are interspecific hybrids. In this study, the majority of interspecific hybrids were sampled from the Lake Erie region, likely explaining the higher levels of phosphorous acid resistance found in this species group.

Vineyards with isolates resistant to three chemical classes were found throughout all regions except Southeastern PA and Central MD, with most vineyards having a 3CCR maximum. Isolates displaying multi-chemical class resistance were most commonly resistant to azoxystrobin in conjunction with either mandipropamid and/or phosphorous acid, while 2CCR isolates resistant to mandipropamid and phosphorous acid were the least common phenotype. Azoxystrobin resistance has been documented for decades (Collina et al., 2005; Corio-Costet et al., 2011; Gisi et al., 2002), compared to the relatively recent appearance of mandipropamid resistance (Baudoin et al., 2008; Feng and Baudoin, 2018; Gisi et al., 2007). This long-term establishment in northeastern vineyards likely explains the high level of multi-class resistance groups containing azoxystrobin resistance, as the G143A mutation is a stable mutation with no known fitness penalties (Corio-Costet et al., 2011). Due to its early and widespread presence, azoxystrobin resistance likely serves as a foundational resistance mutation, providing a foothold for resistant populations. The emergence of resistance to other chemical classes, such as mandipropamid and phosphorous acid, may build upon these already-established populations, leading to the multi-class resistance profiles observed in this study.

Given the high levels of resistance, it may no longer be effective to use azoxystrobin, as sequencing revealed that every isolate displayed the G143A mutation, with no new mechanism of resistance detected (Baudoin et al., 2008; Zhang et al., 2023). This study also marks the first report of phosphorous acid resistance in North America, which has spread rapidly through Pennsylvania and Maryland. Additionally, a possible alternative mechanism of resistance to mandipropamid has been observed, with resistant phenotypes lacking the G1105S mutation.

Interestingly, all but one isolate containing this trait were obtained from the same vineyard. Currently, the recessive G1105S and G1105V mutations in the CeSA3 gene are the only known mechanisms of resistance to mandipropamid in *P. viticola* and *P. infestans* (Blum, Boehler, et al., 2010; Blum, Waldner, et al., 2010).

In the Mid-Atlantic, Clade *aestivalis* dominates, which is consistent with previous reports, where clade *aestivalis* was the most prevalent throughout the United States and only clade found in Maryland. While clade *riparia* has been reported in the Lake Erie region of both Pennsylvania and New York, no occurrences of the clade were found in isolates sequenced in this study (Rouxel et al., 2014). Clade *riparia* is reported to have temporal distribution in Canada, being the dominant clade found on cultivated hybrid grape cultivars earlier in the season before being outcompeted by clade *aestivalis* (Carisse et al., 2021; Mouafo-Tchinda et al., 2022). The earliest date in which samples were collected from the Lake Erie region was not until late July, which may have been too late to observe the resistance profile of the less competitive clade *riparia*. The presence of only one clade limits the opportunity to tailor spray programs based on the activity of other clades. However, this approach could be viable in more northern areas, where there is more diversity in clades found if resistance profiles differ among clades. Further exploration into the resistance profiles of other *P. viticola* clades could potentially aid in DM management, especially in the Lake Erie region where resistance levels are prevalent. Doing so provides the potential for growers to re-integrate chemicals that are currently viewed as non-effective, increasing the number of available chemicals for effective DM control.

To address the challenges posed by this study, it is essential to target spray programs more effectively, possibly utilizing disease risk models to predict infection timing and reduce the number of spray applications per season. An experiment conducted by Cosseboom and Hu effectively reduced the number of spray applications targeting ripe rot in grapes caused by *Colletotrichum sp.* by half while maintaining control efficacy by utilizing a ripe rot prediction model in conjunction with canopy temperature and leaf wetness sensors (Cosseboom and Hu, 2023). Two new predictive models for grapevine downy mildew were published in 2023 and 2024 (Puelles et al., 2024). Incorporation of similar models may help reduce the number of fungicide applications targeting DM in a season, effectively reducing the selection pressure on resistant populations.

While prediction models have the potential to aid in reducing the number of chemical applications made per season, having a robust rotation of fungicides belonging to different FRAC groups is essential for managing both disease and resistance development. With decreasing efficacy of current labeled chemicals and increasing regulation on those commercially available, chemicals available to grape growers are becoming extremely limited and potentially “threaten the sustainability of growing highly susceptible cultivars in humid regions such as those found in the eastern US. Mancozeb, a multi-mode of action fungicide is a staple among grape growers in the Eastern US and the backbone of many grower’s spray programs. In June 2024, the EPA published a proposed registration review decision (PID) for Mancozeb that will restrict the use of the chemical on grapes, further limiting the chemicals available for grape growers (Office of Chemical Safety and Pollution Prevention, 2024). In this study, Oxathiapiprolin showed 100% efficacy and could potentially be labeled for Downy Mildew.

In summary, leveraging disease risk models, a deeper understanding of *P. viticola* population dynamics, and exploring alternative methods such as biological controls and resistance breeding is essential for Mid-Atlantic wine grape production. Further exploration and implementation of these measures may enhance the effectiveness of spray programs while reducing the environmental impact and cost for growers, leading to a more sustainable future for grape production.

## Summary

This study underscores the growing challenges of managing grapevine diseases in the Mid-Atlantic wine grape industry, driven by the increasing prevalence of fungicide-resistant *Plasmopara viticola* populations and the complex interactions between cultivar susceptibility, canopy management practices, pathogen biology, and environmental conditions. Resistance variability among vineyards highlights the critical impact of grower-specific spray programs, with high-frequency fungicide use contributing to resistance selection. The stable G143A mutation underpins widespread azoxystrobin resistance, while mandipropamid resistance suggests alternative mechanisms beyond the G1105S mutation. Notably, this is the first instance of phosphorous acid resistance in North America, particularly affecting susceptible cultivars like

‘Chardonnay’, which may further exacerbate resistance development and disease pressure. Environmental factors, such as rainfall, irrigation, and cultivar selection, play pivotal roles in shaping resistance dynamics.

In conclusion, my research emphasizes the urgent need for integrated vineyard disease management approaches that combine fungicide resistance management with tailored cultural practices. Strategies such as optimizing fungicide rotations, utilizing disease risk models to improve spray timing, and exploring alternative methods like biological controls and resistance breeding can help alleviate selection pressure on resistant populations. Additionally, employing site-specific canopy management practices, such as denser canopies to mitigate ripe rot and open canopies to manage powdery mildew and Botrytis, can significantly improve disease outcomes. By understanding the nuanced relationships among pathogens, cultivars, and environmental conditions, growers can develop sustainable practices to enhance vineyard productivity and health in the Mid-Atlantic region.

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