Fungicide Sensitivity of *Erysiphe necator* and *Plasmopara viticola* from Virginia and nearby states

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ABSTRACT

This study was undertaken to determine the sensitivity of grape downy mildew (DM, *Plasmopara viticola*) and powdery mildew (PM, *Erysiphe necator*) to commonly used single-site fungicides in Virginia and nearby states. DM and PM isolates were collected from 2005 to 2007. In grape leaf disc bioassays, 92% of the DM isolates were QoI (azoxystrobin)-resistant, but none were resistant to mefenoxam. Eighty-two percent of the PM isolates were QoI-resistant, but none were resistant to boscalid and quinoxyfen. The frequency of the G143A point mutation, which confers high levels of QoI resistance, was quantified in DM and PM isolates by real-time PCR. Most of the QoI-resistant DM and PM isolates contained >95% of the 143A allele. QoI-sensitive DM isolates contained less than 1% of 143A. One out of 145 and 14 out of 154 QoI-resistant DM and PM isolates (able to grow on azoxystrobin concentration ≥ 1 µg/ml), respectively, contained less than 1% 143A. Most PM isolates exhibited reduced sensitivity to five DMI fungicides when compared to a sensitive subgroup (n=9) and compared to published reports for unexposed populations; the resistance factor (median EC50 of the entire isolate collection divided by median EC50 of sensitive subgroup) was highest for tebuconazole (360) and myclobutanil (350), followed by triflumizole (79), triadimefon (61), and fenarimol (53). Sensitivities to all five DMI fungicides, but also azoxystrobin, were moderately to strongly correlated (pairwise r-values ranging from 0.60 to 0.88).

DEDICATION

To my adviser, for being the BEST adviser.

To my friends, for all the fun times of hanging out, being silly, and enjoying life.

To my parents, sister, and brothers, for all the support, prayer, unconditional love, and being there for me always. I love you.

To God, for the gift of life, love, and salvation. Praise and glory to you forever.

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CHAPTER 1

Historical Background

Powdery mildew (PM, *Erysiphe (Uncinula) necator* [Schw.] Burr.) and downy mildew (DM, *Plasmopara viticola* [Berk. & Curt.] Berl. & de Toni) are the two most important fungal diseases of grapes. DM (Lafon and Clerjeau 1988) and PM affect almost all green tissues of a grapevine including the leaves, petioles, stems, clusters (Pearson and Goheen 1988), and buds (Rumbolz and Gubler 2005). Infection of grapevines by PM can reduce the quality of the berries, the winter hardiness of canes and buds, and the quality of wines produced from infected berries, and result in lower yield (Pool et al. 1984). DM infection may lead to browning, drying, and dying of shoot tips, petioles, tendrils, inflorescences, and berries, defoliation that results in the reduction of sugar accumulation in fruits, and decreased hardiness of overwintering buds (Lafon and Clerjeau 1988). Both diseases may cause great damage if left controlled.

Since the most valuable grape cultivars are highly susceptible to these diseases, the primary measure used to control these diseases is the use of chemicals. But some of these chemicals may or may not have risk of resistance development. Fungicide resistance may be defined as reduced sensitivity of a pathogen to a fungicide, especially when this trait is stable and heritable (McGrath 2004). Prior to the 1960s, chemicals used for grape disease control, such as sulfur, copper, captan, and mancozeb, had a multi-site mode of action and no history of resistance. Multi-site fungicides have multiple targets in the metabolic pathways of a fungus. For many years, sulfur was commonly used by growers to control PM (Ypema et al. 1997). However, sulfur has disadvantages such as phytotoxicity at higher temperatures and the necessity of frequent applications to provide good control (Gubler et al. 1996). Thus, growers switched to the new fungicides that came into the market. Many of the new chemicals that have been introduced since the 1960s have single-site modes of action, and target only one critical point in the metabolic pathway of a fungus. A modification of such a single site is much easier to accomplish by a fungus without affecting its viability than modification of multiple targets, and single-site fungicides often have a moderate to high risk of resistance development by the pathogen, which can turn into a serious problem (Brent and Hollomon 2007).

1.1. Fungicides used to control PM and DM

PM or DM can be controlled by several groups of single-site fungicides, e.g. quinone outside inhibitors (QoI), sterol demethylation inhibitors (DMI), and methyl benzimidazole carbamates. The QoIs are a group of fungicides that can be used to control either PM or DM or both. QoIs control plant pathogens by inhibiting mitochondrial respiration leading to ATP deficiency (Gisi et al. 2002). QoIs were introduced into the market in 1996 (Gisi et al. 2002; Ma and Michailides 2005). Of the QoIs, azoxystrobin was the first to be introduced in the US grape market in 1997, followed by kresoxim-methyl in 2000, trifloxystrobin in 2001, pyraclostrobin in 2003, and famoxadone in 2008.

PM can also be controlled by other fungicides such as DMIs, boscalid, and quinoxyfen. DMIs prevent ergosterol biosynthesis in the target pathogen by inhibiting the demethylation of eburicol which is an intermediate in the biosynthesis of ergosterol (Ma and Michailides 2005; Gisi et al. 2000). Triadimefon was the first DMI that was registered for grape PM control in 1982 (Miller and Gubler 2004; Ypema et al. 1997; Gubler et al. 1996). In 1989, another two DMI fungicides, myclobutanil and fenarimol were registered to control PM, and several years later two additional DMI fungicides, tebucanozole and triflumizole, entered the market. Boscalid which was introduced in 2003 has a mode of action not previously available in grape fungicides. It is effective in controlling PM by inhibiting succinate dehydrogenase in the mitochondrial electron transport chain (FRAC 2007; Stammler et al. 2007). Quinoxyfen, another fungicide that has a mode of action different from any other grape fungicide and can also control PM, is known to interfere with the germination or pre-infection development of PM but its exact site of action has not yet been determined (Green and Gustafson 2007).

DM can also be controlled by other fungicides, such as mefenoxam/metalaxyl which specifically controls *P. viticola* by inhibiting its ribosomal RNA polymerase (FRAC 2005; Gisi and Cohen 1996).

Being single-site inhibitors, all the fungicides mentioned that can either control PM or DM or both have either high (e.g. QoIs and mefenoxam) or medium (DMIs, boscalid, and quinoxyfen) risk of resistance development (FRAC 2007). Once fungicide

resistance develops, it can turn into a serious problem because it can lead to unexpected epidemics and severe crop losses.

Of all the fungicides mentioned above that can control PM and DM, resistance to QoI in PM and DM is the most serious problem because it has become widespread in Europe and the US (Baudoin et al. 2008; Baudoin and Baldwin 2006; Wilcox et al. 2003; Sirven and Beffa 2003; Toffolatti et al. 2007) and very high levels of resistance have been observed in field isolates (Baudoin et al. 2008). Metalaxyl or mefenoxam resistance in DM has been detected in Europe in the early 1980s and in Australia in 2005 but not in the US. PM isolates collected from California (Gubler et al. 1996), New York (Erickson and Wilcox 1997), and Ontario Canada (Northover and Homeyer 2001) with reduced sensitivity to DMIs have been documented. At present, no boscalid or quinoxyfen resistance in grape PM has been documented, although boscalid resistance in *Alternaria alternata* (Avenot et al. 2008) and *Didymella bryoniae* (Stevenson et al. 2008) and quinoxyfen resistance in *Blumeria graminis* f.sp. *hordei* (Hollomon et al. 1997) have been detected.

1.2. DMI Resistance

Reduced performance of triadimefon against *E. necator* was first noticed in California in 1985 and 1986, three years after the first use of triadimefon in California vineyards, and documentation became available in 1986 (Gubler et al. 1996, Ypema et al. 1997). According to Gubler et al. (1996), the widespread use of triadimefon under severe disease pressure may have favored isolates with elevated resistance level and contributed to a rapid increase in resistance in the population. Miller and Gubler (2004) theorized that the rampant use and misuse of triadimefon hastened resistance development. Under high disease pressure, the repeated use of triadimefon as the sole chemical control for powdery mildew may explain the high frequency of resistant individuals and the high EC_{50} values (Gubler et al. 1996). It was suspected, and later confirmed, that cross resistance exists among the DMI fungicides (triadimefon, myclobutanil, fenarimol) (Miller and Gubler 2004; Ypema et al. 1997), although there are inherent differences in the activity of triadimefon, myclobutanil, and fenarimol against grape PM.

E. necator isolates from New York, California, and Ontario Canada have been studied for their sensitivity distribution to DMI fungicides (Gubler et al. 1996; Erickson and Wilcox 1997; Northover and Homeyer 2001, Ypema et al. 1997). Analysis of the EC_{50} values of isolates showed a continuous distribution which is indicative of multigenic, quantitative resistance that is expressed as a range of phenotypic sensitivities. The sensitivity of *E. necator* was highest to fenarimol, intermediate to myclobutanil, and lowest to triadimenol (the active form of triadimefon) (Erickson and Wilcox 1997) or triadimefon (Gubler et al. 1996; Table 1). Gubler et al. 1996 confirmed that application of triadimefon favored resistant *E. necator* isolates because the EC50 values of the isolates collected prior to triadimefon application were significantly lower than the EC50 values of isolates collected after the last application of triadimefon in the season (Table 1).

Table 1. Mean EC50[5] values (mg/l) for DMI fungicides in California and New York.

Year –month	Triadimefon	Myclobutanil	Fenarimol	Metric tons of triadimefon used in California[3]
California				
1990 – not exposed[1]	1.40	0.15	0.13	
1990 – exposed Jan[1]	21.35	3.16	0.14	13.00 (1990)
1990 – exposed May[1]	33.35	3.27	0.69	
1990 – exposed Sep[1]	54.41	7.94	0.94	
1990 – exposed (based on cleistothecia sample) [1]	32.20	3.52	0.43	
1991				28.00 (1991)
1992[2]				57.00 (1992)
1993[2]	38.10	6.07	0.86	
1994[2]	28.10	3.17	0.58	
1995	18.10	3.35	0.58	
2002[3]	8.80 – 12.80			0.32 (2002)
New York, 1995[4]				
Not exposed	0.06	0.03	0.03	
Exposed	1.90	0.23	0.07	
Ontario, Canada[5] 1999/2000				
Early in season		0.24		
Late in season		0.54		

[1]Gubler et al. 1996; [2]Ypema et al. 1997; [3]Miller & Gubler 2004; [4]Erickson & Wilcox 1997; [5]Northover and Homeyer 2001; [5] It is the effective fungicide concentration that can inhibit 50% of PM growth

Interestingly, Ypema et al. (1997) observed that EC_{50} values of California *E. necator* isolates for triadimefon, myclobutanil, and fenarimol declined from 1993 to 1994 and triadimefon from 1994 to 1995. One possible reason for the decrease in EC50 values was the reduced usage of triadimefon in the vineyards where the isolates were collected.

1.3. Mechanisms of DMI resistance

The mechanisms for DMI resistance include point mutations and overexpression of *CYP51* (Délye et al. 1997; Joseph-Horne and Hollomon 1997; Marichal et al. 1997b; Délye et al. 1998; Butters et al. 2000; Hamamoto et al. 2000; Schnabel and Jones 2001; Albertini et al. 2003; Fraaije et al. 2007) and overexpression of ABC transporters (Hayashi et al. 2002; Zwiers et al. 2002). *CYP51* in *E. necator* was cloned and sequenced by Délye et al. (1997a). The sequence showed that the gene is interrupted by two short introns and encodes for a protein of 544 amino acid units. A single mutation in *CYP51* causes a lower resistance factor than multiple mutations in *CYP51* (Gisi et al. 2000). It was discovered that *E. necator* isolates resistant to triadimenol, and with a resistance factor higher than 5, possessed a single-point mutation on position 136 leading to the substitution of phenylalanine for tyrosine (Y136F) (Délye et al. 1997b). This mutation was also found to be responsible for DMI resistance in another plant pathogen, *B. graminis* f. sp. *hordei* (Délye et al. 1998). However, this mutation was not observed in DMI-resistant isolates of *Mycosphaerella graminicola* (Gisi et al. 2000), *Penicillum digitatum* (Hamamoto et al. 2001), *Tapesia yallundae* (Wood et al. 2001), *Venturia inaequalis* (Schnabel and Jones 2001), and in triadimenol-sensitive or weakly triadimenol-resistant (resistance factors below 5) *E. necator* and triazole-resistant *Tapesia acuformis* isolates. This indicated that resistance to DMI in these organisms may not be related to the Y136F mutation in *CYP51* and that other mechanisms might be involved. *T. acuformis* possessed a Y180L mutation (tyrosine substituted with leucine in position 180) (Ma and Michailides 2005) instead of Y136F. Another organism, *Candida albicans,* a clinical isolate resistant to azole antifungal agents, was discovered to possess five different mutations which were correlated with the occurrence of azole resistance. These mutations were: G129A, Y132H, S405F, G464S, and R467K (Sanglard et al. 1998; Gisi

et al. 2000). Recently, Fraaije et al. (2007) discovered and reported that isolates with point mutation I38V in the *CYP51* of *M. graminicola* were selected by the azole fungicides tebuconazole and difenoconazole, but were negatively selected by another azole fungicide (prochloraz). This suggests that rotating tebuconazole or difenoconazole with prochloraz might be beneficial for resistance management.

A second possible mechanism conferring DMI resistance is the overexpression of the *CYP51* (Ma and Michailides 2005). Three possible ways by which the *CYP51* gene can be overexpressed have been described: 1) increased the copy number of *CYP51,* which was observed in *Candida glabrata* (Marichal et al. 1997); 2) five times tandem repetition of a 126-bp sequence in the promoter region of the *CYP51* in *P. digitatum* (Hamamoto et al. 2001); and 3) a 553-bp insertion in the promoter region of the *CYP51* which was detected in *V. inaequalis* (Schnabel and Jones 2001). A third type of mechanism causing DMI resistance has been found in DMI-resistant phytopathogenic fungi that had neither a single-point mutation nor overexpression of *CYP51* (Ma and Michailides 2005). This mechanism involves the overexpression of the ABC transporter gene. DMI-resistant *M. graminicola* (Zwiers et al. 2002) and *Botrytis cinerea* (Hayashi et al. 2002) isolates created in the laboratory were found to have overexpressed ABC transporters. ABC transporters may affect the uptake/efflux balance of DMI fungicide (Ma and Michailides 2005).

1.4. QoI Resistance

QoI-resistance in *P. viticola* was first detected in Italy in 2000 (Brunelli et al. 2002; Latorse and Gonzalez-Hilt 2003; Collina et al. 2006) and was discovered in Virginia, USA, in 2005 (Baudoin and Baldwin 2006; Baudoin et al. 2008). QoI-resistance in *E. necator* was first detected in New York, USA, in 2003 (Wilcox et al. 2003) and Hungary and Austria in 2006 (FRAC 2006). It has been confirmed that the G143A mutation in the cytochrome *b* (cyt *b*) confers QoI resistance in both *P. viticola* and *E. necator* (Sirven and Beffa 2003; Sierotski et al. 2005; Collina et al. 2006; Grasso et al. 2006; Baudoin et al. 2008)

1.5. Mechanisms of QoI resistance

Resistance to QoI is mostly based on point mutations in two "hot spot" regions of the cyt *b* gene (Ma and Michailides 2005; Gisi et al. 2000, Gisi et al. 2002; Sierotski et al. 2005). These point mutations result in single amino acid changes in positions 127-147 (cd loop) and 275-296 (ef loop) of the amino acid sequence. Amino acid changes that occur in the cd loop usually express higher resistance factors than the ones that occur in the ef loop. Two mutations have been identified to cause amino acid change in the cd loop that confer fungicide resistance: G143A (changes glycine amino acid to alanine) and F129L (changes phenylalanine to leucine). G143A is the major mutation in the cd loop because it causes a higher level of resistance (Ma and Michailides 2005; Gisi et al. 2000, Gisi et al. 2002; Sierotski et al. 2005). The G143A amino acid change is the cause of the fungicide resistance in several pathogens including *P. viticola, B. graminis* f. sp. *tritici, P. cubensis, M. fijiensis, S. fuliginea, P. grisea, and D. bryoniae* (Barlett et al. 2002; Sirven and Beffa 2003). The mutation F129L confers a lower resistance level than G143A (Barlett et al. 2002; Sirven and Beffa 2003), reduced fitness (Toffolatti et al. 2007), and occurs at lower frequencies in populations of *P. viticola* (Sierotski et al. 2005). This second mutation was detected in *Pyricularia grisea* and *Pythium aphanidermatum* which are turfgrass pathogens (Barlett et al. 2002; Grasso et al. 2006), *Alternaria solani* (Sierotski et al. 2005), and *P. viticola.*

There are conflicting findings whether the QoI-resistant pathogens suffer fitness penalties or not. Genet et al. (2006) showed that QoI-resistant *P. viticola* reverted to full sensitivity after consecutive transfers to untreated plants, which suggests that the resistant phenotypes are less fit than sensitive individuals. Toffolati et al. (2007) observed that *P. viticola* that are QoI-resistant seem to be less competitive than sensitive strains. In contrast, Ma and Michailides (2005), Gisi et al. (2000), Gisi et al. (2002), and Sierotski et al. (2005), reported that the G143A mutation does not affect enzyme activity and does not compromise the fitness of a pathogen. Avila-Adame and Koller (2003) reported that QoI-resistant *Magnaporthe grisea* with the G143A mutation did not suffer any detectable reduction in fitness.

1.6. Cross resistance within DMIs and QoIs and multiple resistance between DMIs and QoIs

Cross resistance is defined as the selection by one compound in a fungicide group also selecting for resistance to another compound with similar mode of action, even without exposure to the second. Multiple resistance in fungicides occurs when a pathogen independently develops resistance to more than one chemical group of fungicides (EPPO 1988). Cross resistance within the group of QoI fungicides (Chin et al. 2001; Ishii et al. 2001; Kim et al. 2003) and DMIs (Gubler et al. 1996; Erickson and Wilcox 1997) has been documented. The existence of cross resistance and multiple resistance among fungicides has great bearing on the decision on what fungicides can be applied and whether fungicides can be rotated or mixed to control PM and DM. If cross resistance to DMI exists in a PM population within a given geographical area, then spray programs might need to exclude DMI fungicides. If multiple resistance to QoI and DMI exists in a PM population, then spray program might need to exclude QoI and DMI altogether and use other fungicides like boscalid and quinoxyfen.

Anecdotal reports from growers indicate that compromised control by some fungicides is not uncommon, but without information on sensitivity of the pathogen, it is difficult to ascertain whether reduced control is due to resistance, or to other factors such as poor application timing or poor coverage. Growers commonly rotate and mix fungicides, increasing the difficulty of determining the effectiveness of individual fungicides. The overall objective of this thesis was to determine the sensitivity of grape powdery and downy mildew populations from Virginia and nearby states to commonly used single-site fungicides; specifically to sterol demethylation fungicides (fenarimol, myclobutanil, tebuconazole, triadimefon, and triflumizole), quinone outside inhibitors (azoxystrobin), boscalid, and quinoxyfen for powdery mildew and quinone outside inhibitors (azoxystrobin) and mefenoxam for downy mildew. Some of the results obtained have been published (Baudoin et al. 2008). The sensitivity of *E. necator* to DMIs will be discussed in Chapter 2 and the sensitivity of *E. necator* to QoI, boscalid and quinoxyfen and of *P. viticola* to QoI and mefenoxam will be discussed in Chapter 3.

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CHAPTER 2

Sensitivity of *Erysiphe necator* to Demethylation Inhibitor (DMI) Fungicides

2.1. INTRODUCTION

The most important disease of grapes worldwide is powdery mildew (PM) (Pearson and Gadoury 1992) which is caused by the fungal ascomycete *Erysiphe necator* (formerly *Uncinula necator*). It affects almost all green tissues of a grapevine, including the leaves, petioles, stems, clusters (Pearson 1988), and buds (Rumbolz and Gubler 2005). Infection of PM in grapevines can reduce the quality of fruit, the winter hardiness of canes, and the quality of wines produced from infected berries, and result in lower yield (Pool et al. 1984). Since common cultivars are very susceptible to PM, it must be controlled by regular application of fungicides.

For several years, sulfur has been commonly used by growers to control PM (Ypema et al. 1997). However, sulfur has disadvantages, such as phytotoxicity at higher temperatures and the necessity of frequent applications to provide good control (Gubler et al. 1996). Thus, growers switched to new fungicides that came into the market because they provided excellent control at low rates and could be applied at longer intervals. However, some of these fungicides (e.g., quinone outside inhibitors and sterol demethylation inhibitors), have a moderate to high risk of resistance development. Thus, it is of utmost importance to detect the emergence of PM isolates that have reduced sensitivity to fungicides in order to determine what fungicides and rates of fungicides to use and how frequently they have to be applied.

One of the most important groups of fungicides used in contemporary agriculture, a group that is commonly used to control PM in the US, is the sterol demethylation inhibitors or DMIs. DMIs inhibit sterol C-14 α-demethylation of eburicol which is a precursor of ergosterol in fungi (Ma and Michailides 2005; Gisi et al. 2000). The first DMI introduced in the US market in 1982 was triadimefon (Ypema et al. 1997). In 1989,

two other DMI fungicides, namely myclobutanil and fenarimol, entered the market, and several years later, tebuconazole and triflumizole were registered.

The sensitivities of plant pathogen populations to DMIs are a continuous spectrum ranging from very sensitive to less sensitive (Köller and Scheinpflug 1987). Given this continuous sensitivity spectrum, an application of DMI to the population will provide selection pressure that will favor the more resistant genotypes. It is expected that continued application of DMI will shift the distribution of sensitivities to the less sensitive end of the sensitivity spectrum and thus practical resistance may occur. Practical resistance has been defined as labeled rates of the compound no longer provide commercially acceptable control of the disease in question (EPPO 1988), and failure of fungicide to control the disease is correlated with increased frequency of resistant strains (Köller and Scheinpflug 1987).

The DMIs have been a very important tool in PM management. However, a serious problem emerged when resistance of PM to triadimefon was first suspected and later confirmed in California after a few years of use in grape vineyards (Gubler et al. 1996). Studies were conducted on DMI sensitivity on PM isolates collected from California (Gubler et al. 1996; Ypema et al. 1997), New York (Erickson and Wilcox 1997; Wong and Wilcox 2002), and Ontario (Northover and Homeyer 2002) vineyards. In all three locations, there were significant shifts in the EC50 of the DMI-exposed PM population relative to the DMI-unexposed (sensitive) PM population, confirming the existence of DMI resistance.

The EC50 of New York and California PM isolates collected from vineyards with and without previous exposure to DMIs was highest for triadimefon, intermediate for myclobutanil, and lowest for fenarimol (Gubler et al; 1996; Erickson and Wilcox 1997). The cause of the difference in the sensitivity of these isolates to the three DMIs is unknown (Ypema et al 1997). It has been documented however, that cross-resistance between DMIs exists in DMI-resistant PM (Erickson and Wilcox 1997; Ypema et al. 1997). Cross resistance is defined as the selection by one compound also selecting for resistance to another compound, even without exposure to the second (EPPO 1988).

Three mechanisms conferring DMI resistance in fungal pathogens and other organisms have been described namely: 1) point mutation in the DMI target gene, *CYP51*

of *E. necator* (Délye et al. 1997), *Blumeria* (*Erysiphe*) *graminis* f. sp. *hordei* (Délye et al. 1998), *Penicillum italicum* (Joseph-Horne and Hollomon 1997); *Ustilago maydis* (Butters et al. 2000); *Tapesia acuformis* (Albertini et al. 2003); and *Mycosphaerella graminicola* (Fraaije et al. 2007); 2) overexpression of *CYP51* (Marichal et al. 1997; Hamamoto et al. 2000; Schnabel and Jones 2001); and overexpression of the ABC transporter encoding gene (Hayashi et al. 2002; Zwiers et al. 2002).

The objective of this study was to determine the sensitivity of grape PM isolates from Virginia and nearby states to sterol demethylation inhibitors.

2.2. MATERIALS AND METHODS

The methods for maintaining PM isolates and performing bioassays were adapted from those described by Gubler et al. (1996), Erickson and Wilcox (1997), Ypema et al. (1997), Miller and Gubler (2004), and Wong and Wilcox (2000 and 2002).

2.2.1. Leaf material and surface disinfestation

Leaf material for isolation, maintenance, and bioassays was obtained from potted Chardonnay plants grown in a greenhouse with temperatures of 35-37^{o}C to prevent the development of PM, and long day lengths (>14 hours) to maintain leaf production through the winter. Young, not yet fully expanded, still shiny leaves were collected from the tips of the most rapidly growing shoots and used for PM.

Leaves were surface-disinfested in 10% bleach with 0.1% Tween 20 for 2 min with regular agitation to ensure uniform coverage, rinsed three times in sterile distilled water, blotted dry between paper towels, and cut into sizes to fit into a 60-mm petri plate with approximately 7 ml of 1.5% water agar (Bacto agar, Difco). Leaves for PM cultures were placed on the agar with the adaxial side facing up.

2.2.2. Collection and maintenance of *E. necator* isolates

Grape leaves infected with PM, were collected between 2005 and 2007 from vineyards in Virginia, Maryland, North Carolina, and Pennsylvania (Figure 2.1) by either researchers or grape growers. An attempt was made to ensure representation of different geographic regions. Infected grape leaves were collected from vineyards, as much as possible from separate locations, and leaves from each location were placed separately in Ziploc bags which were mailed (if collected by growers) or placed in a cooler and transported (if collected by the researchers) immediately to the laboratory.

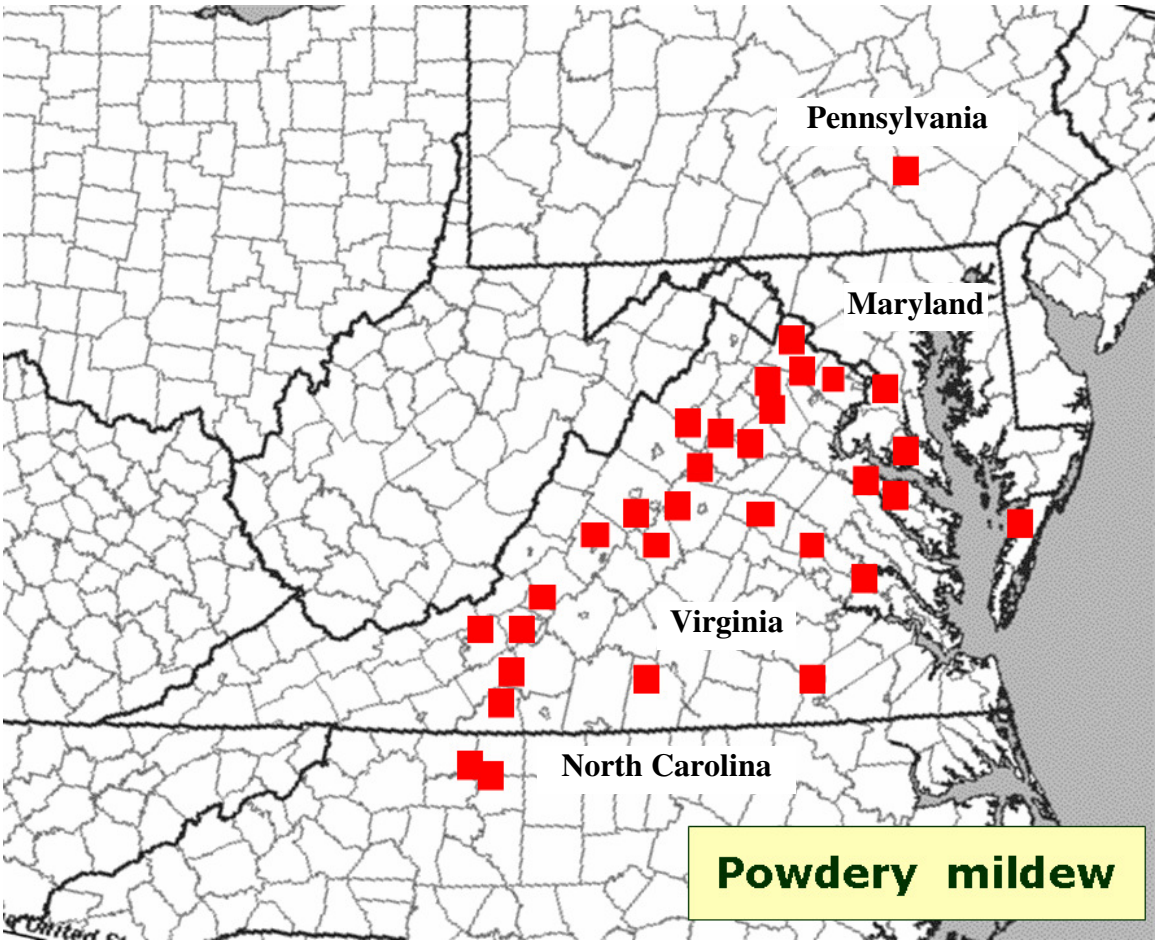

Figure 2.1. Map of Virginia, Maryland, North Carolina, and Pennsylvania showing the locations where PM isolates were collected.

Upon arrival in the laboratory, samples were immediately examined using a dissecting microscope with side illumination to determine the presence of sporulating *E. necator.* Leaves with PM infection that appeared to have viable PM conidia were rub-inoculated (initial transfer) onto the adaxial surface of grape leaves. The plates were placed in a transparent plastic box and incubated in alternating 12-hr light and 12-hr darkness at room temperature for 5-7 days.

After incubation, the grape leaves were observed under a dissecting microscope at 40X magnification to check for PM growth and sporulation. Individual PM isolates were obtained from the initial transfers. A single chain of conidia was picked up using a sterile camel hair attached to a 5 ¾" Pasteur pipette. The camel hair was sterilized by soaking in 70% ethanol for 10 min, dipped in 95% ethanol, shaken to remove excess ethanol, and air-dried for at least 5 minutes. The transfer of a single chain of conidia was repeated three consecutive times to ensure the purity of each isolate. Inoculated leaves were incubated as described above. Isolates were transferred after 10-14 days or as frequently as necessary. Sterile acupuncture needles were used in the succeeding transfers of the PM isolates because they were more convenient and easier to use than the camel hair.

2.2.3. Biological assay

2.2.3.1. Fungicides and fungicide concentrations

Stock solutions of five DMI fungicides (fenarimol, myclobutanil, tebuconazole, triadimefon, and triflumizole) were prepared by dissolving the appropriate amount of a technical grade fungicide into acetone and were stored in the freezer until use. Serial dilutions were prepared by diluting an aliquot of stock solution in sterile distilled water containing 0.005% Tween 20. Fungicide dilutions were prepared the day before use in the bioassay.

The PM isolates were tested against all five fungicides at concentrations of 0.3, 1, 3, and 10 µg/ml. As a basis for comparison, a fungicide-sensitive subgroup of ten isolates from two locations that were thought to have had no or very little exposure to single-site fungicides was selected. One location was a new vineyard in Halifax County, VA at least

10 miles from any existing vineyards; the other consisted of spontaneous powdery mildew infections on potted grape plants in Blacksburg, VA, also with no commercial vineyards nearby. The selected ten fungicide-sensitive PM isolates did not grow at concentrations of 0.3, 1, 3, and 10 µg/ml of any of the tested fungicides. Since they did not grow at 0.3 µg/ml of any of the fungicides, they were further tested with the same fungicides but at lower concentrations listed in Table 2.1. The median EC50 of the subgroup sensitive to the DMI fungicides was used as the "baseline EC50" to which the EC50 of all PM isolates were compared to determine if there were significant shifts in their EC50 distribution.

To determine the reproducibility of the bioassay, nine PM isolates (eight isolates were part of the sensitive subgroup of ten) were tested four to five times against all five DMI fungicides at the concentrations listed in Table 2.1.

Table 2.1 Fungicide and fungicide concentrations used to determine the EC50 of the sensitive PM subgroup (n=10) and the reproducibility (n=9) of the leaf bioassay.

Fungicides	Concentrations (µg/ml)
Fenarimol	0.001, 0.007, 0.04, 0.15
Myclobutanil	0.001, 0.007, 0.04, 0.15, 0.3
Tebuconazole	0.001, 0.007, 0.04, 0.15, 0.3
Triadimefon	0.001, 0.007, 0.04, 0.15, 0.3
Triflumizole	0.001, 0.007, 0.04, 0.15, 0.3

2.2.3.2. Preparation of leaf discs for biological assay

The leaves (the type of leaves used for PM were the same as described previously) were surface disinfested as described above and cut into 13-mm leaf discs using a sterile, #9 cork borer. Cut discs were mixed to randomize them and soaked in fungicide solutions for 1 hr with gentle and continuous shaking in an IKA® VIBRAX

VXR shaker at 200 rpm. They were then blotted dry with a paper towel. Six to ten discs were placed in each water agar plate. Leaf discs were placed in the petri dish with the adaxial surface facing up.

2.2.3.3. PM inoculation and evaluation

Inoculation for the PM sensitivity tests was accomplished using a settling tower (to provide a uniform spore deposit on the leaf discs), after the design of Reifschneider and Boiteux (1988). Before inoculation, the tower was disinfected by spraying the inside with 95% ethanol, thoroughly wiped with paper towels, and then allowed to dry for 10 min. Leaves infected with 14-21-days old PM cultures were used as inoculum. Some of the plates containing the PM inoculum had contaminating fungi growing on the agar and on deteriorating parts of the leaves; therefore, the contaminated parts of the leaves were cut off and the non-contaminated parts were transferred to a clean petri dish before inoculation to prevent spreading the contaminants in the bioassay plates during inoculation. When the inoculation tower had dried, the PM inoculum was placed on the stage of the tower and the uncovered plates with fungicide-treated leaf discs were placed on the floor of the tower. Then air pressure in the tower was reduced by switching on the vacuum pump (a small, hand-held vacuum cleaner) and the cover above the stage was removed quickly to allow the spores to be distributed by the sudden rush of air into the tower. The cover was replaced, the air pump switched off, and the tower was left to stand for at least 7 min to allow the spores to completely settle down on the leaf discs. Then the inoculated plates and the plate with inoculum were covered and removed from the inoculation tower. The vacuum was turned on again for at least 30 seconds to remove remaining airborne spores from the tower, and the inside of the tower was sprayed with 95% ethanol, wiped, and allowed to dry before the inoculation with the next isolate. Inoculated plates were incubated in a transparent box in the conditions mentioned above. After 10 days, individual leaf discs were evaluated for disease incidence (disease incidence, DI, percentage of leaf discs with sporulating colonies) and disease severity (DS, the percent surface area of each leaf disc with PM growth). Evaluation was done

with the aid of a dissecting microscope at 40x magnification. Ratings of DS ranged from 0-100%.

The relative growth (RG) of the isolates was determined by calculating the mean of the ratings obtained as a percentage of the mean rating of the control. In order to estimate the EC50, the relative growth on the treated discs was regressed on ln-transformed fungicide concentration. Only the linear portions of the response were used to estimate the EC_{50} which was computed using Microsoft Excel and the formula (after Miller and Gubler 2004 but with /m rather than /-m):

$$EC_{50} = e^{([50-b]/m])}$$

from the regression equation:

$$y = m.1n\ (concentration) + b$$

2.2.3.4. Statistical analyses

The range, mean, median, standard deviation, and 95% confidence intervals of the EC50 values of the sensitive subgroup, as well as for all isolates, were calculated using JMP 7, a statistical analysis program (SAS Institute, Cary NC). The calculated EC50 values were log_{10}-transformed and used to develop the frequency distributions for the five DMI fungicides.

To determine the reliability of the bioassay, the mean, variance, coefficient of variation (CV), and 95% confidence intervals were calculated in Microsoft Excel using formulas in Table 2.2 (Wong and Wilcox 2002):

Table 2.2 Formulas used in the computation of mean, variance, coefficient of variation, and lower and upper confidence intervals.

Mean	$e^{\mu+\sigma 2/2}$
Variance	$e^{2(\mu+\sigma 2)} - e^{2\mu+\sigma 2}$
Coefficient of variation	$e^{2(\mu+\sigma 2)} - e^{2\mu+\sigma 2} / \mu+\sigma 2/2$
Confidence interval upper limit	$e^{\mu+\sigma 2/2+(\sigma H1-\alpha/\sqrt{n-1})}$
Confidence of interval lower limit	$e^{\mu+\sigma 2/2+(\sigma H\alpha/\sqrt{n-1})}$

We wanted to determine if there was a shift and calculate the magnitude of the shift (resistance factor) of the average EC50 of all PM isolates compared to the average EC50 of the sensitive subgroup. The frequency distributions of all PM isolates and the sensitive subgroup were not log normally distributed, but somewhat skewed. Thus, the Wilcoxon/Kruskal-Wallis non-parametric test was performed using JMP 7 to determine whether the frequency distributions of the entire PM isolates and the sensitive subgroup to the DMIs were significantly different.

2.3. RESULTS AND DISCUSSION

2.3.1. Reproducibility of the bioassay tests

The CV for the individual isolates ranged from 10% to 64% with a mean of 26%-37% for the different fungicides (Table 2.3), which shows only moderate variation in the repeated bioassays, indicating that the bioassay is reproducible. The computed CVs for the repeated PM assays for myclobutanil and triadimenol were similar to the values obtained by Erickson and Wilcox (1997) and Wong and Wilcox (2002).

Table 2.3. Reproducibility of the biosassays of *E. necator* (n=9) against fenarimol, myclobutanil, tebuconazole, triadimefon, and triflumizole.

	Fenarimol			Myclobutanil			Tebuconazole			Triadimefon			Triflumizole		
Isolate	Mean (µg/ml)	CV (%)	95% CI	Mean (µg/ml)	CV (%)	95% CI	Mean (µg/ml)	CV (%)	95% CI	Mean (µg/ml)	CV (%)	95% CI	Mean (µg/ml)	CV (%)	95% CI
BL-P1	0.005	12	0.005-0.007	0.011	12	0.010-0.014	0.020	38	0.013-0.096	0.014	32	0.010-0.035	0.016	31	0.011-0.041
BL-P4	0.013	50	0.010-0.052	0.023	42	0.018-0.050	0.031	27	0.025-0.048	0.020	24	0.017-0.030	0.025	37	0.021-0.043
MV-P1	0.014	26	0.013-0.020	0.007	21	0.006-0.008	0.009	10	0.008-0.010	0.017	34	0.015-0.025	0.013	26	0.012-0.017
MV-P10	0.004	39	0.004-0.008	0.004	36	0.004-0.006	0.006	28	0.006-0.008	0.005	28	0.004-0.007	0.008	21	0.007-0.010
MV-P2	0.004	15	0.004-0.005	0.005	28	0.005-0.006	0.001	44	0.0005-0.001	0.028	32	0.026-0.040	0.018	25	0.017-0.021
MV-P5	0.007	26	0.007-0.009	0.006	25	0.006-0.008	0.009	13	0.008-0.009	0.019	57	0.018-0.033	0.007	19	0.006-0.008
MV-P8	0.012	40	0.011-0.017	0.001	16	0.0006-0.001	0.005	60	0.007-0.104	0.015	29	0.015-0.021	0.013	44	0.013-0.025
M-VP9	0.002	37	0.002-0.004	0.005	30	0.005-0.006	0.004	34	0.004-0.005	0.035	29	0.033-0.043	0.007	20	0.007-0.008
PB-P1	0.006	25	0.005-0.006	0.013	35	0.013-0.018	0.032	31	0.031-0.039	0.031	65	0.060-1.353	0.086	13	0.083-0.092
Mean (µg/ml)	0.008	30		0.008	27		0.013	32		0.020	37		0.021	26	

2.3.2. Powdery mildew sensitivity to the DMIs

The range and mean EC50 of the isolates in the sensitive subgroup are shown in Table 2.4. These data show that there are differences in the intrinsic activities among the DMIs, and that fenarimol and myclobutanil had greater activity than tebuconazole, triadimefon and triflumizole. The data confirm the observation by Erickson and Wilcox (1997), Gubler et al. (1996), and Ypema et al. (1997) that sensitive PM isolates without prior exposure to DMIs are more sensitive to myclobutanil and fenarimol than to triadimefon.

Table 2.4. The mean and median EC50 of all PM isolates and of the sensitive subgroup for the five DMI fungicides.

Fungicide	Mean EC50 of the sensitive subgroup (µg/ml)	Median EC50 of the sensitive subgroup (µg/ml)	Mean EC50 of all PM isolates (µg/ml)	Median EC50 of all PM isolates (µg/ml)
Fenarimol	0.008	0.006	0.85	0.32
Myclobutanil	0.009	0.006	4.20	2.10
Tebuconazole	0.014	0.010	7.03	3.60
Triadimefon	0.017	0.016	1.72	0.98
Triflumizole	0.014	0.015	4.73	1.18

The number of PM isolates tested against the DMI fungicides ranged from 143-147, except that only 110 isolates were tested against triflumizole. The number of PM isolates tested from each vineyard varied; some vineyards were represented by more than 10 isolates while a few vineyards had only one isolate.

The median EC50 values of our sensitive isolates for fenarimol, myclobutanil, and triadimefon were closer to the EC50 values obtained in New York than in California. The published median EC50 values of the New York powdery mildew population without prior exposure to DMIs were 0.03 µg/ml, 0.03 µg/ml, and 0.06 µg/ml for fenarimol, myclobutanil, and triadimefon, respectively (Erickson and Wilcox 1997). These values were 5 to 6 times higher than the EC50 values of our sensitive subgroup tested for fenarimol, myclobutanil, and triadimefon (Table 2.4). It is possible that part of these differences is due to differences in the methods used in the bioassay, in addition to the fact that the number of isolates in our sensitive subgroup was small. Erickson and Wilcox (1997) inoculated each individual leaf disc in its center with a conidium and measured the colony growth with an ocular micrometer. In our tests, we inoculated all leaf discs in an inoculation tower which deposited PM spores more or less uniformly onto each of the leaf discs, and visually estimated percent coverage as described earlier. Although the EC50 values of the unexposed New York PM population and our sensitive subgroup were different, the following similarities were observed: 1) the fact that the EC50 values of the sensitive/unexposed PM isolates for myclobutanil and fenarimol were similar and 2) the EC50 values for triadimefon were higher (three-fold in NY and two-fold in our sensitive subgroup) than for myclobutanil and fenarimol. Tebuconazole and triflumizole data were not included in these earlier studies because they did not become registered for use on grapes until later.

In California (Gubler et al. 1996), the baseline mean EC50s of unexposed PM populations bioassayed were 1.40 µg/ml, 0.15 µg/ml, and 0.13 µg/ml for triadimefon, myclobutanil, and fenarimol, respectively. These values were 82-fold, 17-fold, and 16-fold higher than the mean EC50 of our sensitive subgroup for triadimefon, myclobutanil, and fenarimol, respectively. The reason for the large difference in the EC50 may relate to differences in the bioassay technique used. Gubler et al. (1996) used heterogeneous *E. necator* subcultures in their bioassay to increase the probability of detecting isolates with

the highest level of resistance. Ypema et al. (1997) compared the mean EC50 of heterogeneous subcultures and monoconidial isolates and observed a statistically significant difference in the mean EC50 for fenarimol but not for triadimefon and myclobutanil. We used single-conidial *E. necator* isolates in our bioassays, as did Erickson and Wilcox (1997). Alternatively or additionally, it is possible that the high EC50 of the PM population in California is really a reflection of their greater tolerance to triadimefon, myclobutanil, and fenarimol at the time of sampling compared to the EC50 of our entire PM collection.

The median EC50s of the DMI-exposed New York PM population for fenarimol and myclobutanil were 0.07 µg/ml and 0.23 µg/ml, respectively (Erickson and Wilcox 1997). The median EC50 (Table 2.4) of our entire PM collection was 5- and 9-fold higher than the EC50 of the DMI-exposed NY PM isolates for fenarimol and myclobutanil, respectively, even though our entire PM collection described included isolates from non-exposed vineyards. Interestingly, the median EC50 of our entire PM collection for triadimefon was 2-fold lower than the median EC50 of 1.9 µg/ml for triadimenol of the exposed New York population (Erickson and Wilcox 1997). Because the differences in the EC50 were not that great, it is possible that they are only due to the differences of the bioassay test used as described earlier. However, it is also possible that these differences are actual differences between the PM isolates from New York and our PM collection. The calculated EC50 for New York PM isolates were obtained 10 years ago. It is probable that those 10 years provided more time for a higher level of resistance to develop, especially for fungicides that have been commonly used.

DMI-exposed PM populations from different regions in California differed in sensitivity, and the highest mean EC50 (16.48 µg/ml) for triadimefon was from the PM population collected from the Central Coast (Gubler et al. 1996). This value is 10-fold higher than the mean EC50 of 1.72 µg/ml of our entire PM collection for triadimefon. However, the mean EC50 values of our entire PM collection were 1.5-fold higher for myclobutanil and 2-fold higher for fenarimol than the highest (from the Central Coast of California) mean EC50 values of 2.83 µg/ml and 0.44 µg/ml for myclobutanil and fenarimol, respectively (Gubler et al. 1996). The lower EC50 values of our entire PM collection to triadimefon were unanticipated because previous reports from California

and New York suggested that it had the greatest resistance problem among the DMIs. Probably in part because of reports of triadimefon resistance, triadimefon has not been used that much recently to control grape powdery mildew, thereby eliminating the pressure for selecting triadimefon-resistant PM isolates which might explain the low EC50 values obtained. We suspect that triadimefon resistance may have a fitness penalty causing triadimefon-resistant PM isolates to be at a selective disadvantage without triadimefon. However, Miller and Gubler (2004) reported that triadimefon resistance in California in 2002 was still high, even after many years of little use.

The mean and median EC50 values of the entire PM collection for all five DMI fungicides were higher than those of the sensitive subgroup (Table 2.4 and Figure 2.2). Wilcoxon/Kruskal Wallis non-parametric tests show that the median EC50 of our entire PM isolates to the five DMIs were significantly higher than the sensitive subgroup ($P>0.0001$). Tebuconazole and myclobutanil had a greater median shift (resistance factor) than the other three fungicides (Table 2.5). Data from the National Agricultural Statistics Service (NASS 1992-2006) for New York, Pennsylvania, and Michigan suggest that the use of triadimefon in the eastern United States has greatly declined since the mid 1990s; that triflumizole has probably not been used much in the eastern US; and that fenarimol may have seen moderate sustained use over the years. DMIs were more important for PM control in the 1980s and 90s, but since the late 1990's, some of the load has been carried by the QoI fungicides, especially perhaps in the eastern US because QoIs also provide control of other important diseases. However, with the advent of QoI resistance, some shift back to DMIs may be expected.

It is unclear to what extent this shift in the EC50 of PM isolates translates into practical resistance in the field. Erickson and Wilcox (1997) observed that a 30-fold difference in the median EC50 for triadimenol between the exposed and unexposed PM population in New York resulted in less than 50% control of PM, while the exposed PM population with 2-fold and 8-fold increase in the median EC50 for fenarimol and myclobutanil, respectively was still controlled by labeled rates of both fungicides. The resistance factors that we have obtained for fenarimol, myclobutanil, and triadimefon were 53, 350, and 61 respectively (Table 2.5). However, the median EC50 for fenarimol, myclobutanil, and triadimefon of our sensitive subgroup that was used to calculate the

resistance factor of all PM isolates was 5-fold lower for myclobutanil and fenarimol and 6-fold lower for triadimefon than the median EC50 of the unexposed (sensitive) New York PM isolates; this may be due in part to the limited number of isolates included (n=10). Based on these considerations, we expect that myclobutanil and tebuconazole will provide poor PM control in vineyards with shifted populations, while fenarimol and triadimefon may still provide moderate or good PM control in many locations.

Table 2.5. Comparison of median EC50, resistance factors of the entire *E. necator* collection and relation to maximum label rates for five DMI fungicides.

	Fenarimol	Myclobutanil	Tebuconazole	Triadimefon	Triflumizole
Median EC50 (µg/ml) (see also Fig 2.2)	0.32	2.10	3.60	0.98	1.18
Median resistance factor	**53**	**350**	**360**	**61**	**79**
Maximum label rate[1]	56	150	135	225	300
Median EC50 as % of maximum label rate	**0.57**	**1.40**	**2.67**	**0.44**	**0.39**

[1]as a concentration, based on a spray volume of 945 liters/ha

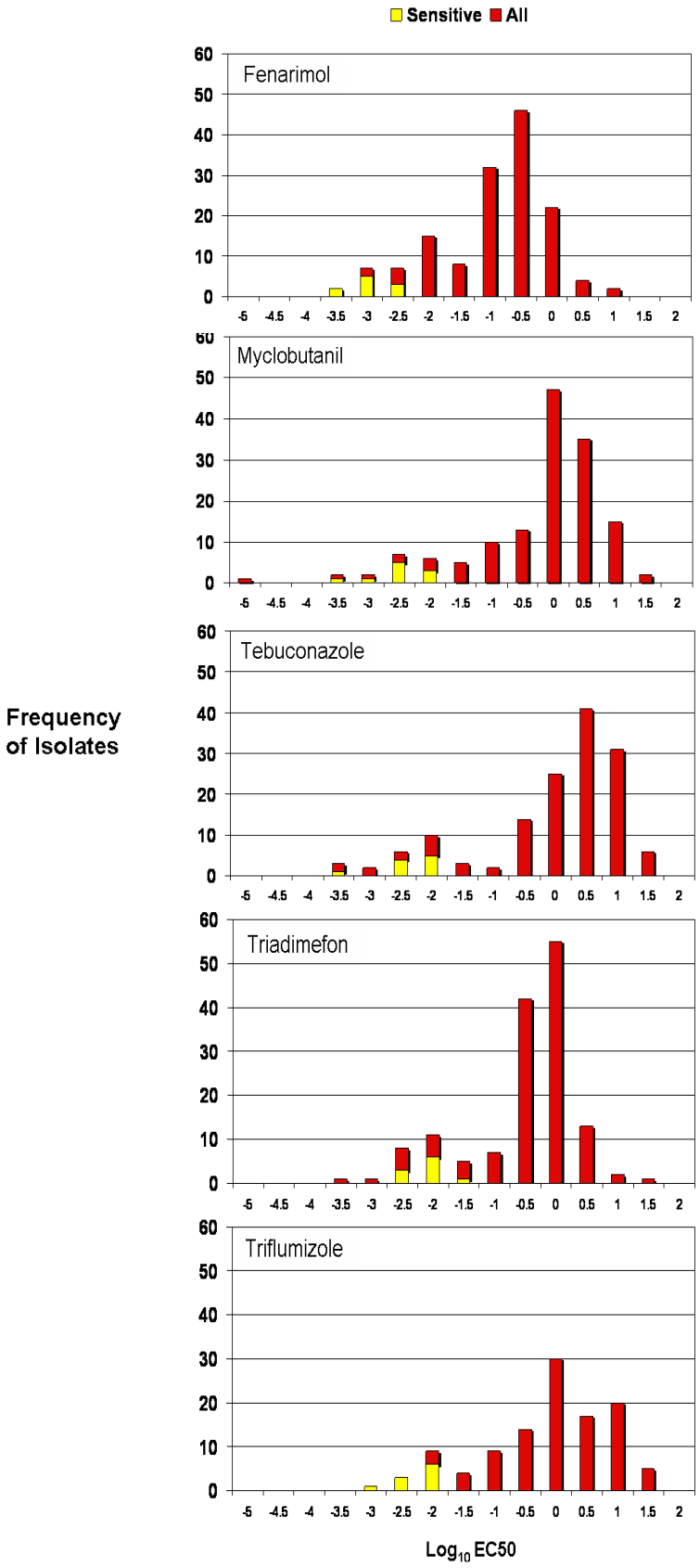

Figure 2.2. Frequency distribution of the $\log_{10}$ EC50 of the sensitive subgroup and all Mid-Atlantic US PM isolates of *E. necator* to fenarimol, myclobutanil, tebuconazole, triadimefon, and triflumizole. (Yellow bars represent a sensitive subgroup, namely isolates from two sites that appear to have had very little or no exposure to single-site fungicides; red bars represent counts for all PM isolates).

Tebuconazole had the highest median EC50 followed by, in decreasing order: myclobutanil, triflumizole, triadimefon, and fenarimol (Table 2.5). When comparing the mean EC50 values (Table 2.5) of these five DMI fungicides, one should take into account that they have different intrinsic activities, which is expected to be reflected in their maximum label rates. Thus, the EC50 was expressed as percent of the maximum label rate (Table 2.5). Although the average of our isolates was not highly sensitive to triflumizole, when expressed in relation to the maximum label rate or as a resistance factor, the resistance shift for triflumizole was similar to that for fenarimol and triadimefon, and less than for tebuconazole and myclobutanil. The results suggest that among the five DMI fungicides, triflumizole, fenarimol, and triadimefon might be expected to provide the best control of grape powdery mildew. However, the use of triadimefon on grapes has recently been discontinued.

Except for two fenarimol-resistant PM isolates that had an EC50 greater than 10 µg/ml (tested only once), all fenarimol-resistant PM isolates had modest reductions of sensitivity to fenarimol. This observed modest shift in the EC50 of fenarimol-resistant PM isolates concurs with the results obtained by Ypema et al (1997) and Erickson and Wilcox (1997). Since the shift is modest considering that fenarimol has been used against PM for almost 20 years now (but in moderate sustained use), it appears that grape PM may have difficulty developing higher level of resistance to it for unknown reasons or that fenarimol resistance may have a fitness penalty.

The results of laboratory bioassays cannot be easily translated into predicted efficacy of individual fungicides in the field. More information is needed on the practical level of PM control that DMI fungicides can still provide in vineyards with reduced sensitivity. The bioassay tests to determine DMI sensitivity of PM are labor-intensive and impractical for growers. It may be possible to develop a practical way of testing PM sensitivity to DMIs by spraying potted grape plants with various rates of DMI fungicides, placing them near vineyards, and evaluating them for PM growth (Baudoin pers. comm.).

2.3.3. EC50 Correlations

Table 2.6 shows the r-values summarizing the correlations of the $\log_{10}$ EC50 of 86 *E. necator* isolates for azoxystrobin (results presented in next chapter) and the five DMI fungicides. Based on the pairwise analysis there were high ($r > 0.70$) and statistically significant correlations in the $\log_{10}$ EC50 for all fungicide pairs, except for the moderate correlation between triadimefon and azoxystrobin (r=0.69), triflumizole and azoxystrobin (r=0.66), fenarimol and triflumizole (r=0.63), and fenarimol and azoxystrobin (r=0.60). These results suggest that the sensitivities of the *E. necator* population to the DMI fungicides are related to each other, but also that the sensitivities between the DMIs and azoxystrobin are related.

Table 2.6. R-values summarizing the correlations of the $\log_{10}$ EC50 of 86 *E. necator* isolates for fenarimol, myclobutanil, tebuconazole, triadimefon, triflumizole and azoxystrobin.

	Fenarimol	Myclobutanil	Tebuconazole	Triadimefon	Triflumizole
Fenarimol					
Myclobutanil	0.7657				
Tebuconazole	0.7532	0.8845			
Triadimefon	0.8332	0.8521	0.8361		
Triflumizole	0.6256	0.8067	0.7378	0.7148	
Azoxystrobin	0.6022	0.7712	0.7347	0.6863	0.6581

Since there is a relationship in the sensitivities of *E. necator* to different DMI fungicides, it is expected that an isolate with reduced sensitivity to one DMI fungicide would also have a reduced sensitivity to other DMI fungicides, a phenomenon known as cross-resistance (EPPO 1988). In this context, cross resistance is defined as the selection by one compound also selecting for resistance to another compound, even without exposure to the second. Cross resistance between DMI fungicides is expected because fungicides within the group have similar modes of action.

Point mutations in *CYP51* in DMI-resistant *B. graminis* f.sp. *hordei,* (Délye et al. 1998), *P. italicum* (Joseph-Horne and Hollomon 1997), *U. maydis* (Butters et al. 2000),

T. acuformis (Albertini et al. 2003), and *M. graminicola* (Fraaije et al. 2007) are responsible for DMI resistance.

Délye et al. (1998) identified a mutation Y136F (single point mutation on position 136 leading to the substitution of phenylalanine for tyrosine) in *CYP51* of triadimenol-resistant *E. necator* with resistance factor higher than 5. A variety of point mutations have been described in a number of pathogens (Délye et al. 1998; Joseph-Horne and Hollomon 1997; Butters et al. 2000; Albertini et al. 2003; Fraaije et al. 2007). We speculate that there may be two types of point mutations conferring DMI-resistance, namely fungicide-specific and group-specific mutations. Fungicide-specific mutations are those that confer resistance to one or several but not all DMI fungicides, whereas group-specific mutations confer resistance to all DMI fungicides. Some of our isolates had high resistance to one DMI fungicide but not to other DMIs. This partial cross resistance between the DMIs could be explained by a mixture of fungicide-specific and group-specific mutations in the population. An example of a fungicide-specific mutation is the I381V DMI resistance mutation described by Fraaije et al. (2007) in *M. graminicola*, which was positively selected by tebuconazole and difenoconazole treatment but appeared to be negatively selected by prochloraz.

A correlation in the sensitivities between DMI and azoxystrobin (two unrelated fungicides) was also observed by Wong and Wilcox (2002) and they referred to it as cross sensitivity. We observed this phenomenon with a much wider range of azoxystrobin sensitivities than was present in Wong and Wilcox's isolates. These authors discussed several mechanisms to explain cross sensitivity or pleiotropic resistance. The first one is an efflux mechanism, which would prevent or reduce the accumulation of several different fungicides in a pathogen (Wong and Wilcox 2002). Another explanation is that if a pathogen develops resistance to a specific fungicide, it predisposes it (through an unknown mechanism) to develop resistance to another fungicide that has a different mode of action. With respect to our results, since both QoI and DMI fungicides have been widely used for a number of years now, another likely explanation for the cross sensitivity of QoI and DMI would be correlated selection pressure. This means that growers inclined to use one group of fungicides (e.g. QoI) intensively also tend to be heavy users of other groups (e.g. DMI); selection pressure may be correlated even if QoI-

and DMI-resistance in the PM population develop independently.

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Chapter 3

Sensitivity of *Erysiphe necator* to QoI fungicides, boscalid, and quinoxyfen, and of *Plasmopara viticola* to QoI fungicides and mefenoxam

3.1. INTRODUCTION

Powdery mildew (PM) and downy mildew (DM), caused by *E. necator* and *P. viticola,* respectively, are the two most important fungal diseases of grapes (Wong and Wilcox 2000; Wong and Wilcox 2001; Northover and Homeyer 2001; Wong and Wilcox 2002). Both diseases may cause great damage if left uncontrolled. Since the most valuable grape cultivars are highly susceptible to these diseases, the primary control measure used to control these diseases is the use of chemicals.

Most of the older chemicals used for grape disease control, such as sulfur, copper, and mancozeb, have a multi-site mode of action. Many of the chemicals that have been introduced since the 1960s have single-site modes of action, and with these kinds of fungicides, resistance development by the pathogen can turn into a serious problem (Brent and Hollomon 2007).

The objectives of this study were to: 1) determine the sensitivity of grape PM from Virginia and nearby states to azoxystrobin, quinoxyfen, and boscalid and 2) determine the sensitivity of grape DM populations from Virginia and nearby states to azoxystrobin and mefenoxam. A portion of the results of this study has been published (Baudoin et al. 2008).

3.1.1. Quinone outside inhibitors (QoI)

The quinone outside inhibitor (QoI) fungicides are effective in the control of both PM and DM. The QoIs are unique in being the first site-specific crop protection compounds that are effective against ascomycetes, basidiomycetes, and oomycetes (Wong and Wilcox 2002). QoIs were introduced into the market in 1996 (Gisi et al. 2002; Ma and Michailides 2005). In 1997, azoxystrobin was introduced in the US grape market,

followed by kresoxim-methyl in 2000, trifloxystrobin in 2001, and pyraclostrobin in 2003. Famoxadone, which has activity only against downy mildew, was registered for use on US grapes in 2008.

The target of the QoIs is the Qo site of the cytochrome *bc1* enzyme complex (Genet et al. 2006; Gisi et al. 2002). Once the QoIs bind to the Qo site, the electron transfer between cytochrome *b* and cytochrome c_1 is inhibited (Ma and Michailides 2005; Gisi et al. 2000; Gisi et al. 2002; Grasso et al. 2006). This results in the disruption of adenosine triphosphate (ATP) production resulting in energy deficiency in the fungus.

The majority of QoI fungicides, but not famoxadone, belong to the subgroup known as strobilurins, which are an agronomically important class of fungicides (Bartlett et al. 2002). They are used in a wide variety of crops, such as cereals, turfgrass, grapevines, potatoes, fruits, and vegetables. Soon after their introduction, the strobilurins became one of the most widely used groups of fungicides. The success story of strobilurins as fungicides is due to their characteristics, which are broad-spectrum activity, control of fungal isolates resistant to other fungicide, low use-rates, and excellent yield and quality benefits (Toffolatti et al. 2007). They have been used as foliar fungicides, for seed treatment, as well as in-furrow treatments for soil-borne diseases. Since they disrupt ATP/energy production in phytopathogenic fungi, they greatly affect the fungal stages that require large amounts of energy. These stages are spore germination and zoospore motility (Bartlett et al. 2002). They have also been observed to inhibit the formation of mature cleistothecia in *E. necator* and formation of mature oospores of *P. viticola* and to reduce their viability (Bartlett et al. 2002).

3.1.2. QoI resistance

The intensive use of QoIs increases the selection pressure that favors and selects resistant strains in plant a pathogen population. Not long after the introduction of QoIs in the market, several QoI-resistant plant pathogens were detected in several crops in different countries (Gisi et al. 2000). Plant pathogens that developed resistance include *Blumeria (Erysiphe) graminis* f. sp. *tritici* on wheat in Germany, France, and UK, *Sphaerotheca fuliginea* and *Pseudoperonospora cubensis* on cucumber in Spain and

Japan, *P. viticola* on grape in Italy, and *Mycosphaerella fijiensis* on banana in Costa Rica (Gisi et al. 2002). Resistant individuals of *V. inaequalis* on apple were also detected in experimental fields in Switzerland and Northern Germany. However, other pathogens like *Mycosphaerella graminicola* on wheat, and *Pyrenophora teres* on barley (Gisi et al. 2000), *S. tritici*, *Rhynchosporium secalis,* and *Helminthosporium teres* (Bartlett et al. 2002) remained sensitive. A complete and updated (December 2006) list of plant pathogens that developed resistance to fungicides can be accessed in the FRAC website (FRAC 2006).

There are at least two groups of mechanisms that have been discovered to cause QoI resistance in vitro. These are mutations of the target site in mitochondrial cytochrome bc_1 and the induction of alternative respiration.

Resistance to QoI is mostly based on point mutations in two "hot spot" regions of cyt *b* gene (Ma and Michailides 2005; Gisi et al. 2000; Gisi et al. 2002; Sierotski et al. 2005). Two point mutations have been identified to cause single-amino acid changes in the protein that confer fungicide resistance: G143A, which changes glycine amino acid to alanine, and F129L, which changes phenylalanine to leucine. G143A is the major mutation because it causes a higher level of resistance (Ma and Michailides 2005; Gisi et al. 2000; Gisi et al. 2002; Sierotski et al. 2005) than F129L. The G143A amino acid change is the cause of the fungicide resistance in several pathogens including *P. viticola, B. graminis* f sp. *tritici, P. cubensis, M. fijiensis, S. fuliginea, P. grisea*, and *Didymella bryoniae* (Bartlett et al. 2002; Sirven and Beffa 2003). The F129L mutation confers a lower resistance level than G143A (Bartlett et al. 2002; Sirven and Beffa 2003), often occurs at lower frequencies in a sample when both mutations are present (Sierotski et al. 2005), and may reduce fitness (Toffolatti et al. 2007). This second mutation was detected in *P. grisea* and *Pythium aphanidermatum* which are turfgrass pathogens (Bartlett et al. 2002; Grasso et al. 2006), *Alternaria solani* (Sierotski et al. 2005), and *P. viticola.*

The fitness cost of the G143A mutation is unclear. A fitness cost was not observed in isolates of *B. graminis* f. sp. *tritici* (Gisi et al. 2002) and *Magnaporthe grisea* (Avila-Adame and Köller 2003) that contained the G143A mutation. However, it is not known if this is true also for other pathogens that contain G143A (Gisi et al. 2002). In contrast, Genet et al. (2006) showed that QoI-resistant *P. viticola* reverted to full

sensitivity after consecutive transfers to untreated plants. Toffolatti et al. (2007) observed that QoI-resistant *P. viticola* seem to be less competitive than the sensitive isolates. Sierotski et al. (2005) reported that the mutation in *P. viticola* declined in test plots in Brazil after QoI use was stopped. This finding indicates that the resistant phenotypes of this particular pathogen are less fit than sensitive individuals.

It is possible that G143A can reduce the fitness in some pathogens (e.g. *P. viticola*) and may not affect the fitness of other pathogens (such as *M. grisea* and *B. graminis* f. sp. *tritici*). Strobilurin-producing fungi, such as *Strobilurus tenacellus, Mycena galopoda,* and *M. viridimarginata*, are believed to be protected from the strobilurin metabolite they produce by five significant amino acid substitutions in their cyt *b* gene (Gisi et al. 2002). These substitutions are: T127I (threonine changes to isoleucine), A153S (alanine changes to serine), S255Q (serine changes to glutamine), N262D (asparagine changes to aspartic acid), and G143A (glycine changes to alanine). Interestingly, the main point mutation developed by phytopathogenic fungi that makes them fungicide resistant is G143A, which is one of the point mutations possessed by the strobilurin producing fungi.

As QoIs are more widely used in many crop/pathogen systems, new mechanisms conferring QoI resistance may develop. For instance the activation of an alternative oxidase (AOX) in *M. graminicola* caused it to be less sensitive to QoI fungicides (Ma and Michailides 2005).

3.1.3. Mefenoxam and Metalaxyl

Metalaxyl (a mixture of two stereoisomers) or mefenoxam (the active stereoisomer) is a fungicide that belongs to the class phenylamides (FRAC 2005; Gisi and Cohen 1996) and has been available in the US grape market since around 1990. It is effective against plant pathogenic oomycetes such as *P. viticola*. It inhibits the ribosomal RNA (rRNA) polymerase of the target pathogen. It is categorized by FRAC (2007) as at high risk of resistance development. Even before the use of metalaxyl in the field, which started in 1977, metalaxyl-resistant *P. viticola* already existed at very low proportions within the wild-type populations (FRAC 2005). Metalaxyl-resistant *P. viticola* isolates

were detected as early as 1983 in France (Leroux and Clerjeau 1985; Gisi and Cohen 1996) and just recently in 2005 in Australia (Wicks et al. 2005). To date there is no report of mefenoxam-resistant *P. viticola* isolates on grapes in the US.

The mechanism conferring resistance to mefenoxam is still unknown (FRAC 2007; Gisi et al. 2000). However, it is suspected that resistance may involve one or two major genes and several minor genes (FRAC 2005). Resistance development to mefenoxam is affected by several factors, such as selection pressure brought about by the use of the fungicide, inheritance of resistance, and fitness and migration of resistant isolates.

3.1.4. Boscalid

Boscalid and quinoxyfen are two fungicides that have been available in the US market to control grape PM since 2003. Boscalid has a broad spectrum of activity (Avenot et al. 2008) and targets the enzyme succinate dehydrogenase (SDH) in the mitochondrial electron transport chain (FRAC 2007; Stammler et al. 2007). It is categorized by FRAC (2007) as medium risk in resistance development. Resistance to boscalid has been detected in *Alternaria alternata* in pistachios (Avenot et al. 2008) and *D. bryoniae* (Stevenson et al., 2008). Several fungal species in the field, as well as laboratory mutants, have been known to contain mutations in the SDH gene conferring resistance to carboxamide, the group of fungicides to which boscalid belongs (FRAC 2007). There are four sub-units in the SDH. Organisms like *Ustilago hordei* (Ben-Yephet et al. 1975), *U. maydis* (Keon et al. 1991), *Aspergillus nidulans* (Gunatilleke et al. 1976), and *Mycosphaerella graminicola* (Skinner et al. 1998) were observed to have reduced sensitivity to a carboxamide fungicide called carboxin. *U. hordei, U. maydis*, and *A. nidulans* were reported to have mutations in the B- sub-unit of the SDH and *U. maydis* and *M. graminicola* had mutations in the C- sub-unit. A bacterium, *Paracoccus denitrificans* resistant to carboxin had mutations in the D- sub-unit of the SDH.

3.1.5. Quinoxyfen

Quinoxyfen belongs to the group of quinolines and its mode of action is still unknown (FRAC 2007; Hollomon et al. 1997). However, it has been proposed that its target is the G-proteins in early cell signaling (FRAC 2007). Quinoxyfen controls powdery mildew of grasses, *Blumeria graminis* f.sp. *hordei* by interfering with either/both germination and formation of appressoria (Wheeler et al. 2003). Like boscalid, quinoxyfen is categorized by FRAC as at medium risk of resistance development. Resistance to quinoxyfen is known to occur (FRAC 2007). Quinoxyfen-resistant *B. graminis* f.sp. *hordei* were generated in the laboratory when quinoxyfen-sensitive *B. graminis* f.sp. *hordei* were exposed to *N*-nitrosoguanidine which is a chemical mutagen (Hollomon et al. 1997). Quinoxyfen-resistant *B. graminis* f.sp. *hordei* isolates were also obtained in the field. It has been observed that these quinoxyfen-resistant isolates were defective and will only survive in the presence of quinoxyfen.

3.2. MATERIALS AND METHODS

The methods for the collection and maintenance and bioassay for the *E. necator* isolates were the same as described in detail in Chapter 2. Single-conidial-chain *E. necator* isolates were maintained on grape leaves placed in water agar plates. They were tested against azoxystrobin, boscalid, and quinoxyfen. The bioassay was done with six leaf discs per treatment, which were inoculated in a settling tower. The settling tower was used because it ensures uniform deposit of spores in the leaf discs.

3.2.1. Leaf materials and surface disinfestation

The youngest fully expanded leaves from growing shoots, which had just lost their shine, were used for DM. The grape leaves used for the maintenance and bioassay

of the DM isolates were collected from greenhouse grown Chardonnay plants, and were disinfested as described in Chapter 2.

3.2.2. Collection and maintenance of *P. viticola* isolates

The DM isolates were obtained from DM-infected grape leaves that were collected from Virginia, Maryland, North Carolina, and Pennsylvania vineyards between 2005 and 2007 by researchers or grape growers. DM-infected grape leaf samples were placed in Ziploc bags which were either mailed if collected by the growers or placed in a cooler and transported immediately to the laboratory if collected by the researchers. The leaf samples were examined microscopically in the laboratory for the presence of sporulating *P. viticola.* Sporangiophores of DM were picked up from grape leaf samples using sterile, fine-tipped tweezers (#5-A), placed (an initial transfer to obtain a clean culture of downy mildew free of fungal contaminants that were present in the leaf samples) into a drop of water on the abaxial surface of grape leaves and incubated overnight in darkness to allow infection. The following morning, the drops of water were removed by holding the plates upside down to tap off the water droplets and the leaves were allowed to dry in the laminar-flow hood. The plates were incubated as described previously.

After incubation, the grape leaves were observed under a dissecting microscope at 40X magnification to check for DM growth and sporulation. Individual single-spore DM isolates were obtained from the initial transfers. A single sporangiophore was picked up with tweezers and transferred to a new leaf as previously described. The single-sporangiophore isolation was repeated three consecutive times to ensure the purity of each isolate. Inoculated leaves were incubated as described above. Isolates were transferred every 10-14 days or as frequently as necessary. For future use, isolates grown in a grape leaf were placed in 2-ml cryotubes, dried overnight in the laminar-flow hood with the fan running, and the tubes stored in a -80 ^{o}C freezer.

3.2.3. Biological assay

3.2.3.1. Fungicides

Fungicide stock solutions and serial dilutions of all fungicides were prepared as described in Chapter 2. The majority of the PM isolates were tested against the fungicides and fungicide concentrations listed in Table 3.1

Table 3.1 Fungicides and fungicide concentrations (µg/ml) used to determine the EC50 of all PM isolates.

Fungicides	Concentration (µg/ml)
Azoxystrobin	0.3, 1.0, 3.0, 10.0, 30.0
Boscalid	0.3, 3.0
Quinoxyfen	0.3, 3.0

The sensitive subgroup that did not grow at 0.3 µg/ml azoxystrobin, boscalid, and quinoxyfen was tested again at lower concentrations 0.001, 0.007, 0.04, 0.15 µg/ml to determine their median EC50 and the repeatability of the bioassays for the three fungicides. In Chapter 2 the median EC50 of this subgroup to the DMI fungicides was used to compare the shift in the mean EC50 of the entire collection of *E. necator* isolates. In this chapter, the mean EC50 to azoxystrobin of this sensitive subgroup will be used to compare and determine the shift in the EC50 of all *E. necator* isolates to azoxystrobin.

The DM isolates were tested against azoxystrobin at 1, 10, and 20 µg/ml and mefenoxam at 1 and 10 µg/ml.

3.2.3.2. Preparation of leaf discs for biological assay

The preparation of the leaf discs for bioassay was the same as described in Chapter 2 except for the type of leaves (specific for DM as described earlier) used and the fungicide

and fungicide concentrations. Leaf discs for DM assays were placed in the petri dish with the abaxial surface exposed. The majority of the isolates were assayed at least twice. When the leaf discs were soaked in azoxystrobin at a concentration above 10 µg/ml (and to a lesser extent at lower concentrations), the edges, and often most or all of the disc, would turn necrotic within 24 hours. To get around this problem, we sprayed Riesling leaves that were still attached to the plant with 10 µg/ml and 30 µg/ml a.i. of formulated azoxystrobin (Abound), allowed them to dry, and collected the leaves and placed them in separate Ziploc bags. The leaves were not disinfested as described earlier but were cut into discs right away and placed into water agar plates. Riesling leaves not treated with fungicide were used as control.

3.2.3.3. DM inoculation and evaluation

Spore suspensions were prepared by placing a grape leaf piece infected with 7-10-days old culture in a tube with sterile distilled water, determining the sporangial concentration with a hemocytometer, and diluting to at least 2 $x10^4$ sporangia/ml. Using a sterile Pasteur pipette, approximately 10 µl of the spore suspension was placed on the center of each leaf disc. The inoculated discs were incubated in the same conditions described above for culture maintenance.

After 7-8 days of incubation, each leaf disc was evaluated for the presence or absence of disease (disease incidence, DI, percentage of leaf discs with sporulating colonies) and disease severity (DS, the percent surface area of each leaf disc with DM growth and the degree of sporulation). Both DI and DS were estimated visually with the aid of a dissecting microscope at 40x magnification. The following scale was used to score each leaf disc for DS.

0 = no disease, no growth of *P. viticola*
1 = 1-20% of the disc's leaf area has growth with no or very little sporulation
2 = 21-40% of the disc's leaf area has growth with limited sporulation
3 = 41-60% of the disc's leaf area has growth with moderate sporulation
4 = 61-80% of the disc's leaf area has growth with abundant sporulation

5 = 81-100% of the disc's leaf area has growth with very abundant sporulation

3.2.3.4. Statistical analyses

The statistical analyses for both *E. necator* and *P. viticola* bioassay were the same as described in Chapter 2.

3.2.4. Molecular Assay

The methods described by Sirven and Beffa (2003) and Baudoin et al. (2008) were adapted and modified to detect the presence of the G143A mutation in the cyt *b* gene.

3.2.4.1. DNA extraction

The DNA that was used as template for the real time polymerase chain reaction (PCR) was extracted from spore suspensions of PM and DM. The DM spore suspension was prepared by putting leaf pieces with abundant sporulation of DM in a 10-ml tube with 2 ml of sterile distilled water. Using a Pasteur pipette, water was flushed onto the leaf to wash off the spores. This was done repeatedly until no spores were left on the leaf upon checking under the dissecting microscope. The spore suspension was thoroughly mixed using a Vortex Jr. Mixer (Scientific Industries Inc.) and the leaf pieces removed from the tube. The concentration of the spores in the suspension was determined using a hemocytometer. The spore concentration used was between 2 and 4 x 10^4 per ml. Spore suspensions were transferred to 2-ml microcentrifuge tubes, which were either stored in the freezer or immediately used for DNA extraction. Spore suspensions were centrifuged at 10,000 rpm for 10 min, after which the supernatant was discarded to obtain the pellet of spores. PM spores were collected by scraping conidial masses off leaves with abundant PM sporulation using a stainless steel spatula, which was thoroughly cleaned and sterilized between isolates.

Two to three glass beads were added to the spore pellet and the tube was placed in liquid nitrogen for quick freezing. Then the spore pellet was bead-beaten using a mini-beadbeater-1 (BioSpec Products, Inc) machine for 30 sec at 2,500 rpm and then placed in liquid nitrogen again. This step was repeated three times. After the bead beating, 300 µl of RLT buffer (provided in the Qiagen Biosprint DNA Plant Kit) was added for the lysis of cells and the tube mixed vigorously using a Deluxe Vortex Mixer (Fisher Scientific). The tube was centrifuged for 5 min at 6,000 rpm. The DNA in the lysate was purified using the Qiagen BioSprint 15 platform by following manufacturer's protocol (Qiagen, Valencia, CA). The DNA extract was transferred into a fresh, sterile centrifuge tube and stored in the freezer until use.

3.2.4.2. SYBR-green real-time PCR

SYBR Green Quantitative PCR (Q-PCR) was used to determine the presence and quantify the percent of the G143A mutation in the cyt *b* gene. The primer sequences used to detect the mutation in the PM and DM mildew isolates were (Sirven and Beffa 2003; Baudoin et al. 2008):

Downy mildew:

Mutant Allele (resistant):	5'-GGACAAATGAGTTTTTGGGC-3'
Wild-type Allele:	5'-GGACAAATGAGTTTTTGGGG-3'
Common Reverse:	5'-ATTATCAACGGCGAATCCAC-3'

Powdery mildew:

Mutant Allele (resistant):	5'- TACGGGCAGATGAGCCTATGCGC -3'
Wild-type Allele:	5'- TACGGGCAGATGAGCCTATGCGG -3'
Common Reverse:	5'- ACCTACTTAAAGCTTTAGAAGTTTCC -3'

The components of each PCR reaction were: 5 µl of DNA extract (between 1.10^{-3} and 1.0^{-6} ng/µl), 2.5 µl each of forward and reverse primer, 2.5 µl of sterile distilled

water, 12.5 µl Bio-Rad 2X iQ™ SYBR® Green Supermix containing SYBR green, nucleotides, and enzyme

Sirven and Beffa (2003) and Baudoin et al. (2008) used different real-time PCR machines and SYBR green mastermixes. Primer-dimers were formed when the real-time PCR parameters used by Baudoin et al. (2008) and Sirven and Beffa (2003) were used in the PCR reactions that used Bio-Rad 2x iQ SYBR green supermix and performed in a Bio-Rad ICycler iQ machine. Therefore, the real-time reaction parameters were optimized for the Bio-Rad supermix and real-time machine, leading to the following modified parameters: 95° C for 4 min, 40 cycles of 95° C for 10 s, 58° C for 15 s and 72° C for 15 s. Data were collected during the 72° C step.

The %G143A in each isolate was calculated using the formula:

$$\%G143A = [1 / 2^{(\text{Mutant Ct} - \text{Wild type Ct})} + 1] \times 100\%$$

3.3. RESULTS AND DISCUSSION

3.3.1. Reproducibility of the bioassay tests

The reproducibility of the powdery mildew bioassay tests for azoxystrobin, boscalid, and quinoxyfen was determined by testing nine isolates, mostly from the sensitive subgroup, four to five times, as described in Chapter 2. The EC50 values of the repeated tests for azoxystrobin, boscalid, and quinoxyfen showed moderate variation (Table 3.2), with coefficients of variation (CV) ranging from 14% to 66% (Table 3.2). The CVs for azoxystrobin were similar to the values computed by Wong and Wilcox (2002), which ranged from 10%-29% with a mean of 31%. The 95% confidence interval ranged from 91%-176% of the mean EC50 for azoxystrobin, 94%-294% for boscalid, and 93%-146% to quinoxyfen.

Table 3.2. Reproducibility of the bioassays of *E. necator* for azoxystrobin, boscalid, and quinoxyfen.

	Azoxystrobin			Boscalid			Quinoxyfen		
Isolate	Mean (µg/ml)	Coefficient of Variation (%)	95% Confidence Interval	Mean (µg/ml)	Coefficient of Variation (%)	95% Confidence Interval	Mean (µg/ml)	Coefficient of Variation (%)	95% Confidence Interval
BL-P1	0.003	47	0.002-0.014	0.006	43	0.005-0.008	0.000	15	0.0003-0.002
BL-P4	0.049	14	0.045-0.058	0.003	34	0.0023-0.005	0.011	40	0.009-0.019
MV-P1	0.003	26	0.003-0.004	0.014	33	0.013-0.019	0.009	14	0.008-0.010
MV-P10	0.007	19	0.007-0.008	0.012	66	0.013-0.126	0.008	16	0.007-0.009
MV-P2	0.005	29	0.005-0.007	0.003	25	0.003-0.004	0.016	62	0.016-0.048
MV-P5	0.007	24	0.007-0.008	0.005	29	0.005-0.006	0.012	26	0.012-0.015
MV-P8	0.017	62	0.017-0.035	0.010	55	0.011-0.031	0.017	21	0.016-0.020
M-VP9	0.018	37	0.017-0.023	0.004	14	0.004-0.004	0.016	21	0.016-0.018
PB-P1	0.041	32	0.040-0.053	0.024	30	0.023-0.029	0.061	42	0.061-0.091
Mean (µg/ml)		32			37			29	

3.3.2. Powdery mildew sensitivity to azoxystrobin

The range of the EC50 of 10 isolates of the sensitive subgroup tested by bioassay against azoxystrobin was 0.0029 to 0.0497 µg/ml (mean = 0.014 µg/ml) (Table 3.3). Sensitive PM isolates from New York had similar EC50 values, which were 0.0037 µg/ml to 0.028 µg/ml (mean = 0.0097 µg/ml) (Wong and Wilcox 2002). Wong and Wilcox (2002) recommended 2 µg/ml as the discriminatory dose for azoxystrobin but in this study we used 1 µg/ml as the cut-off, categorizing isolates with EC50 < 1 µg/ml as sensitive and ≥ 1 µg/ml as resistant (the same criterion used by Baudoin et al. 2008).

Table 3.3. EC50 of the sensitive subgroup (n=10) and of our entire *E. necator* collection from Virginia and nearby states to azoxystrobin, boscalid, and quinoxyfen.

	Sensitive subgroup (µg/ml)		Entire PM collection (µg/ml)	
Fungicide	Median	Mean	Median	Mean
Azoxystrobin	0.007	0.014	10.8	13.0
Quinoxyfen	0.012	0.011	-	-
Boscalid	0.004	0.007	-	-

Figure 3.1 shows the locations from which the *E. necator* isolates were collected and their reaction to azoxystrobin. The frequency distribution of the log-EC50 values of 154 PM isolates was broad and continuous ranging from $\log_{10}$ -2.5 to $\log_{10}$ 2.0 (Figure 3.2). Since, the median EC50 of the sensitive subgroup was 0.007 (µg/ml) we believe that 1 (µg/ml) is a valid discriminatory dose to classify whether an isolate has a distinct degree of QoI resistance. Out of the 154 *E. necator* isolates, 28 (18%) were QoI-sensitive and 126 isolates (82%) were QoI-resistant. EC50 values ranging from 1-10 were classified as intermediate resistance while EC50 values greater than 10 were classified as high resistance. Seventy-nine of the QoI-resistant (63%) isolates were highly resistant and 47 isolates (37%) had intermediate resistance (Fig 3.2). Resistant isolates were collected from all geographic areas sampled (Figure 3.1) indicating that QoI resistance is widespread in Virginia and nearby states.

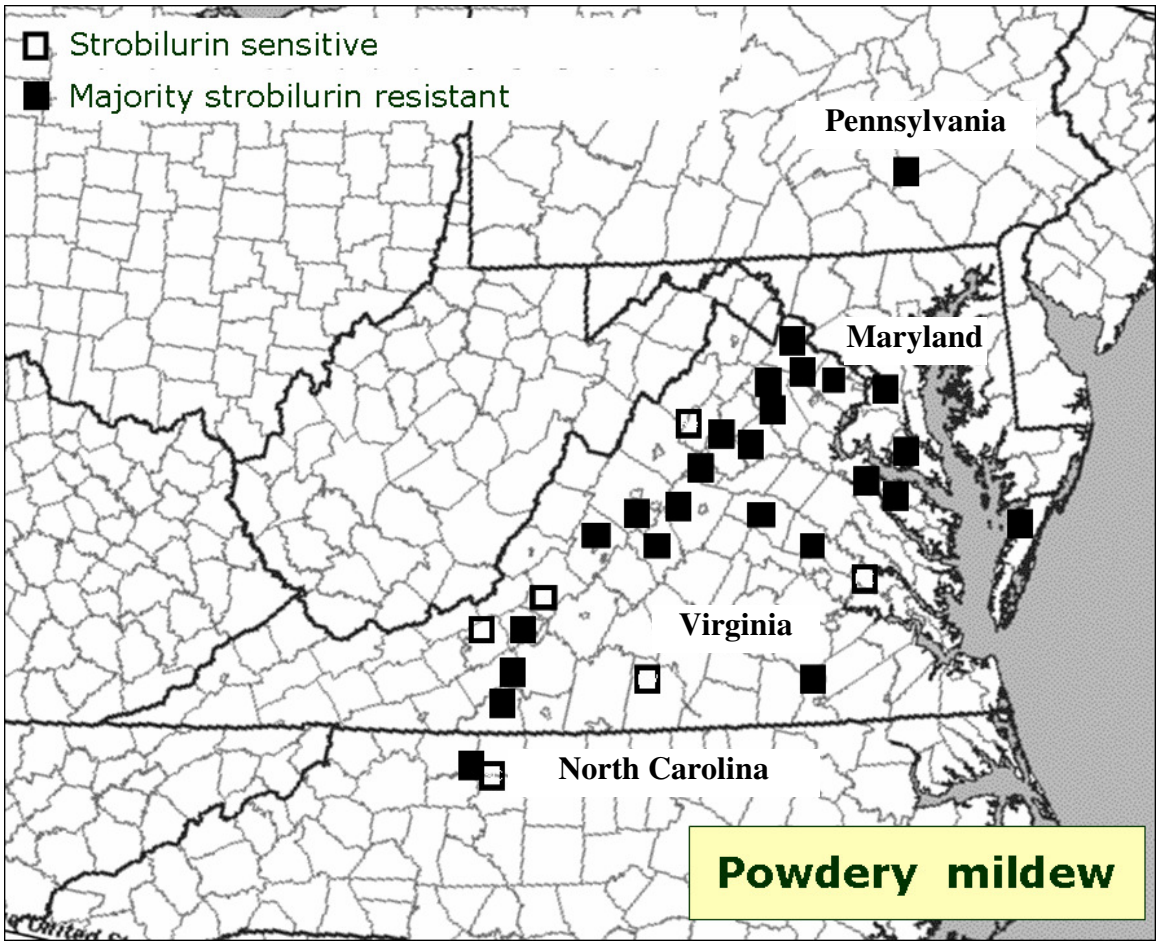

Figure 3.1. Map of Maryland, North Carolina, Pennsylvania, and Virginia showing the locations where PM isolates were collected and the predominant reaction of the isolates to azoxystrobin. Note that for some of the "sensitive" locations, only one or two isolates were available. QoI-sensitive isolates are represented by open squares. QoI-resistant isolates are represented by solid squares.

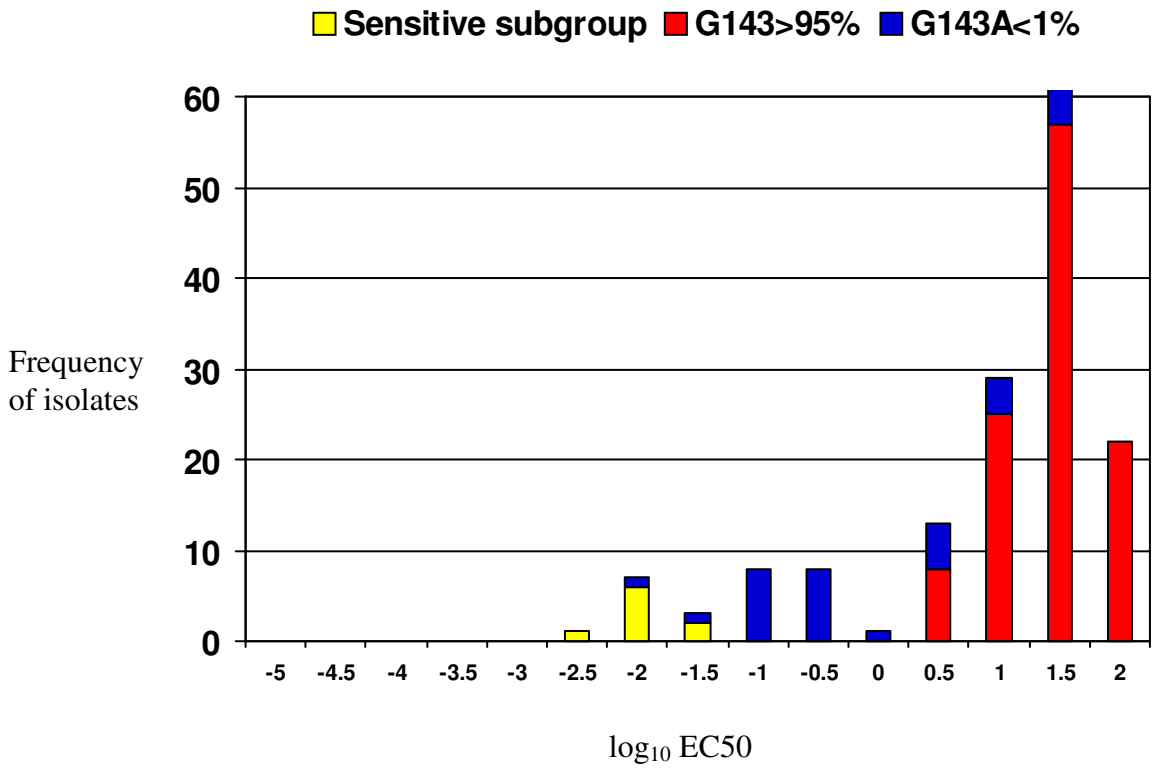

Figure 3.2. Frequency distribution of the log10 EC50 of *E. necator* to azoxystrobin. Yellow portions of bars represent the azoxystrobin-sensitive subgroup (n=10), red portions of bars represent isolates with G143A>1% mutation (n=112), and blue as well as yellow portions of bars represent isolates with G143A<1% (n=42).

There was a significant shift in the median EC50 for azoxystrobin of all isolates of *E. necator* compared to the sensitive subgroup (Figure 3.2). The median EC50 of the sensitive subgroup was 0.007 µg/ml compared to the median EC50 of all isolates, which was 10.8 µg/ml (Table 3.3). The log EC50 ranged from -2.5 to 2.0 while the sensitive subgroup ranged from -2.5 to -1.5 (Figure 3.2). The magnitude of the shift of the median EC50 of all PM isolates was 1526-fold (3.2 log units). Since the frequency distributions were somewhat skewed, and therefore not log normally distributed, the non-parametric Wilcoxon/Kruskal Wallis test was performed to determine whether the distribution of the entire population differed from that of the PM subgroup sensitive to azoxystrobin. The difference was highly significant ($P<0.0001$).

Based on the SYBR-green real time PCR results, all the PM isolates in the sensitive subgroup had G143A <1%. One hundred twelve (73%) out of the 154 PM isolates tested by real time PCR and bioassayed had G143A>1%; they had a broad range of EC50 values ranging from 1 µg/ml to greater than >10 µg/ml (Fig. 3.2). The remaining 42 (27%) PM isolates had G143A<1%. Twenty eight of the 42 isolates had EC50 values less than 1 µg/ml and the remaining 14 had EC50 values above 1 µg/ml (classified as QoI resistant based on our criterion). QoI-resistance of the 14 isolates with less than 1% G143A may be conferred by other mutations such as F129L or other mechanisms which were not explored in this study. Thus, it is recommended that the *cyt b* of PM isolates that were resistant to QoI but contained <1% G143A should be sequenced to determine if mutations other than G143A were present that may also be responsible for QoI resistance.

3.3.3. Downy mildew sensitivity to azoxystrobin

A portion of QoI-resistance of DM experiments has been published (Baudoin et al. 2008). Some of the DM isolates had resistance factors over 100 and were either poorly or not controlled when sprayed with label rates of the QoI fungicides azoxystrobin and pyraclostrobin. A majority (91.5%) of the 153 DM isolates tested against azoxystrobin at 1 and 10 µg/ml were resistant (they grew at concentrations >1 µg/ml). QoI-resistance was geographically widespread (Fig. 3.3). The EC50 of the resistant DM isolates was difficult to determine accurately because when the leaf discs used for the bioassay were soaked in a higher dose of azoxystrobin, they died due to phytotoxicity. This phenomenon has previously been reported by Baudoin et al. (2008) for azoxystrobin and by Wong and Wilcox (2000) for two other QoI fungicides, namely kresoxim-methyl and trifloxystrobin at 0.5 µg/ml and 3 µg/ml, respectively.

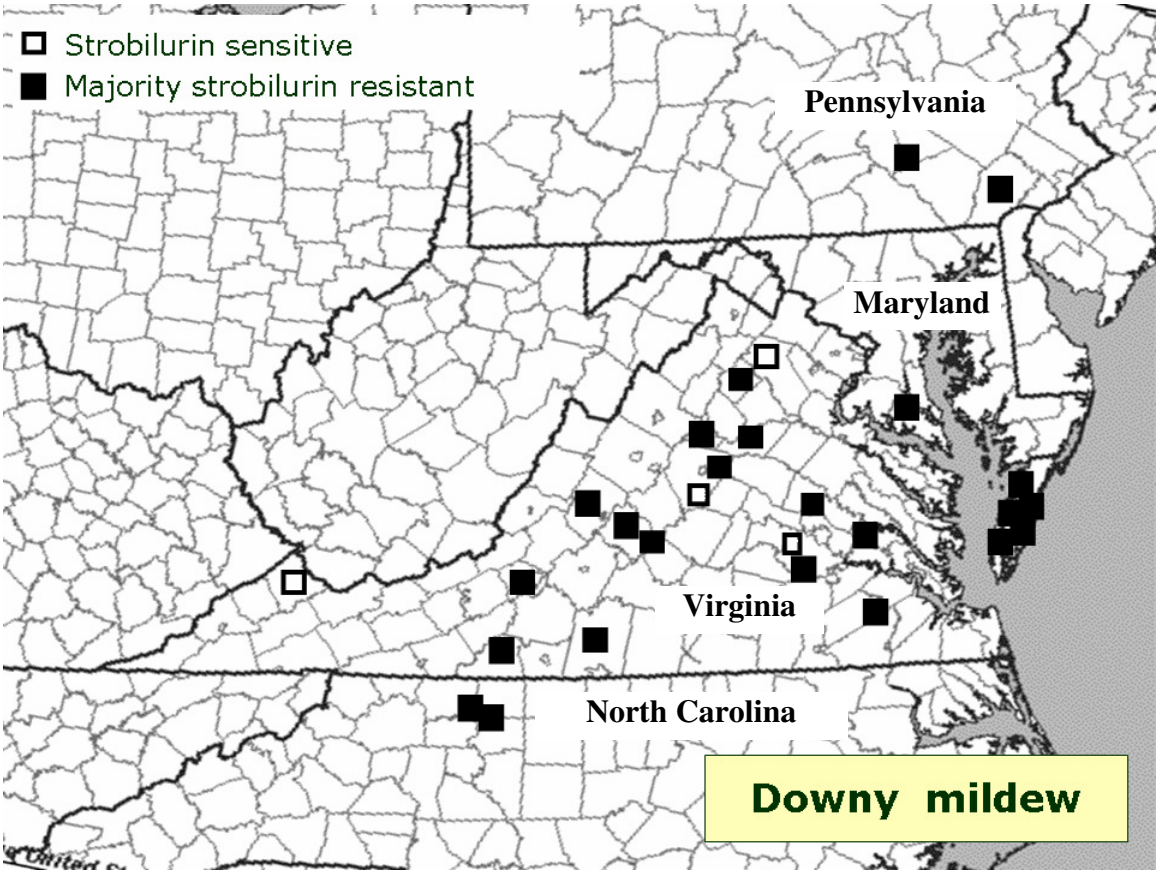

Figure 3.3. Map of Virginia and nearby states showing the locations where DM isolates were collected and the predominant reaction of the isolates to azoxystrobin. QoI-sensitive isolates are represented by open squares. QoI-resistant isolates are represented by solid squares.

It has been found in previous studies that QoI resistance in DM is conferred by a point mutation called G143A in the cyt *b* gene (Baudoin et al. 2008; Collina et al. 2005; Sierotski et al. 2005; Sirven and Beffa 2003) which can be detected using SYBR green real-time PCR. The conventional bioassay and real-time PCR can be combined to screen for QoI-resistance in DM. Based on the results of the bioassay and PCR tests, the isolates were divided into three groups namely: Group I (QoI resistant with G143A >1%), Group II (QoI-sensitive with G143A<1%,) and Group III (QoI-resistant with G143A <1%).

The majority of the DM isolates fell into Group I (92%), followed by Group II (7.6%). (1.4%) (Figure 3.1 and Table 3.3). One isolate was QoI resistant in the bioassay but contained less than 1% G143A. This result clearly shows that resistance in the DM isolates was largely due to the presence of a very high percentage of G143A mutation in the cyt *b* gene. The % of the G143A in each resistant isolate was >95% while the %G143A in the sensitive isolates was less than 1%. Since QoI-resistance is correlated with >1%G143A, the real-time PCR method can be used to quickly screen DM isolates for QoI resistance. DM isolates that have <1% G143A can be tested by the conventional bioassay to determine their sensitivity to QoI. One µg/ml of azoxystrobin can be used as a discriminatory dose to determine QoI resistance.

Although there were indications of somewhat different intrinsic activities among QoIs (Wong and Wilcox 2000), a strong cross-resistance was detected in the pathogens that have been tested against QoI fungicides (Chin et al. 2001; Ishii et al. 2001; Kim et al. 2003). The G143A mutation is key to QoI resistance in *P. viticola* (Gisi et al. 2000). As shown in the findings of Kim et al. (2003) for *P. grisea*, it appears that the mutation G143A and not F129L confers cross-resistance between the QoIs. Since the majority of the QoI-resistant DM isolates tested possessed G143A, it is expected that these isolates are also resistant to other QoI fungicides like kresoxim-methyl, trifloxystrobin, and others which they have not been tested against. We believe that QoI fungicides will no longer provide control of *P. viticola* in some vineyards in Virginia and nearby states and therefore other fungicides like mefenoxam should be used instead.

3.3.4. Downy mildew sensitivity to mefenoxam

Bioassays with 153 DM isolates from 26 vineyards in Virginia, Maryland, North Carolina, and Pennsylvania (Figure 2.4) provided no evidence of any mefenoxam resistance. None of the isolates grew on leaf discs treated with 10 µg/ml. Some DM growth would commonly develop on discs treated with 1 µg/ml; apparently, the effects of mefenoxam dissipated after lengthy incubation, because when leaf discs were floated on mefenoxam suspensions of 1 µg/ml, these isolates did not grow (Baudoin, pers. comm.). However, metalaxyl-resistant DM isolates from France (Leroux and Clerjeau 1985; Gisi

and Cohen 1996) and Australia (Wicks et al. 2005) had 100% leaf discs sporulation at metalaxyl concentration of 10 µg/ml.

According to Gisi and Cohen (1996), naturally occurring metalaxyl-resistant *Phytophthora infestans* isolates already existed even before the use of metalaxyl in the late 1970s, and it is possible that low proportion of mefenoxam-resistant *P. viticola* exists in Virginia and nearby states but went undetected in this survey. It might be helpful to focus sampling in vineyards with relatively intensive use of mefenoxam, if they exist, but we have insufficient information on fungicide use practices in individual vineyards to do so. However, our results indicate that there is no widespread mefenoxam resistance problem; apparently, mefenoxam resistance management practices which limit this compound to four applications per season, and only in mixtures with copper or mancozeb are effective. In addition, mefenoxam is relatively expensive which discourages overuse. To date no mefenoxam resistance in *P. viticola* has been detected in the US. However, since mefenoxam has a high risk of resistance development, it should be monitored closely in order to detect resistance early.

3.3.5. Powdery mildew sensitivity to boscalid and quinoxyfen

All PM isolates (n=160) tested against boscalid and quinoxyfen were sensitive; they did not grow on leaf tissue treated with a concentration of 0.3 µg/ml. The sensitive PM isolates (n=10) tested against quinoxyfen and boscalid had an EC50 range (Table 3.3) of 0.00061 µg/ml to 0.01906 µg/ml (mean=0.01118 µg/ml) and 0.00047 µg/ml to 0.01504 µg/ml (mean=0.00667 µg/ml), respectively. These data can be used in the future to compare with isolates that will be tested to determine if the PM isolates collected from the Virginia and nearby states will have a shift in their EC50 values for boscalid and quinoxyfen. According to Green and Gustafson (2006), the leaf disc bioassay can effectively and accurately detect quinoxyfen-sensitive PM isolates but may overestimate the frequency of PM isolates with reduced sensitivity to quinoxyfen. Thus, quinoxyfen-resistant PM isolates detected through leaf disc bioassay must also be tested on plants or in the field to confirm resistance.

The range of EC50 of grape PM isolates tested by Green and Gustafson (2006) to quinoxyfen ranged from 0.03 µg/ml to 2.6 µg/ml which was broader than the range of EC50 of the ten isolates in our sensitive PM subgroup. To date, quinoxyfen resistance in *B. graminis* f.sp. *hordei* is the only example of quinoxyfen resistance reported (Hollomon et al. 1997; FRAC 2006). Quinoxyfen-resistant *B. graminis* f.sp. *hordei* have been generated in the laboratory and collected in the field. The EC50 of the resistant isolates was unclear but they grew at 0.16 µg/ml. It has been observed that these quinoxyfen-resistant isolates were defective and will only survive in the presence of quinoxyfen, thus, would not have been detected by our procedures where isolates were cultured repeatedly on untreated leaf tissue prior to bioassay.

The calculated range and mean of the EC50 of the boscalid-sensitive PM isolates was lower than the mean and range of EC50 of sensitive isolates from California which were 0.0039-0.052 mg/liter (mean=0.022 µg/ml) (Wong et al. 2003). To date no boscalid-resistant PM isolates have been reported in the US. However, boscalid resistance was detected in another pathogen, namely *A. alternata* in pistachio, with the resistant isolates having a mean EC50 >100 µg/ml (Avenot et al. 2008).

The Fungicide Resistance Action Committee (FRAC 2007) has classified both boscalid and quinoxyfen as medium risk and azoxystrobin as at high risk of resistance development (FRAC 2007). Boscalid and quinoxyfen became registered for use on US grapes in 2003, and the time available for resistance to develop has been limited. Since PM resistance to the QoIs is now widespread and many PM isolates also had lower sensitivity to the EBIs (Chapter 2), growers may increase their use of boscalid and quinoxyfen. Thus, it is worth continuing to monitor the PM population to detect any resistance development early.

Since quinoxyfen and boscalid have medium risk of resistance development, a continued monitoring for quinoxyfen and boscalid resistance in PM should be done. An early detection of the emergence of resistance to the three fungicides mentioned will be useful in deciding what management practices should be implemented by the growers and what fungicides should and should not be used in the control of PM.

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