Characterizing resistance of the grapevine powdery mildew *Erysiphe necator* to fungicides belonging to quinone outside inhibitors and demethylation inhibitors

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ABSTRACT

Practical resistance of *Erysiphe necator* to quinone outside inhibitors (QoIs) is now widespread, and resistance to demethylation inhibitors (DMIs) has also developed. The goal of this research was to characterize fungicide resistance by elucidating resistance mechanisms and determining its stability. QoI resistance persisted for several years in a field population after QoI application ended. Resistant isolates were highly competitive in mixed populations in competition assays under laboratory conditions, indicating a lack of fitness cost. In one competition trial under field conditions, resistance frequency declined, possibly due to spore migration and influx of background inoculum, but in a second trial, it did not decline. Double resistance to QoI and DMI was detected and DMI application may have been partially responsible for maintaining QoI resistance in the field. One isolate with QoI resistance but an undetectable level of the major QoI mutation was shown to be heteroplasmic – resistant strains could be selected from this isolate.

DMI resistance mechanisms in *E. necator* included the Y136F mutation in CYP51 and *cyp51* over-expression. The first mechanism was present in almost all isolates with substantial levels of resistance, and *cyp51* expression level was correlated with resistance level. Three *cyp51* genotypes were detected. Wildtype isolates with the TAT genotype were sensitive to DMIs, while isolates with increased resistance had either a TTT or TWT genotype; TWT indicated the presence of both wildtype and mutant alleles. *Cyp51* was expressed 1.4 to 19 times more in mutants than in wildtype. It is not known whether the significant differences in *cyp51* expression level among isolates and among genotype groups are due to gene copy number variation. DMI resistance was found to decline after years of subculturing, and the decline appeared to occur after a few culture transfers of field samples on fungicide-free host leaves. The observed decline, together with the finding that isolates could be "trained" to increase resistance, and may be slightly induced in *cyp51* expression when successively challenged to grow in increasing fungicide concentration, indicate instability of DMI resistance.

DEDICATION

From Whom all blessings flow,
the Source of wisdom and strength,
You are indeed the Great “I AM”.

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TABLE OF CONTENTS

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendix Tables

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendix Figures

CHAPTER 1

Background on DMI and QoI Resistance in *Erysiphe necator* (Burr.)

1.1 Fungicide resistance problems in *E. necator*

Grapevine powdery mildew caused by *Erysiphe necator* [syn. *Uncinula necator* (Schw.) Burr.] is a major disease in grapes affecting vine vigor, fruit yield, wine quality and winter-hardiness of canes (33, 48, 93). Grapevine susceptibility varies but no commercially grown cultivars of the European grape species *Vitis vinifera* are known to be resistant to *E. necator* (33, 34, 101). Disease management relies heavily on the use of protective and systemic fungicides in conjunction with aggressive cultural practices.

Among the widely employed fungicides against grapevine powdery mildew are the quinone outside inhibitors (QoIs) and the demethylation inhibitors (DMIs). In the United States, pathogen resistance to QoIs and DMIs developed not long after such chemistries were introduced into the market (8, 84, 104, 105). For instance, in 1985-1986, only three years after initial use, a decline in disease control for triadimefon (DMI) was observed in California (51, 110). QoI resistance of *E. necator* was recorded first in New York in 2002 (104) then in Virginia in 2005 (7, 8). Since reports came out starting in 2005, it is uncertain how quickly resistance developed from first introduction of azoxystrobin in the US grape market in 1997 (49), but it was probably rapid due to the fungicide characteristics and the biological nature of the pathogen. Rapid resistance development to QoIs have been observed in other pathosystems (54, 58). In fact, practical QoI resistance is now a problem in several pathogens of a variety of crops (42-44, 57).

The risk of resistance development is a function of the mode of action of the fungicide (single-site vs. multi-site), the selective pressure arising from its application, the local conditions for disease development, and the evolutionary potential of the pathogen (83). Fungicides with a single-site mode of action such as QoIs allow for the rapid development of resistance in pathogens, hence they are classified into the high resistance risk group (45). On the other hand, the gradual development of resistance to DMIs observed among pathogens qualifies the risk as moderate.

Our laboratory has documented QoI resistance of powdery mildew isolated from vineyards in the mid-Atlantic region of the United States. The shift in sensitivity for the QoI azoxystrobin from a median EC_{50}=0.007 $\mu g.ml^{-1}$ of the sensitive sub-group to a median EC_{50}=10.8 $\mu g.ml^{-1}$ for the resistant group was large (20). The majority of isolates from commercial vineyards were also QoI-resistant. Moderately reduced DMI sensitivity of powdery mildew isolates from the same region in the United States was documented as well. Among DMI resistant isolates, mean EC_{50} values for myclobutanil and tebuconazole ranged from 260 to 350 times greater than the DMI-sensitive group. It should be noted that the sensitive group was even more sensitive than those reported in other regions of the United States (37, 51). For example, median values reported by Colcol (20) for her sensitive subgroup were only 11 (fenarimol) and 70 (myclobutanil) times higher than median values reported for an unexposed population by Erickson and Wilcox (37). Overall, the above findings suggest a loss of efficacy of QoI fungicides and reduced efficacy of DMI fungicides. For a grower, it is often difficult to detect chemistries with lowered field performance because of the practice of tank mixing and rotation.

Fungicides are expected to continue to be an integral component of the viticulture industry in the near future. It is thus important to continuously develop strategies that will maximize the benefits and minimize the disadvantages (such as pathogen resistance build-up) derived from the use of these fungicides if their useful life is to be extended. Understanding the molecular basis of resistance and the accompanying genetic changes as well as the dynamics of resistance in the pathogen population presents ways to improve risk assessment of resistance development, optimize resistance management and support product development (78).

1.2. Resistance mechanisms to DMIs

DMIs inhibit the cytochrome P-450 sterol 14α-demethylase ($P\text{-}450_{14DM}$) which is encoded by *cyp51* and is a key enzyme for sterol biosynthesis (47). DMIs have been in use against grape powdery mildew since 1982 in the United States, with triadimefon as the first labeled chemical, followed by fenarimol and myclobutanil, and others (51, 84, 110). In North America, reduced sensitivity of grapevine powdery mildew has been reported in New York (37, 105), California (84, 110), Ontario (90), and Virginia and nearby states (8, 21). To alleviate the selection pressure towards fungicide resistance, DMIs are often used as rotational partners with QoIs for improving control (13, 14, 46, 55).

Mechanisms of resistance to DMIs generally fall into three categories: (1) target site modification, (2) over-expression of the *CYP51*, and (3) increased activity of efflux transporters, (10, 74). Single-point mutations conferring amino acid changes in *CYP51* have been described for a number of fungal pathogens (17, 28, 29, 68). Generally, many of the mutations are clustered in the αB′C loop involved in forming the substrate-binding cavity of eukaryotic CYP51. Based on protein modeling of different eukaryotic CYP51, these mutations may also be responsible for either expanding or reducing the binding cavity, which in turn affects interactions with azole molecules (10).

Based on protein modeling studies, Xiao et al. (108) proposed an explanation why some mutations confer greater levels of resistance than others. The binding of certain fungicides with the altered target molecule occurs in a specific site that cannot accommodate other fungicides. This stabilizes the interaction between the bound azole and the protein. Becher and Wirsel (10) expounded further on the effects of target site modifications in fungal CYP51 using homology modeling of related structures from other biological origins. They summarized three possibilities for amino acid changes resulting in DMI resistance: (1) removal of amino acids interacting with the docked azole ligand, by substitution or spatial displacement; (2) structure rearrangements in the binding cavity, including the position of the heme, that disrupt the interaction between the azole and the heme iron, or (3) disturbing the access of the drug to the active site.

For *E. necator* and the cereal powdery mildews *Blumeria graminis* f.sp. *hordei* (*Bgh*) and *B. graminis* f. sp. *tritici* (*Bgt*), the Y136F mutation leading to a change from tyrosine to phenylalanine in the CYP51 protein is associated with DMI resistance. The mutation is found within the highly conserved CR2 domain of CYP51, which has a role in substrate recognition (28). It has been proposed that this mutation decreases the affinity of the enzyme for its inhibitor (azole) because of increased hydrophobicity of the active site. Phenylalanine has a non-polar side chain while tyrosine has a polar side chain. Délye et al. (1998) (28) showed a high degree of similarity (>70%) between *E. necator* and *Bgh* CYP51 amino acid sequences, indicating that they belong to the same family of P45014DMs (88). In addition, in *Bgh* a combination of Y136F

and another substitution K147Q was identified in highly resistant isolates, with K147Q absent from isolates with lower levels of resistance (107).

Y136F was the only mutation reported to be associated with DMI resistance and was found in *E. necator* isolates with resistance factor (RF) to triadimenol higher than 5 (30). In Europe, two groups of *E. necator* with different susceptibility to azoles have been described (31, 36, 82). Group B was found to be less susceptible to azoles than Group A. These groups were suggested to have different ecological niches based on their differences in prevalence over the growing season and mode of reproduction. Amino acid changes specific to each group might be responsible for this difference. The Y136F mutation was also found mainly in field isolates of group B, which correlated with high levels of azole resistance; these accumulated in French vineyards with prevalence reaching 100% in some plots. Overall, %Y136F was low, suggesting that DMI resistance in French vineyards was still restricted (36).

The Y136F mutation in *E. necator* corresponded to the same substitution in highly and moderately resistant laboratory mutants of *Penicillium italicum*, a common spoilage pathogen of fruits and vegetables; thus, it has been suggested that Y136F is probably sufficient to confer high resistance (30). A similar amino acid change (tyrosine to histidine) in the yeast *Candida albicans* ERG11 (corresponding to CYP51) at codon 132 also resulted in a higher resistance level to fluconazole (61, 85). Earlier, it was reported that the Y136F mutation occurred in a low-resistance strain of *Bgh* (12). Similarly, not all highly resistant isolates tested by Délye's group possessed the Y136F mutation. This indicates that in these species, there could be additional mutations in other regions of the genome or alternative mechanisms that resulted in higher levels of resistance.

Y136F is also equivalent to Y137F in *Mycosphaerella graminicola*, the causal agent of septoria leaf blotch in wheat (68). This mutation was said to have no correlation with reduced sensitivity to triazoles (22). However, the Y137F mutation was found in a 'Tri R3' azole resistance phenotype characterized as having high resistance to triadimenol and flusilazole, intermediate resistance to propiconazole and epoxiconazole and sensitivity to triflumizole and fluquinconazole (67). Moreover, a gradual sensitivity shift to DMIs in European isolates of *M. graminicola* was found to correlate well with a stepwise accumulation of a number of other amino acid substitutions (68). The contribution of these mutations to resistance was found to be complex. Alterations in five different codons (459, 460, 461, 316, 317) were found only in isolates with low resistance levels, whereas a change from isoleucine to valine at position 381 (I381V) in combination with some of the mutations (459-461) provided the highest resistance levels to most of the DMIs tested (67). A group of isolates possessing several mutations and a 6-bp deletion (ΔY459/G460) but lacking I381V also exhibited reduced sensitivity. Modeling of the CYP51 molecule of *M. graminicola* revealed various functional effects of these mutations, such as loss of triadimenol sensitivity with Y137F and lower sensitivity to tebuconazole with I381V (87). Multiple mutations correlating with propiconazole resistance were also found in *M. fijiensis*, the black sigatoka pathogen in banana (18).

The findings in *M. graminicola* also led Brunner and colleagues (16) to investigate the evolutionary mechanisms of the CYP51-based mutations. They hypothesized that the gradual shift towards reduced sensitivity of the pathogen to azole fungicides in Europe is a result of a rapid and widespread selective replacement of the "old" alterations by "newer" mutations conferring increased resistance. This phenomenon was supported by the known population

biology of the pathogen, i.e. regular recombination within the population and little genetic differentiation among populations from the same regions coupled with long-distance gene flow due to wind dispersal of ascospores.

Some mutations that have been described are considered to be of no practical importance. A conserved phenylalanine residue found in *Oculimacula yallundae* (syn. *Tapesia yallundae* (cereal eyespot fungus) and in all known *CYP51* proteins from other filamentous fungi was replaced by a leucine at codon 180 in another closely related species, *Oculimacula acuformis* (syn. *T. acuformis*), that is known to be naturally resistant to DMI triazoles (74). However, the F180K mutation and other laboratory-induced mutations have not been detected in field isolates, so their impact on field resistance development is considered low.

Modifications in the expression of *CYP51* are associated with DMI resistance in some pathogens. Overexpression may result from chromosomal duplication such as in the case of the human pathogen *Candida glabrata* (76), or transcriptional enhancement by producing a tandem-repeated sequence in the promoter region in *Penicillium digitatum* (52), or an upstream insertion of various truncated derivatives of a LINE-like retrotransposon in *Blumeriella jaapii* (75).

In *Venturia ineaqualis*, causing apple scab, high expression levels of *cyp51* were correlated with a 553-bp insertion with predicted promoter sequences upstream of the gene, suggesting that this insertion may be a transcriptional enhancer (97). However, expression analysis of isolates from orchard populations showed that majority of field strains without the insertion could still over-express *cyp51*. Resistant isolates that did not over-express *CYP51* were also recovered from these commercial orchards. These findings suggest that other mechanisms contribute to myclobutanil resistance in *V. inaequalis.*

Recently, three amino acid substitutions in the *cyp51* gene of *Cercospora beticola* were found in some triazole-resistant isolates from sugar beet fields (89) and none of these mutations have been recorded previously for DMI resistance. A three-dimensional model of an isolated fragment of the *cyp51* gene further showed that these mutations do not significantly affect inhibitor binding to the active site. Instead, it was proposed based on expression analysis by real-time PCR that overexpression may well be the mechanism of high levels of resistance in this pathogen.

Cyp51 over-expression was also demonstrated to be a common mechanism for DMI resistance in *Monilinia fructicola*, the causative agent of brown rot of peach (72). A short repetitive element ("MONA") with a putative promoter was closely associated with DMI resistance and found only in isolates that over-expressed the gene (71, 72). Further molecular analysis of a larger collection of isolates revealed that the Mona element is widespread among resistant isolates of *M. fructicola*.

The occurrence of two or more copies of the genes of the ergosterol pathway, including *cyp51* is a common phenomenon in fungi. It has been suggested that duplicate copies may allow for differential expression at different environments, may provide the same function but in different cell localization, may allow for different substrate affinities, or for gene amplification (79).

Becher et al. (9) found that the *cyp51* gene in several species belonging to the subphylum Pezizomycotina of the Ascomycota exists as exists as one to three copies, designated *CYP51A, CYP51B* and *CYP51C*. In *Aspergillus fumigatus*, a human pathogen, there are two homologous CYP51 proteins – CYP51A and CYP51B. The two genes encoding these proteins were proposed to have different functions if those have indeed arisen by gene duplication (79). A phylogenetic analysis of the various *cyp51* sequences clustered *A. fumigatus cyp51A* with *P. italicum* and *A. nidulans* (79). Meanwhile, *A. fumigatus cyp51B* was more closely related to that of *E. necator, B. graminis*, and *Botrytis cinerea*. Both A and B variants in *A. fumigatus* were expressed during growth in submerged culture. Knock-out mutants lacking the A copy were more susceptible to itaconazole, fluconazole and ketoconazole than the wild-type organisms but did not incur morphological defects, changes in ergosterol content, or increased *cyp51b* expression, indicating that CYP51A is not essential for viability (81). Since the CYP51B copy was still active in these mutants and *A. fumigatus* is intrinsically resistant to the azoles tested, the authors suggested that the B form could be more susceptible to the DMIs. Further analysis of *cyp51A* in additional clinical isolates showed that a short tandem repeat in the promoter region plus a L98H amino acid substitution resulted in resistance phenotypes observed in clinical isolates. Both target site modification and overexpression could be necessary for increased resistance to triazoles (80).

Two *cyp51* genes were also found in *A. parasiticus*, one of the mycotoxin-producing fungi that contaminate agricultural products. The nucleotide sequences had high similarity with *cyp51A and cyp51B* of *A. fumigatus* (35). Only *cyp51A* possessed a mutation (G54W) that correlated with flusilazole resistance. However, resistance in this species was not solely due to target site modification, since over-expression of *cyp51A* and an ABC transporter gene *mdr1* was demonstrated for resistant strains.

The activity of drug transporters (also known as efflux pumps) may also confer resistance to fungicides among plant pathogens. These pumps secrete compounds and prevent their accumulation to toxic levels within the cell (26). The primary role of these transporters in plant pathogens is known to be in the secretion of pathogenicity factors and expulsion of plant defense compounds (27).

The two main groups of efflux proteins are the ATP-binding cassette (ABC) and the Major Facilitator Superfamily (MFS) transporters (19). ABC transporters have a broader range of substrates and are driven by ATP hydrolysis. The MFS transporters utilize the proton-motive force across the plasma membrane. The up-regulation of genes encoding these efflux pumps may be involved in fungicide resistance. A microarray analysis of transcriptional responses of *Fusarium graminearum*, causing fusarium head blight in cereals, revealed that a number of ABC transporter genes were up-regulated upon tebuconazole treatment (9). An ABC-like transporter gene was also characterized in *M. fructicola* whose expression in transformed yeast was induced by the DMI fungicides myclobutanil and propiconazole (96). Over-expression of ABC transporters have been associated with DMI resistance in laboratory mutants of *B. cinerea*, the grey mold fungus (53), and field isolates of *M. graminicola* (111). However, it was suggested that multiple mechanisms, including up-regulation of the ABC transporter gene *MgAtr1*, may be responsible for cyproconazole resistance in *M. graminicola* (111). Meanwhile, the activity of efflux pumps in *B. cinerea* is the basis for multiple drug resistance (MDR). In French vineyards, three MDR phenotypes have been increasing in frequency and these were correlated with

increased drug efflux activity and overexpression of two efflux transporters (65). Andrade et al. (1) also proposed, based on the lowered cellular accumulation of fenarimol in *atrB*-overexpression mutants of *A. nidulans*, that fenarimol resistance could be due to increased efflux activity of the AtrB multidrug transporter. Similarly, in a postharvest pathogen of citrus, *Penicillium digitatum*, a MFS transporter encoded by the *PdMfs1* gene conferred resistance to imazalil when overexpressed in a *PdMfs1*-disruption mutant (103). In addition, *PdMfs1* was found to have a role in virulence of the pathogen.

1.3. Resistance mechanisms to QoIs

QoIs were introduced in the US market in 1996, and azoxystrobin was the first strobilurin to be registered for grape powdery mildew in 1997 (5, 86). The other strobilurins registered for grape powdery mildew are pyraclostrobin, kresoxim-methyl and trifloxystrobin. Strobilurins were shown to have limited curative effects likely due to mycelium collapse (66), and preventative activity primarily due to their inhibitory effects on spore germination and zoospore motility (in downy mildew) (6, 105).

QoIs were the first fungicides developed with a protein target site encoded by a mitochondrial gene (14). Strobilurins target the cytochrome *b* gene and inhibit mitochondrial respiration by binding the outer quinol-oxidation site of the cytochrome bc_1 enzyme complex (39). This prevents the transfer of electrons between cytochrome *b* and cytochrome c_1, which in turn, disrupts the energy supply.

Practical resistance among plant-pathogenic fungi and oomycetes to strobilurins is now rampant (39, 45, 57). Mutations in the cytochrome *b* gene (*cyt b*) are associated with QoI resistance, resulting in amino acid substitutions that can disrupt fungicide binding. In yeast mutants (32) and strobilurin-producing fungi (64), 11 point mutations were described in two regions of cytochrome *b*, corresponding to amino acids 127-147 and amino acids 275-296 (100). Two of these mutations have been demonstrated to occur in naturally QoI-resistant pathogens, a mutation leading to a change in position 143 from glycine to alanine (G143A) and a mutation leading to a change in position 129 from phenylalanine to leucine (F129L) (74). The G143A mutation is associated with high resistance factors (49).

The F129L substitution, associated with lower levels of resistance (91, 102), has been detected in pathogens such as *Pyricularia grisea* (62), *Pythium aphanidermatum* (Edson) Fitzp. (49), *Pyrenophora teres* and *P. tritici-repentis* (98), and *Alternaria solani* (92). It has not been reported in any of the powdery mildews so far (38, 99). More direct evidence of a correlation between the mutations and levels of QoI resistance was provided in mutational studies of *Saccharomyces cerevisiae* whose residues in the cytochrome *b* Q_o site were modified to mimic that of the binding site in some fungal and oomycete pathogens (40). The G143A mutation generated high resistance to myxothiazol, azoxystrobin and pyraclostrobin, while a different pattern was observed for the F129L and L275F mutation found in resistant *Phytophthora* sp. These findings further support G143A as a major mechanism of QoI resistance.

Multiple point mutations relevant to QoI resistance can occur in the same species. In addition to G143A and F129L, a third mutation G137R was found at a very low frequency in *P. tritici* (98). G143A had the strongest impact on disease control in the field based on its association with high resistance factors and the failure of controlling G143A-carrying isolates by

QoI applied at a full rate. Recently, both G143A and F129L mutations were also found in field isolates of *Pestalotiopsis longiseta*, the causal agent of gray blight of tea plants in Japan (109). While F129L was correlated with moderate resistance, it allowed isolates to overcome the inhibitory effect of azoxystrobin in leaf bioassays. In contrast, in greenhouse experiments, *P. tritici-repentis* and *P. teres* isolates carrying F129L were effectively controlled by field rates of QoI fungicide. However, in populations of these pathogens in certain parts of Europe, F129L frequencies continued to increase in a three-year survey (98). It is not yet clear whether this increase in frequency will later pose practical problems. However, field studies on the early blight pathogen *Alternaria alternata* populations dominated by F129L revealed that this mutation could reduce the efficacy of strobilurins (91).

Data in our laboratory also support the correlation between resistance and G143A (20). In addition, two categories of strobilurin resistance were described based on the frequency of the G143A mutation measured by qPCR using the SYBR Green chemistry and the EC_{50} from leaf disc bioassay: (1) highly resistant isolates (EC50 $\geq$10 $\mu g.ml^{-1}$) with G143A >95%; and (2) moderately resistant isolates (EC50 = 1-15 $\mu g.ml^{-1}$) with G143A <1%. All members of a sensitive subgroup (EC_{50} <1 $\mu g.ml^{-1}$) had G143A <1%. It is not yet known if what we call "moderate" resistance is conferred by a different amino acid change in the target protein.

Some pathogens may be heteroplasmic, i.e., cells harbor both wild-type and mutant *cyt b*. The heteroplasmic state was hypothesized to allow for a loss of QoI resistance after transfer on fungicide-free media of field and laboratory mutants of *Botryotinia fuckeliana* (anamorph *B. cinerea*) (25) and single-spored isolates of *Bgt* (41). In *Podosphaera leucotricha* (apple powdery mildew), differences in sensitivity to trifloxystrobin correlated with the proportion of the mutation, indicating that the level of mutant mitochondria affects the level of resistance to this fungicide (69).

Grasso *et al.* (50) investigated the *cyt b* gene structure and the occurrence of point mutations associated with resistance to QoI fungicides in 14 species of agronomically important plant pathogens. A type I intron was found directly after the codon for glycine at position 143, and this is believed to affect the occurrence of point mutations in this codon. This was confirmed in pathogens (e.g. rusts and *A. solani*) in which resistance due to the G143A mutation had never been reported. This intron was absent in pathogens where QoI resistance is known to be due to the G143A mutation (*A. alternata, B. graminis, M. grisea, M. fijiensis, M. graminicola, V. inaequalis* and *P. viticola*). It was proposed that the presence of the G143A mutation hinders intron splicing, leading to a lethal condition with a deficient cytochrome *b*; thus, pathogens carrying the intron directly after position 143 are least likely to evolve the G143A mutation. An intron between the 143rd and 144th codons in the cytochrome *b* was also found in field isolates of *B. cinerea* (4). The proximity of this intron to the 143rd codon and its presence only in isolates that were QoI-sensitive and that lacked the G143A mutation suggest that isolates with this type of cytochrome *b* would also least likely develop the G143A-based resistance. However, the type 1 intron was suggested to be of no consequence to QoI resistance in *P. longiseta* (109). This intron could be present or not in QoI-resistant isolates with the G143A or F129L mutation.

Resistance mechanisms other than the G143A mutation have been determined for a number of phytopathogenic ascomycetes and oomycetes. For example, a new mutation at position 137 replacing glycine with arginine (G137R) was found at low frequency in field isolates of *P. tritici-repentis* but not in *P. teres* (98). This mutation conferred similar levels of

resistance as F129L. Gene analysis in the two species showed different structures. *P. teres* possessed an intron directly after position 143 while *P. tritici-repentis* lacked this intron. This is consistent with what is known so far about the non-co-existence of the G143A mutation and the type I intron adjacent to the codon. In *P. teres*, the G143A mutation is least likely to occur as it could be detrimental to the pathogen. A stable G143S (glycine to serine) mutation was also found in a laboratory-generated QoI-resistant mutant of *Magnaporthe grisea* (3) but this mutation has not been reported for field isolates, thus, it might have no practical importance in the field. Nevertheless, G143S in *M. grisea* was associated with equally high resistance factors but lower conidial production under laboratory conditions compared to the G143A mutant.

Another mechanism of QoI resistance is an alternative respiratory pathway which is sustained by an alternative oxidase (AOX) (38, 39). Despite arguments that this mechanism plays a minor role in resistance, it has been demonstrated to aid the pathogen once infection progresses but is unable to interfere with strobilurin action during germination (106). During the post-germination stage, a lower demand for energy could result in insensitivity towards the fungicide. The alternative oxidase could explain the poor eradicant activity of QoI fungicides on many fungi, i.e., control is lost once visible symptoms appear.

Efflux transporters that operate for fungicide resistance have been characterized in a few fungal pathogens such as *M. graminicola*, *P. tritici-repentis*, and *Colletotrichum* sp. A structural change in the Rieske protein, an integral component of the catalytic site of the cytochrome *b* has also been suggested for resistant isolates such as *P. fusca* where other mechanisms have been ruled out, but no evidence has been put forward as yet (38). Other possibilities such as pesticide biotransformation (e.g. detoxification of the fungicide which is more common in insects and plants) or decreased activation in the case of triadimenol may be operational (15).

1.4. Stability and fitness cost of DMI resistance

The fitness of resistant individuals in a field population is critical to their persistence once they are selected for (74). Fitness is the survival and reproductive success of an allele, individual or group (94). A lowered fitness among resistant isolates will consequentially reduce their survival in the absence of the selection pressure. If resistant isolates are as fit as sensitive isolates, they will persist in the population even after fungicide application is stopped.

Experimental results generally show that vegetative and reproductive fitness costs associated with fungicide resistance may be highly specific to the fungal species and fitness parameters, as studies indicate no consistent correlations with regard to fungicide resistance and fitness (24). To determine fitness cost, experiments usually involve isolates with different sensitivities to the fungicide and/or competition experiments where a resistant population is paired with a sensitive population and grown in the absence of the fungicide. Some fitness attributes, usually two or more of the following - sporulation, germination, and germ tube length, latent period, virulence and mycelial growth (for non-obligate pathogens) - are measured, then compared. In general, the fungicide in question can be an important determining factor in the stability or instability of resistance in certain pathogens. For example, benzimidazole-resistant isolates of *C. beticola* did not exhibit any fitness penalty, but strains resistant to fentin fungicides were less competitive than sensitive strains (60).

It has commonly been observed that DMI-resistant fungal strains are less fit than sensitive strains (63); hence, DMIs are considered to be of moderate risk for resistance development in pathogens (43). The presence of fitness costs associated with resistance development was cited for the pathogens *C. beticola*, *P. italicum*, *P. digitatum*, *Bgt*, and *Pseudocercosporella herpotrichoides* but its absence in *Podosphaera fusca* (syn. *P. xanthii*, *Sphaerotheca fuliginea*, *S. fusca*), *Nectria haematococca* var. *cucurbitae*, *Bgh* and *P. teres* (60). A fitness cost was also demonstrated for fenhexamid-resistant laboratory mutants of *Botrytis cinerea* possessing a mutation in the target gene *erg27*, suggesting that the spread and development of strains with this type of fenhexamid-resistance will be restricted in this pathogen (11). Observations on the decreased fitness under *in vitro* experimentation of DMI-resistant cucurbit powdery mildew (*P. fusca* syn. *Sphaerotheca fuliginea*) initially led the authors to suspect that DMI-resistance would not persist in this pathogen. However, since no fitness disadvantage was observed in competition assays in the greenhouse, the authors hypothesized that DMI resistance could develop gradually with continuous exposure to the fungicide (95). In fact, a decline in disease control due to widespread resistance to fenarimol and triadimenol has been observed from pathogen isolates sampled in 2002-2004 from the main cucurbit-growing areas in south central Spain (70).

Application of DMI fungicides to control pathogen growth can lead to the selection of resistant members with higher fitness through fitness-modifying genes (60). Alternating or tank-mixing of fungicides with different modes of action is a recommended guideline in resistance management. However, the combination of fungicides used may not be an easy decision to make because the interactions between the components vary depending on their nature and the individual doses (77). In addition, mixture effects could improve control but lead to an increase in selection for resistance (77). In *M. fructicola*, disease control did improve when a mixture of propiconazole and sulfur was used as a protective application against propiconazole-resistant isolates of *M. fructicola* (56). This strategy was recommended in situations where some level of resistance has developed but not to the level of disease control failure.

1.5. Stability and fitness cost of QoI resistance

The G143A substitution was claimed not to affect enzyme activity; thus, resistant individuals may not suffer from a significant fitness penalty (49) as demonstrated for *Magnaporthe grisea* (2). In contrast, a fitness penalty was described for a number of other pathogens (23, 59, 73). For example, in *M. oryzae* causing gray leaf spot of perennial ryegrass, fitness costs of resistance were measured in competition experiments using mixed populations with various ratios of sensitive and resistant strains in the absence of the selection pressure (73). Although conidia production was the same for all ratios tested, the proportion of resistant spores became lower after infection, indicating a competitive advantage of sensitive strains over the resistant ones. In pathogens with heteroplasmic mitochondria, i.e., carrying both mutant and wildtype alleles, a fitness cost can be deduced from genetic shifts in culture in the absence of QoI selection pressure. In wheat powdery mildew *Bgt*, a reversion to a homoplasmic state after 30 generations was observed, suggesting the lowered fitness of the mutant allele (41). Also, in untreated field plots located beside QoI-treated plots, G143A remained below 10% in the population during the experimental period, whereas the frequency in QoI-treated plots reached an average of 58% after just three sprays. Resistant spores from treated plots could migrate to untreated plots, but no substantial increase in the resistant fraction was obtained in the control.

This suggests that, if migration did occur, the G143A mutation compromised the pathogen's ability to grow in the absence of the selection pressure.

Reports in the literature indicate that fitness costs may or may not be present. This led us to ask whether the G143A mutation conferring a high level of QoI fungicide-resistance in our *E. necator* isolates carries a fitness penalty that reduces the ability of resistant populations to establish over time and cause disease when the fungicide is withdrawn. In disease management, knowing the fitness of resistant variants and predicting subsequent changes through selection have implications in chemical control measures. If fitness costs are associated with fungicide resistance, withdrawal of the fungicide will be followed by a decreasing frequency of resistant isolates (39). Fungicides can thus be reintroduced or occasionally used in areas where resistant strains are not highly fit. Otherwise, strategies that can eliminate or slow down resistance development must be in place. This can happen when fungicides from different cross-resistance groups are alternated since the reduced selection pressure also decreases the rate of mitochondrial inheritance of the A143 allele (41). In the long run, however, the selection for the mutant allele may be difficult to counteract if there is continuous exposure to the fungicide. Currently, little is known regarding fitness compromise and resistance stability in QoI-resistant *E. necator* isolates. The conflicting reports on fitness of QoI-resistant pathogens suggest that the development and stability of QoI-resistance is species-specific, dictated by the pathogen's genetic background and conditions of disease development.

1.6. Research objectives

1. determine a fitness cost for QoI resistance in *E. necator*,
2. determine a molecular mechanism for observed QoI resistance in isolates that lack a high proportion of G143A,
3. determine the role of the Y136F mutation and other mechanisms in DMI resistance, and
4. characterize the stability of DMI resistance.

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CHAPTER 2

Fitness of G143A-Based Resistance to QoIs

in *Erysiphe necator* Isolates and Field Populations

Abstract

Quinone outside inhibitors (QoIs) were widely used against grapevine powdery mildew (*Erysiphe necator*). However, disease control has eroded due to resistance development in some grape-growing areas. To determine resistance stability, we monitored QoI resistance in one vineyard where no QoIs were used for four years. Resistant populations were present in all years and the frequency was still about 25% in the fourth year, with higher frequency (36%) in a hotspot section of the vineyard. All QoI-resistant populations with >5%G143A also harbored the Y136F mutation in the *cyp51* gene that confers some resistance to demethylation inhibitors (DMI), another fungicide group used for powdery mildew control. Such double resistance may jeopardize the use of DMIs and QoIs as rotational or tank mix partners in the spray program. The use of DMIs could also select for QoI resistance and foster its persistence. Competition assays were performed on fungicide-free grape plants by cycling mixtures of QoI-resistant and sensitive isolates characterized as genetically diverse based on microsatellite analysis. Changes in the population were tracked by qPCR analysis of the proportion of the G143A mutation in the fungal cytochrome *b* gene. Under laboratory conditions, there was no decline in %G143A; in fact, it increased significantly, indicating high competitiveness of the resistant fraction. In field inoculation trials done in two growing seasons, the proportion of the G143A tended to decrease, most likely because background infection (year 1) and spore migration caused gradual mixing of populations. Nevertheless, QoI resistance persisted for four weeks under field conditions, indicating some degree of persistence and stability.

Keywords: QoI, resistance, *Erysiphe necator*, G143A, stability, fitness cost, competition, field inoculation, double resistance, DMI

2.1. Introduction

Quinone outside inhibitors (QoIs) belong to a fungicide class used for disease control in many crops including cereals, potatoes, turf grass, grapes, vegetables and ornamentals (3). This fungicide class represented a breakthrough in chemical control because it targeted several fungal pathogens, with its protectant, eradicant and systemic activities as a group (33). However resistance developed in pathogens not long after introduction of QoIs in the United States. For example, practical resistance was reported in the grapevine powdery mildew (*Erysiphe necator*) in New York and Pennsylvania (37). Later, resistance was found to be widespread in Virginia (4, 8) and Michigan (31). This has challenged the agro-chemical industries and growers to devise strategies that could extend the usefulness of QoIs as an important component of disease management in grapevines.

The binding of the QoI molecule to the outer quinol-oxidation site of the cytochrome *bc1* enzyme complex inhibits mitochondrial respiration (14). The transfer of electrons between cytochrome *b* and cytochrome c_1 is prevented, consequently disrupting ATP production. Resistance to QoIs is mainly attributed to an amino acid change from glycine to alanine in position 143 of the cytochrome *b* (2). This mutation, called G143A, has been detected in a number of pathogens (18) including US isolates of *E. necator* (4, 8, 31). Although it is claimed to have no effect on enzyme activity, suggesting that resistant individuals may not suffer a significant fitness penalty (1, 6, 10, 23, 25, 28, 29). QoI resistance can compromise fitness in some pathogens (14, 16, 17, 22, 30, 35, 38). Constructs of the Qo domain of the cytochrome b of *V. inaequalis* further showed that the introduction of the G143A in *Saccharomyces cerevisiae* reduced enzyme activity which represents a fitness cost for QoI resistance (16). Conflicting reports on fitness of QoI-resistant pathogens (10, 22, 35) suggest that the development and stability of QoI-resistance may be species-specific, dictated by the pathogen's genetic background and conditions of disease development.

Knowing the fitness of resistant variants and predicting subsequent changes through selection have implications in chemical control. Withdrawal of the fungicide may be followed by a decreasing frequency of the resistant isolates if fungicide resistance is associated with a fitness penalty (14). The fungicide can then be reapplied or occasionally used where resistant strains are not highly fit. Otherwise, anti-resistance strategies such as alternating fungicides with different modes of action may only be able to slow down resistance development (19). Alternation of fungicides or the use of mixtures has long been known to combat evolution of resistance in pathogens (7).

Currently, little is known regarding fitness compromise and resistance stability in *E. necator*. The primary objective of this study was to determine if there is a fitness cost associated with G143A-based resistance in *E. necator*. This was determined using competition assays where artificially mixed populations of resistant (A143-genotype) and sensitive isolates (G143-genotype) arbitrarily selected from various locations, were inoculated on grape plants that were not sprayed with QoIs. We hypothesized that %G143A declines through time if a fitness cost is associated with QoI resistance. The competition assay was done under controlled and field conditions. The persistence of resistance in one commercial vineyard that has stopped using QoIs was monitored for several years. Our findings provide evidence for a lack of a significant fitness cost in QoI-resistant *E. necator*.

2.2 Materials and Methods

2.2.1. Sources and maintenance of isolates. The cultures were part of a collection of isolates taken from various locations mostly in Virginia and a few from neighboring states (Table 2.1). These isolates were collected in the period 2005-2010, and were maintained as single-spore cultures on fungicide-free grape leaves. For culture transfers, young, susceptible leaves were harvested from greenhouse-grown Chardonnay grape plants, and disinfested by soaking leaves for 2 min in 20% commercial bleach with 0.01% Tween 20. After rinsing three times with sterile distilled water, leaves were blotted dry and individually plated on 0.7% water agar in 60 mm-petri dishes. Several single spores from one colony for each culture were inoculated on the leaf. Plates were incubated at 25^{o}C under a 14-hr light regimen for 15 to 21 days before the next transfer.

2.2.2. Isolate phenotyping and genotyping. The resistance phenotype for each isolate was checked by qPCR (see section on real time PCR) and bioassay using azoxystrobin. For bioassays, the EC_{50} was obtained by the settling tower method (8). Three microsatellite primer sets developed for the *E. necator* loci *EnMS1*, *EnMS3* and *EnMS7* (20) were also used to analyze the genetic diversity of the isolates used in the competition assays. The method employed a forward primer with an M13 (5′-CACGACGTTGTAAAACGAC-3′)-specific sequence at the 5′-end. The other primer components included the reverse primer and an M13 primer with a fluorescent dye (FAM, NED or VIC) attached at the 5′-end. The 25-µL PCR reaction consisted of 0.32 µM reverse primer, 0.16 µM M13-forward primer, 0.4 µM M13-dye primer and 1x of the 2x Taq Pol Master Mix (NEB). Reactions were done in the MasterCycler (Eppendorf) with the following cycling conditions: initial denaturation at 95^{o}C for 2 min, 35 cycles of denaturation at 95^{o}C for 30s, annealing at 55^{o}C for 30s and extension at 72^{o}C for 30s, and a final extension step at 72^{o}C for 5 min. Products from the three PCR amplifications were pooled. Two microliters from the pool were added to 9.8 µL of HIDI formamide (Applied Biosystems) and 0.2 µL of the GeneScan Liz500 size standard (Applied Biosystems), then heated at 95^{o}C for 5 min. Fragment analysis was conducted in the 3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were analyzed using the SoftGenetics GENEMARKER software v.1.60. The tri-loci allele sizes were tabulated to determine similarities and differences across the isolates. Each unique multilocus genotype was assigned a haplotype number.

2.2.3. Plant materials for competition assays. Overwintering canes (cv. Chardonnay) were cut around 5 in or at least 2 internodes in length, then placed in water with a rooting hormone until shoots and roots formed. Rooted plants were transferred in soil mix and grown under the mist for one week in the greenhouse. Canes were moved out of the mist when roots were well established and canes were watered regularly. The shoots were cut back weekly until inoculation to maintain a plant height of about 8 in from the potting mix surface. The resulting small plants were used for competition assays under controlled conditions and as test plants in the competition trial under field conditions. Large plants grown for at least one year in 3- and 5-gallon pots were used for the competition trial under field conditions.

2.2.4. Field survey of G143A. A vineyard in Virginia was selected because: (i) it had withdrawn QoIs from the spray program for several years; (ii) powdery mildew infection occurred every sampling year; (iii) QoI resistance was documented in this powdery mildew population in the early years right before QoI use was stopped. This vineyard was sprayed from

2 to 4 QoI applications (azoxystrobin, pyraclostrobin, or trifloxystrobin) per season prior to 2008, the year QoIs were withdrawn from the spray program. DMI sprays were applied 1 to 4 times per season without interruption. Samples of infected leaves were mailed to the laboratory by the vineyard manager in Ziploc bags protected by a cardboard box. Samples for this study were taken from 2008-2010 growing seasons. Initial data from an earlier report (8) were included. For 2008, four single spore cultures were established and used for qPCR to quantify the resistant allele. Samples from 2009 included leaf populations in addition to two single-spored isolates. The 2010-2011 samples consisted of leaf populations only. To generate samples from leaf populations, infected leaves were gently pressed or rubbed on susceptible leaves disinfested as described before. Care was taken to sample all growths on infected leaves. The new growth per inoculated leaf was considered as one 'leaf population'. Spores were harvested and the DNA was extracted for qPCR. All leaf populations were also subjected to a genotyping protocol for the Y136F mutation associated with demethylation inhibitor (DMI) resistance. The assay included *EnCyp51*-specific forward (5′- ACTAATTTAACA ACTCCGGTCTTTGGA-3′) and reverse (5′-ACTCGACCATTTACGGAC CTTTTT-3′) primers and two allele-specific TaqMan MGB probes (Applied Biosystems), each labeled with a different reporter dye at the 5′-end (VIC-TTGGACAATCAAATACAAC-3′ and FAM- TTTGGACAATCATATACA AC-3′). One probe detects the mutation in the *Cyp51* gene, while the other detects the wild-type. Each reaction consisted of 10 µL of the 2x Taqman Universal PCR Master Mix (no AmPErase UNG, Applied Biosystems), 0.5 µL of the 40x Taqman CYP51 Genotyping Assay mix (containing the primers and probes, Applied Biosystems) and 2 µL DNA template (5 to 8 ng ml^{-1}). The total reaction volume was brought up to 20 µL. Reactions were carried out in the StepOne Plus instrument (Applied Biosystems). The fluorescence of the reporter dyes was normalized to the fluorescence of the passive reference dye (ROX). The StepOne Plus Real-time PCR software plots the normalized intensities (Rn) of the reporter dyes in each sample well on an Allelic Discrimination Plot. In the plot, the reporter dye intensities for the probe can be differentiated. A clustering algorithm is used by the software to assign a genotype call according to the position on the plot. A sample can be pure wild-type ('homozygous allele1/allele1'), pure mutant ('homozygous allele2/allele2') or mixed ('heterozygous allele1/allele2').

2.2.5. Growth rate measurement and competition assays under controlled conditions. The growth rate (mm/day) was compared for six sensitive and five resistant isolates. This was obtained by inoculating at least five spore chains on each of three to four fungicide-free grape leaves for each isolate. The diameter of colonies that developed on the leaves was measured daily from six to eight days of incubation. The mean diameter was obtained from each leaf for each isolate, and growth rate was calculated as mm/day for the 6- to 7-day-rating and the 7- to 8-day-rating. A total of 20 measurements were generated for resistant isolates and 28 measurements for sensitive isolates.

Two types (paired or bulked) of inoculation were done for the competition assays. In the first inoculation type, nine unique pairs of resistant ($EC_{50} \geq 10$ µg.ml-1azoxystrobin, ≥99% G143A) and sensitive (EC50 ≤1 µg.ml-1 azoxystrobin, ≤1% G143A) isolates were selected. No two isolates coming from the same source were matched. For bulked inoculation, 3 to 5 resistant isolates were pooled and designated as R inoculum. The same was done for 3 to 5 sensitive isolates, then various ratios of R and S inocula were used. A total of six independent experiments were conducted, with two to three replications per inoculation.

Preparation of spore inoculum. Spore suspensions were prepared from heavily sporulating leaves. Leaves (three per isolate) were placed in 20 ml sterile water with 0.005% Tween 20 (STW). The spore suspension was adjusted to a concentration of 10^4 spores ml^{-1}. Five milliliters of this suspension were sprayed on each disease- and fungicide-free grape plant. Different spore ratios of resistant and sensitive isolates (5:95, 40:60 and/or 50:50) were used. These initial spore ratios did not produce the same ratio for the mutant and wildtype alleles in the inoculum when analyzed by qPCR; therefore, for baseline comparison, the actual mutant:wildtype allele ratios were used instead of the initial spore ratio. The sprayed leaves were allowed to dry before placing the plant inside a 10cm-plastic cylinder with a top lid that has a hole inserted with a 1/8 x 3/16 in Tygon tubing attached to the air pump. Plants were incubated on a 12-hr light cycle for the first 24 hr, then constant air flow was introduced also on a 12-hr cycle thereafter for two weeks. The remaining volume of the inoculum was concentrated for DNA extraction.

Spore harvest. Spores were harvested after 14 days of incubation. All leaves with powdery mildew growth were detached, placed in a sterile flask with 30 ml STW and shaken to dislodge the spores. Five milliliters of the resulting spore suspension were applied on a new cane to start another cycle. The remaining spore suspension was concentrated by vacuum filtration through a 0.22-µm-PES membrane filter (Scientific Tisch), re-suspended in 0.5 ml STW in 2-ml conical tubes, then frozen at-50^{o}C or directly used for DNA extraction. Spores were harvested at 14 day-intervals to make one to four cycles, depending on the experiment.

DNA extraction. To extract DNA, frozen fungal material was thawed, then centrifuged at 10,000 rpm for 10 min. Six sterile 2.5-mm-glass beads (Biospec Products) were added to the resulting pellet. Spore disruption was done three times using the FastPrep Bead Beater (MP Biomedicals) with tube immersion in liquid nitrogen after the first and second bead-beating. The disrupted spores were re-suspended in 300 µL Buffer RLT (Qiagen) immediately after the third bead-beating. DNA extraction was performed using the Biosprint 15 DNA Plant Kit (Qiagen) in the Biosprint 15 workstation (Qiagen) with the program set for plant DNA according to the manufacturer's protocol. The resulting DNA solutions were stored at -20^{o}C.

Real-time PCR of the G143A allele. Amplifications were performed in the iCycler iQ instrument (Bio-RAD) using the SYBR Green I Fluorescent dye detection method (4, 8). Reactions were prepared in 25-µL volumes containing 12.5 µL of 2x SYBR Green Mix (Biorad), 5 µL template DNA, and 2.5 µL of each primer (1 µM). Negative controls included water in place of DNA template. The forward primer for the wild-type allele (G143) was PMWd (5'-TACGGGCAGATGAGCCTATGCGG-3'), and for the mutant allele (A143) was PMMt (5'-TACGGGCAGATGAGCCTATGCGC-3'). The reverse primer PMR (5'-ACCTACTTAAAGCTTTAGAAGTTTCC-3') was the same for both reactions. For each DNA sample, at least two qPCR reactions were prepared. Three-step PCR amplifications were done using the following parameters: pre-heating at 95^{o}C for 4 min, followed by 35 cycles at 95^{o}C for 10s, 58^{o}C for 15s and 72^{o}C for 15s. Data collection was carried out at 72^{o}C. The absence of non-target products was confirmed by automated melting curve analysis. The G143A frequencies were computed from C_T (threshold cycle) values for each DNA sample using the equation:

$$\%G143A = \frac{1}{1 + 2^{C_T(\text{mutant}) - C_T(\text{wildtype})}} \times 100$$

2.2.6. Competition assay under field conditions. Field inoculation trials were conducted in 2011 and 2012 at the Virginia Tech Glade Road Research Center in Blacksburg. The field trial in 2011 was done from June 13 to September 29. Five sites were established for the treatments, with three large plants (in 2-, 3- or 5-gallon pots) per site. The treatments consisted of three controls (non-inoculated, resistant/R inoculum only and sensitive/S inoculum only) and mixed or R/S inoculum. Two sites were reserved for the mixed inoculum treatment. Inoculation was done on site using a Preval Sprayer (Precision Valve Corporation) with a spore suspension (150 ml, 10^4 spores ml^{-1}) of three pooled resistant isolates, three sensitive isolates or a mix of the six isolates. All the isolates came from different locations in Virginia. Five heavily sporulating leaves for each isolate were used to prepare the inoculum. For mixed inoculation, a 10R:90S spore suspension was used. Water was sprayed on the non-inoculated control plants. Sites were separated by over 100 m, and the locations were chosen to maximize barriers such as bushes, trees and buildings to minimize cross-infection by wind-blown spores. Percent G143A (%G143A) was measured for the inoculum by qPCR as described before, and from growths on infected leaves in two sampling periods (July 4-8 and September 19-27), about three and sixteen weeks after inoculation. New plants were added one or two times to each site to provide additional leaf tissue for infection. Powdery mildew growth was rubbed onto clean grape leaves with or without detaching the source infected leaf from the plant. Rubbed leaves were incubated in the laboratory as described before until profuse sporulation was obtained (about 14 to 20 days). Because the rubbing method generated heavy contamination and hastened leaf necrosis, growth was further transferred by picking spores from all sporulating colonies and depositing on fresh leaves. Surface growth was scraped with a sterile spatula and deposited in a 2-ml cryofuge tube. The remaining growth was collected by flushing with 1.5 ml STW, and added to the tube. Fungal material was frozen at -50^{o}C until DNA extraction. DNA was subjected to qPCR to measure %G143A from the spore inoculum and the samples from the two sampling periods.

The 2012 field trial was conducted from May 31 to June 29, 2012. The procedure was the same except for the following modifications.

(1) The spore ratio was increased to 50R:50S consisting of the same resistant and sensitive isolates deployed in 2011.
(2) A spore suspension volume of 250 ml was sprayed on both sides of the leaves of four large grape plants for each treatment (50R:50S, R, S, or no inoculation) were also used. The four plants inoculated with the mixed inoculum were divided over two sites.
(3) The inoculated leaves were allowed to dry, then the plants were wrapped in perforated plastic bags for 16 hr to promote spore germination.
(4) Three small grape plants (grown in Styrofoam cups) per site were included in the inoculation and received the same treatment as the test plants. After un-bagging, two of the small plants were enclosed in a plastic cylinder, transported back to the laboratory and incubated under laboratory conditions to check for growth and phenotype of the inoculum. The third small plant was incubated for one week in the field before further incubation in the laboratory. The onset of visible powdery mildew and disease severity were closely monitored by visual inspection of leaves five days after inoculation, and every three days thereafter until disease rating.

(5) New plants were also added one week before the first sampling on June 15 to provide for new infection courts. The second sampling was conducted on June 28-29 strictly from the infection on the second batch of plants.

Leaf populations were also collected by rubbing and further transferred to new leaves whenever necessary (due to heavy contamination or leaf necrosis). Three leaves per population were inoculated to ensure enough material for DNA extraction. Twenty leaf populations were obtained for each sampling date from each treatment. DNA was extracted from fungal matter (spores, hyphae and cleistothecia in some cases) collected from the leaf surface, then subjected to qPCR.

2.2.7. Statistical analysis. To determine whether there were significant differences in the growth rate between QoI-resistant and sensitive isolates, the isolates were treated as a random effect in a two-factorial analysis of variance where phenotype was the first factor and rating date (6 to 7 days or 7 to 8 days) as the second factor. The analysis was done in JMP version 9.0 (SAS Institute Inc.).

To address if there was a change over time in %G413A in the competition assays under laboratory conditions, we fit the following generalized linear mixed model in SAS 9.2, assuming that %G413A follows a β-distribution (15).

$$\log\left(\frac{E(Y_{ijk})}{1-E(Y_{ijk})}\right) = \mu + \alpha_i + \alpha_{j(i)} + X_{ijk}\beta,$$

$$\alpha_i \sim N(0, \sigma_1^2)$$

$$\alpha_{j(i)} \sim N(0, \sigma_2^2)$$

where μ is the overall mean of G413A at day zero, α_i is the effect for population i of %G413A, $\alpha_{j(i)}$ is the effect for replication j within population i, X_{ijk} is kth measurement of time in days for rep j within population i, β is the effect of time on G413A, σ_1^2 is the variance of the distribution of possible populations for G143A, and σ_2^2 is the variance of the distribution of possible replications from each population. Note, σ_2^2 was assumed to be the same for all populations.

2.3. Results

2.3.1. Genetic diversity and phenotypic stability of single spore isolates. To ascertain the resistance phenotype, the percent G143A was checked for each isolate. All resistant isolates still possessed very high levels of the mutation (>95%), and all sensitive isolates with very low levels of G143A (<1%), after two years of subculturing on fungicide-free host leaves (Table 2.1). This indicates that the QoI-resistance phenotype based on the mitochondria-borne G143A is stable in *E. necator* cultures maintained on fungicide-free host.

Microsatellite analysis was performed to provide a background of the genetic diversity of the isolates. The allele sizes and number of alleles obtained in this study fell within the expected range (20) with some exceptions (Table 2.2). For the *EnMS7* locus, for example, Frenkel's team

reported a size range of 187-210 bp but we obtained alleles of 187-211 bp in length. There were a few other 'new' fragments recorded for all three loci in Virginia isolates that were one base pair-different from those reported by Frenkel's group (Table 2.2). The *EnMS1* locus was also confirmed to be relatively hypervariable as reported before (20). We retained this in our analysis since it differentiated several of the isolates that shared alleles for the other two loci. In general, the diversity among the isolates was very high as the analysis generated 24 unique multi-locus haplotypes for 29 isolates tested (Table 2.1). There were 13 haplotypes among the resistant group and 12 haplotypes among the sensitive group. Three sensitive isolates (BLP4, BLP6, CB9-1) obtained from Blacksburg and Christiansburg, Virginia, shared the same alleles for the three loci; the same was true for three other sensitive isolates (PBP1, SHP6, VIP6) from different sources. A QoI-resistant isolate (VAHP4) also shared the same haplotype with a sensitive isolate (GRP1) taken from a different location.

2.3.2. Persistence of QoI resistance in a vineyard population. A vineyard in Nelson County, Virginia was selected as the survey site because it satisfied the three requirements for this analysis, namely, documentation of QoI resistance in the area in the last year (2007) of QoI application, the withdrawal of QoI fungicides from the spray program starting in 2008, and the presence of powdery mildew infection in the 2008 to 2011 sampling years. Only a few isolates were obtained and tested for QoI resistance in 2007 (8), the last year that QoIs were applied to the vines, according to the spray record provided by the vineyard manager. All four 2007 isolates were resistant, with the G143A >95% (Table 2.3). In 2008 to 2009, the first two years that QoI was not included in the spray program, six isolates were tested to be QoI-resistant. More intensive sampling was done in 2009 to 2011 with the collection of a large number of leaf populations. The percent G143A for these leaf populations was a range of values from 0% to 100%. Some leaves harbored completely resistant populations (100%), while others were infected with a completely sensitive population (0%). Heterogeneous populations were also found. In all sampling years, high percentages of G143A were obtained, and more leaf populations with high G143A than with low G143A were obtained in 2009 to 2010. These findings support the persistence of QoI resistance in the pathogen population even without QoI selection pressure.

The Y136F mutation in CYP51 was also detected in populations with >5% G143A and in all completely QoI-resistant populations (>99% G143A) (Table 2.3). Meanwhile, the mutation was absent in all QoI-sensitive populations in all sampling years. Since the Y136F mutation is correlated with DMI resistance (9), we concluded that resistance to this other fungicide group also developed in the vineyard. Furthermore, the findings point out the occurrence of double resistance in *E. necator*.

A physical mapping of the leaf samples taken in 2011 (Fig. 2.1) revealed that resistant sub-populations were confined up to the 14th row, and these rows were also closest to the 'hotspot' section where powdery mildew outbreaks showed up yearly according to the vineyard manager. The percent G143A in leaf populations sampled from row 18 to 31 was nil. The decreasing proportion of resistance away from the hotspot region supports the idea of a focal origin in this vineyard.

2.3.3. Growth rate and competitiveness under laboratory conditions. For the first six days after inoculation, powdery mildew growth on grape leaves was sparse for many of the inoculated spores, making measurements difficult. The diameters of colony growth were thus

measured only starting day 6 from the time of inoculation. A significant interaction was found between phenotype and rating date (P=0.03), thus, the two growth rates for each isolate were not averaged. The mean growth rate measured from 6 to 7 days after inoculation was higher for sensitive isolates (1.2 mm day^{-1}) than resistant isolates (1.0 mm day^{-1}) but the difference was not significant (Table 2.4). An opposite trend was observed in the growth rate taken from 7 to 8 days after inoculation, with a higher mean growth rate for resistant isolates (0.88 mm day^{-1}) than for sensitive isolates (0.78 mm day^{-1}). Again, the difference was not statistically significant.

The persistence of QoI resistance in the vineyard population led us to test the hypothesis that it does not carry a fitness cost; hence, the competition assays. In the laboratory, mixed cultures of powdery mildew sprayed as spore suspensions at a concentration of 10^{-4} spores ml^{-1} were able to grow on fungicide-free grape plants. Sporulation was generally abundant after 14 days of incubation. This permitted the collection of spores from infected leaves for cycling to new plants and for DNA analysis.

In paired inoculations (Fig.2.3A-B), the %G143A tended to increase in almost all the populations cycled on grape canes incubated under controlled conditions. A decline in %G143A in bulked inoculations was rarely observed (Fig.2C-F). Also, some populations did not change appreciably in the final cycle (see Fig. 2A, D-F). These were populations with an initially low resistant fraction (5-10% G143A). In one inoculation (Fig.2E), the population, starting out at about 60% G143A reverted to about 20% G143A after one cycle, suggesting that less fit resistant strains could exist. Overall, by fitting all the data into a Generalized Linear Mixed model with the assumption of a beta-distribution for the %G143A, an increase in %G143A in mixed populations was observed through time in the absence of the fungicide (P=0.03, Fig. 2.4). This indicates the competitiveness of QoI-resistant isolates in the absence of the selection pressure under the conditions employed in this study.

2.2.4. Competitiveness under field conditions: 2011 trial. To determine the responses of the mixed inoculum under field conditions, we conducted the competition assay on potted plants set outdoors. Starting with a low proportion of resistance (5% G143A), there was no drastic increase in the %G143A after one cycle of infection in R/S-inoculated plants (Fig. 2.2). In one site, the resistant fraction increased to 14% but was followed by a drop to a sensitive range (mean = 0.1%) after another 10 to 11 weeks of infection. An opposite pattern was observed in the second site, with the population decreasing first to a 0.1% followed by an increase back to 5% on the second sampling. The S population remained low at <1% G143A. Unexpectedly, the %G143A in the resistant population quickly dropped to a sensitive range (mean=0.1) in the field. Spore migration between treatments and immigration from outside sources may be responsible for this decline. The infection on non-inoculated control plants, while remaining low at <1% on the average, gave at least one leaf population with a 3% G143A (data not shown), suggesting that spore migration from one inoculated site to another was possible. The non-inoculated plants developed powdery mildew somewhat later than inoculated plants, but disease development was still vigorous by mid-summer. There are no known vineyards nearby, and few known grapevines, but they are likely to exist in residential neighborhoods several 100 meters to a kilometer away. Mere mixing of inoculum among treatments would have been expected to result in approximately 30% G143A while the actual percentage in July-September was clearly lower than 30%. It appears plausible that the decline

in the resistant population was due to background infection from outside sources such as backyard and wild grapevines.

2.2.5. Competitiveness under field conditions: 2012 trial. To determine the extent of background infection and migration, the competition trial was repeated in 2012. Visible powdery mildew was first observed 11 days after inoculation only on inoculated plants for both test canes (data not shown) and field plants (Table 2.5). This also indicates that the sprayed inoculum was able to cause infection under field conditions. The absence of powdery mildew on non-inoculated plants suggests that spore migration of the introduced inoculum or background infection from nearby sources had not occurred yet during that period. The initial infection observed on the inoculated plants most likely represented the competing populations from the sprayed inoculum. Severity ratings almost two weeks after inoculation were generally higher for plants inoculated with resistant population (R) than with sensitive population (S), suggesting a higher inoculum deposition for the former (Table 2.5).

The non-inoculated control plants were checked regularly for the onset of infection. The final evaluation was on July 15, and only a few leaves showed visible powdery mildew growth (<1% surface area, data not shown). Late June to mid-July samples taken from the non-inoculated plants did not have the G143A (0%) except for one leaf sample (5% G143A, Table 2.6), which indicates that spore migration did occur in the field over 100 m despite the physical barriers. The R and S populations remained the same throughout the infection season, except for one R and one S leaf population, with 70% and 13% G143A, respectively (Table 2.6). This further supports the occurrence of spore migration in the field within four weeks of infection.

The small and large plants receiving the mixed population consisting initially of 63% G143A developed a heterogeneous infection (27%-100% G143A, Table 2.6). On the average, the powdery mildew populations on the small plants harbored 84% and 93% G143A. On field plants, these were 95% and 100% G143A two weeks after inoculation, and 85% and 75% another two weeks later, for the first and second sites, respectively. Throughout the monitoring period, resistance (>95% G143A) did not disappear from the population.

2.4. Discussion and Conclusions

E. necator isolates harboring >99% G143A and therefore QoI-resistant retained the phenotype even after more than fifty transfers on fungicide-free grape leaves, indicating the stability of the resistance phenotype when grown in isolation. This means that the G143A-carrying mitochondria in the resistant fungal cell are not lost through repeated sub-culturing unlike in *Blumeria graminis* f. sp. *tritici* which lost the mutation and regained the wild-type allele after several transfers on fungicide-free media (17). The persistence of resistant populations of *E. necator* was observed for four years (2008 to 2011) in a vineyard that has withdrawn strobilurins (QoI) from the spray program. We do not know if QoI resistance will persist longer than that in this vineyard without the selection pressure. If strobilurin is re-applied to this population, it will probably take only one or a few fit resistant cells to evade fungicide effects, reproduce rapidly and spread the mutant allele in a large-scale area within a short period of time.

Vineyard leaf samples collected before 2011 came solely from a ‘hotspot’ section which suffered powdery mildew infection yearly, according to the vineyard manager. Pathogen leaf

populations harboring high % G143A were detected from this section every sampling year without QoI application. It appears that there was a fair amount of mixing in the pathogen population in the vineyard as shown by very heterogeneous populations throughout the sampling years even from the hotspot section. Sensitive leaf populations (<1% G143A) were regularly detected as well. These populations either remained as persistent as the resistant populations or were constantly introduced from outside sources. Wild grapes are known to grow in the wooded area adjacent to the vineyard, and these may be sources of wild-type powdery mildew. We have successfully cultured powdery mildew isolated from a wild variety on *Vitis vinifera* cv. Chardonnay, indicating that gene flow can occur from one species to another. In *E. necator*, little host specialization has been found among *Vitis* species, except for *V. rotundifolia* (5, 21). A vineyard map of our leaf sample data indicates that resistance was confined within the first 14 rows (Fig. 2.1), close to the hotspot section (row 1). The back rows (rows 18-31) harbored only sensitive populations. Immigration of sensitive populations from wild grapevine or other sources may be responsible for this pattern of distribution.

It is also not clear how the hotspot section came about initially and why infection persisted in this area every year. Strobilurins were not applied starting in 2008, but DMIs were still part of the disease control program in this vineyard through all years of sampling. DMI resistance in *E. necator* operates under a different mechanism and, at least in part, involves a mutation (Y136F) in the CYP51-target molecule (9, 12). We hypothesized further that the DMIs selected for DMI-resistant strains that were also QoI-resistant. A SNP genotyping method (Chapter 4) was employed to detect the presence of Y136F. Indeed, all the populations that harbored some level of G143A including 100% G143A-populations also carried Y136F, and those lacking G143A also lacked Y136F. Therefore, in this vineyard, *E. necator* strains with double resistance did exist. This is not surprising as the population has received several exposures to DMI and QoI formulations for more than five years, and in several years even both, allowing for the continuous selection of QoI- and DMI-resistance. Double resistance to DMI and QoI in *E. necator* was first reported in Europe (13). DMI-resistant isolates may be predisposed to accelerated development of QoI resistance as demonstrated for *Monilinia fructicola* (27). This type of resistance can limit the use of DMIs and QoIs as rotational or tank mix partners. It is very likely that the yearly incidence of disease in the hotspot area despite the withdrawal of QoIs was due to a decline in disease control efficacy of DMIs. The durability of QoI resistance in this population was probably further enhanced by DMI-selection of the double mutants.

The persistence of resistance in the vineyard population also prompted the testing of fitness of QoI-resistant *E. necator*. Fitness of resistant individuals in a field population is critical to their persistence once they are selected for (29). It is the survival and reproductive success of an allele, individual or group (32). A lowered fitness among resistant isolates will consequentially reduce their survival in the absence of the selection pressure. If resistant isolates are as fit as sensitive isolates, they are bound to persist in the population even after the fungicide application is stopped. Experimental results generally show that vegetative and reproductive fitness costs associated with fungicide resistance vary with fungal species and with fitness parameters measured (11). To determine fitness cost, competition experiments are done where a resistant population is paired with a sensitive population and grown in the absence of the fungicide. Some fitness attributes, usually two or more of the following - sporulation, germination, and germ tube length, latent period, virulence and mycelial growth - are measured,

then compared between resistant and sensitive isolates. The fungicide in question can be an important determining factor in the stability or instability of resistance in certain pathogens. For example, benzimidazole-resistant isolates of *C. beticola* did not exhibit any fitness penalty but strains resistant to fentin fungicides were less competitive than sensitive strains (26).

In our competition experiments, we followed the same principle that Schepers (34) applied in his fitness testing. We utilized many pairs and bulked combinations of resistant and sensitive isolates collected from the field in order to reduce side-effects brought about by different genetic backgrounds among the isolates. Fitness is a result of interacting factors in the whole genome, which implies that not all observed differences in fitness can be attributed to the resistance phenotype. Ideally, two genetically identical isolates which differ only in fungicide sensitivity are needed. This approach cannot be done with *E. necator* because of the lack of such isolates and the lack of an approach to generate resistance under controlled conditions; hence the use of several isolates with different genetic backgrounds in fitness testing. In general, our microsatellite analysis supported the findings of Frenkel's group about the high genetic diversity of *E. necator* in the eastern part of the United States (20). The high genetic diversity among our resistant isolates supports the idea that at some point in time, G143A arose at low frequency in genetically diverse populations from different locations and subsequently increased along with exposure to strong selection pressure.

Collectively, in this present study, there was no difference in the growth rate between resistant and sensitive isolates on host tissue incubated in the laboratory. This means that the G143A in the cytochrome *b* has no harmful effect on the growth of *E. necator* under controlled conditions in the absence of the fungicide. It is, however, difficult to predict the overall fitness of one population relative to another based only on one fitness parameter; a combination of parameters factored into a fitness index is more informative (10). Another way of determining fitness costs is by allowing the isolates to compete in mixed populations, hence, our competition trials using genetically diverse isolates with or without G143A-based resistance. Under laboratory conditions, our resistant isolates tended to be more competitive than sensitive isolates through several generations in the absence of selection pressure, indicating a lack of significant fitness penalty to QoI resistance. It is possible that a fitness cost is compensated for by second-site mutations in another locus (or other loci) to restore or even increase fitness (24). In *Saccharomyces cerevisiae*, the G143A mutation was shown to have lower respiration and growth (36). However, a secondary mutation in *cyt b* and over-expression of certain nuclear genes with mitochondria-related function restored the respiratory fitness of the resistant mutant. This compensatory effect could possibly occur in plant pathogens since heterologous expression of one of the nuclear genes from *Botrytis cinerea* in *S. cerevisiae* produced the same result.

Under field conditions, the competitiveness of resistance was not obvious. In the 2011 field inoculation, there was neither a consistent decline nor increase in %G143A for mix-inoculated plants. In plants inoculated with only resistant spores, the %G143A unexpectedly declined from 100% to <10% (Fig. 2.2). Powdery mildew collected from the non-inoculated plants (background infection) remained low in %G143A, suggesting that the drop on the R-inoculated plants could be largely due to the influx of sensitive strains and to some extent, spore migration from one treatment to another. This drop was not observed in the 2012 field inoculation trial. The effect of background infection may have been more pronounced in the first field trial because of the longer interval between inoculation and sampling (4 weeks), and

between first and second sampling periods (5 weeks), allowing for more time for gradual mixing of populations. Although the persistence of resistance for at least four weeks under our field conditions was demonstrated in the 2012 trial and the vineyard survey, there was also evidence that the sensitive fraction can also persist. We expected that if the resistant strains are competitive, the vineyard population would have evolved into 100% QoI-resistance. However, this never happened through a long period of time in our vineyard survey, suggesting that the sensitive populations may be as fit as the resistant populations.

Overall, QoI resistance in *E. necator* did not disappear rapidly when the selection pressure was removed. Thus, relying on selection against resistance is not a practical approach to resistance management. Once resistance develops to high frequencies and is not associated with fitness penalties, using QoIs even in mixtures, a commonly recommended strategy to slow down resistance, could still maintain the resistant allele in the population. It is also important to determine if resistance to other chemistries used in the vineyard is present before mitigating the situation. In the case of our vineyard with persistent QoI resistance, DMI resistance has also developed, which could explain the yearly occurrence of powdery mildew infection despite application of DMI sprays and the withdrawal of QoIs.

Tables and Figures

Table 2.1. *Erysiphe necator* isolates used in the competition experiments and their QoI resistance profile and genetic diversity based on three microsatellite loci.

Isolate	Location	%G143A[a]	EC50[b]	Haplotype[c]
AMP1	Nelson Co, VA	99.9	33.8	1
AVP7	Loudon Co, VA	99.9	>30.0	2
AW9-1	Franklin Co, VA	99.9	2.6	6
BPP1	Accomack Co., VA	99.9	5.5	7
GRP20	King George Co, VA	99.9	>20.0	13
HUP1	Halifax Co, VA	99.9	-	3
IVP11	Westmoreland Co, VA	99.9	23.4	8
JRP4	Hanover Co, VA	99.9	>60.0	9
LIP3	Fauquier Co, VA	99.9	9.1*	4
MDMRP4	Prince George Co, MD	99.9	>3.0	10
PRP4	Madison Co, VA	99.9	>30.0*	11
ROP6	Rockbridge Co, VA	99.9	56.3	12
VAHP4	Patrick Co, VA	99.9	>3.0	5
WI9-1	Frederick Co, VA	99.9	>1.0	-
BLP4	Montgomery Co, VA	0.02	0.03	18
BLP6	Montgomery Co, VA	0.01	0.01*	18
BLP11	Montgomery Co, VA	-	-	17
CB9-1	Montgomery Co, VA	0	<0.1	18
CL9-3	Montgomery Co, VA	0.02	-	19
CMP1	Floyd Co, VA	0.02	0.03	23
GRP1	King George Co, VA	0	<0.3*	6
FH9-1	Rappahannock Co, VA	0.01	<0.1	15
HCGP4	Anne Arundel Co, MD	-	0.06	-
LI9-1	Fauquier Co, VA	0.02	<0.1	20
MVP9	Halifax Co, VA	0.1	0.06	21
PBP1	New Kent Co, VA	0.03	0.06	22
SCCP4	Surry Co, NC	0.02	0.1	16
SHP6	Surry Co, NC	0.02	<0	22
SHP7	Surry Co, NC	0	0.14	13
SNP1	Augusta/Albemarle Co, VA (county line)	0.03	<0.01	24
SUP9	Greene Co, VA	0	0.5*	-
VIP6	Fairfax Co, VA	0.01	0.2	22

[a] Measured by qPCR; values>99%=resistant; <1%=sensitive

[b] Values with asterisk (*) are data from Colcol 2008; rows with dash (-) indicate no data because culture was lost before assay

[c] Based on microsatellite analysis for three loci *EnMS1*, *EnMS3*, *EnMS7*; primers and method adapted from Frenkel et al. (2011). The numbers refers to the haplotype coding arbitrarily assigned by the authors; rows with dash (-) indicate no data because culture was lost before assay

[d] Diameter is equivalent to the number of divisions subtended by the colony on the ocular micrometer

Table 2.2. Microsatellite alleles and their frequencies in 29 *Erysiphe necator* single-spore isolates.

EnMS1 locus		*EnMS3* locus		*EnMS7* locus	
Size	Frequency	Size	Frequency	Size	Frequency
239	0.29	236	0.35	193*	0.29
251	0.16	233	0.23	196*	0.23
266	0.16	222	0.19	199*	0.13
236	0.10	228*	0.10	187*	0.10
252*	0.10	231*	0.03	211*	0.06
269	0.06	239	0.03	198	0.06
245	0.03	242	0.03	190*	0.06
250*	0.03	243*	0.03	208*	0.03
254	0.03			202*	0.03
257	0.03				

*alleles not reported by Frenkel et al. (20).

Table 2.3. Percent G143A profile of and occurrence of CYP51 genotypes in *Erysiphe necator* leaf populations in one vineyard that has withdrawn QoI fungicides since 2008.

	2007[c]	2008	2009		2010		2011	
No. resistant[a] isolates/ # tested	5/5	4/4	2/2		-		-	
			% G143A	CYP51[d]	% G143A	CYP51[d]	% G143A	CYP51[d]
%G143A profile of leaf populations[b]	-	-	99	mix	100	Mix	100	mix
			95	mix	100	mix	100	mix
			55	mix	100	mix	100	mix
			42	mix	100	mix	68	mix
			21	mix	100	mix	65	mix
			5	-	99	mix	65	mix
			5	WT	90	mix	59	mix
			0.1	WT	43	mix	43	mix
					27	mix	23	mix
					5	WT	22	-
					1	WT	16	mix
							10	mix
							7	-
							5	-
							2	WT
							0.01	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT
							0	WT

[a] Resistant= 99.9% G143A

[b] Leaf population=all powdery mildew growth on the leaf; each %G143A value represents a leaf population

[c] Reported by J. Colcol (2008)

[d] Based on a SNP genotyping method using multiplex PCR with Taqman chemistry; 'mix' has both Y136 wildtype allele and F136 mutant allele; WT= only wildtype allele Y136 was present

Table 2.4. Mean growth rate (mm day^{-1}) of QoI-resistant and sensitive isolates grown on fungicide-free grape host leaves.

Phenotype	Rating Date (days after inoculation)	n	Mean growth rate (mm day^{-1})
Sensitive	6-7	24	1.20±0.05a
Resistant	6-7	18	1.04±0.06ab
Resistant	7-8	18	0.86±0.05bc
Sensitive	7-8	24	0.78±0.05c

Table 2.5. Disease severity ratings (percent leaf surface area*) of powdery mildew on grape plants inoculated with different populations of *Erysiphe necator* and situated in sites at least 100 m-distance away from each other, for the competition trial under field conditions, rated June 13, 2012.

Plant	Control-site 1	R/S-site 2**	R/S-site 3	R-site 4	S-site 5
A	0	33		27	14
B	0	29		48	20
C	0		17	17	8
D	0		26	36	10
Mean/site	0	31	22	32	13

* Mean of rating from 5 leaves.

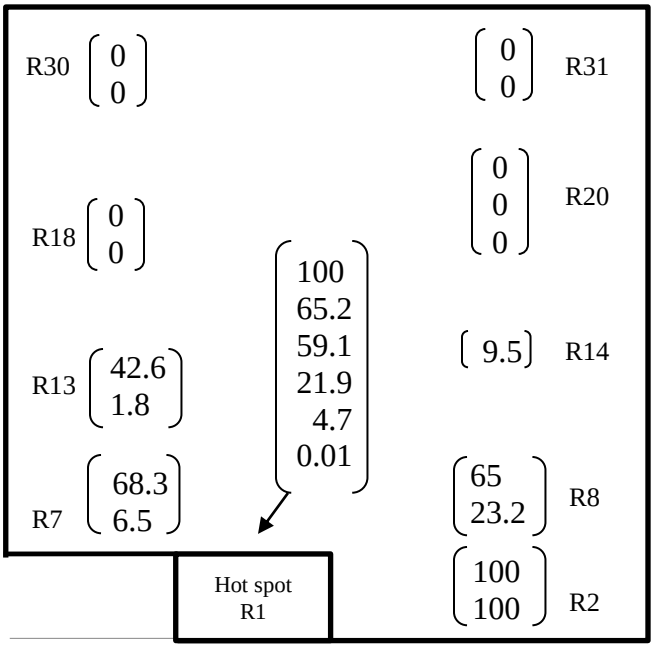
** R/S-mixed inoculum consisting of resistant (R) and sensitive (S) isolates at 50:50 ratio.

Table 2.6. Changes in QoI resistance (%G143A) in mixed resistant and sensitive *Erysiphe necator* populations cycled on fungicide-free grape plants under 2012 field conditions.

Source	%G143A[a]				
	Resistant	Sensitive	Mixed Population		Control[b]
May 31					
Spore Inoculum 1	100	0	61.3		
Spore Inoculum 2	100	0	63.4		
Spore Inoculum 3			63.5		
Mean	100	0	62.7		
June 1			Site 2-R/S	Site 3-R/S	
Test cane 1	100	0	27-100	73-100	-
Test cane 2	100	0	100	100	-
Test cane 3	100	0	80-100	65-100	-
Mean	100	0	84.2	93.2	
June 15					
Field plant A	100	0-0.01	73-100		-
Field plant B	100	0	81-100		-
Field plant C	100	0		96-100	-
Field plant D	72-100	0-0.69		100	-
Mean	98.5	0.001	95.4	99.9	
June 28-29					
Field plant E	100	0-12.6	67-100		0-5
Field plant F	60-100	0	62-100		(July 15)
Field plant G	100			45-91	
Field plant H	100	0		51-99	
Mean	96.6	3.2	86.4	74.2	0.5

[a] Five leaf populations per test cane and field plant

[b] Ten leaf populations from the non-inoculated control plants; sampling on June 21, 29 and July 15. Rows with dash (-) means no analysis because there was no visible powdery mildew growth.



Fig, 2.1. Map of the pattern of QoI resistance in a vineyard surveyed from leaf populations sampled in 2011. Only rows (R) where samples were taken are numbered. Values in brackets indicate % G143A. The number of values represents the number of leaf populations tested by qPCR.

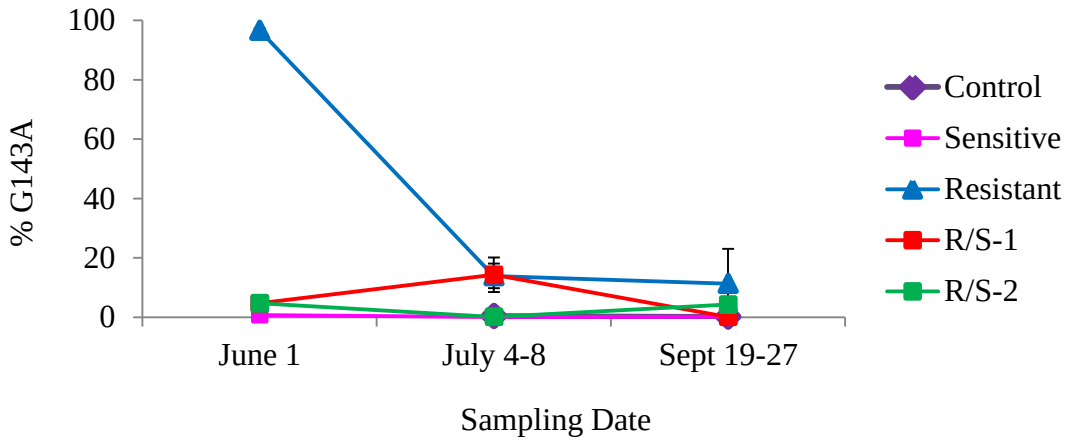


Fig.2.2. Population changes in the percent G143A in mixed inoculations of QoI-resistant and sensitive *Erysiphe necator* isolates on fungicide-free potted grape plants under field conditions. Initial %G143A was based on the spore inoculum (June 1). Field samples were collected on July 4-8 and Sept 19-27, 2011.

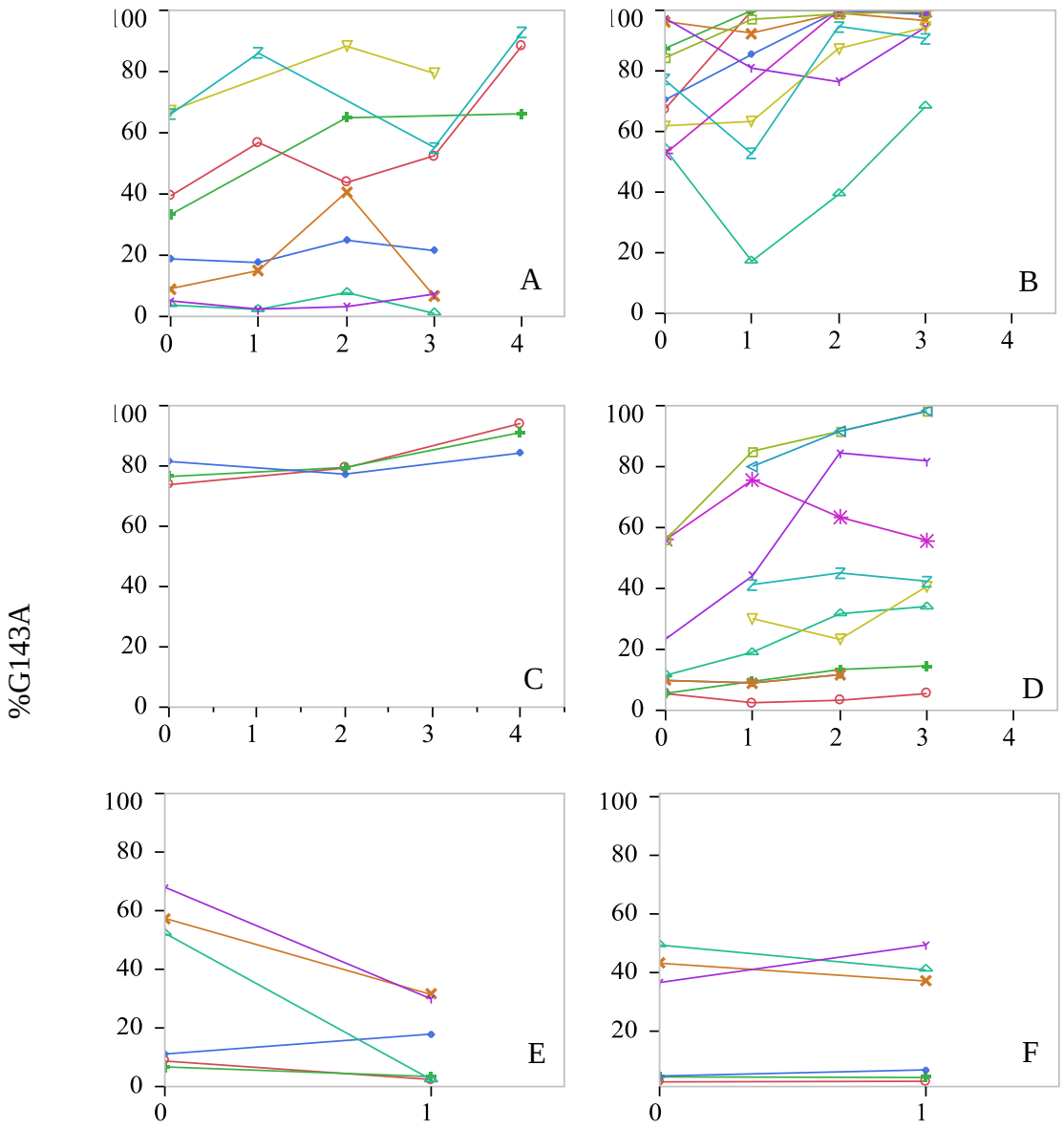


Fig. 2.3. Changes in percent G143A in several mixed (QoI-resistant and QoI-sensitive) populations of *Erysiphe necator* cycled in fungicide-free grape plants. Cycle numbers are indicated as CO, C1…C4. One cycle is equivalent to 14 days of incubation. Each graph (A to F) are independent experiments using artificially mixed inoculum consisting of QoI-resistant ad sensitive isolates. Each line in A and B refers to a paired inoculum consisting of a unique combination of one resistant and one sensitive isolate. Graphs C to F include data from competition assays employing bulked inoculum (at least 3 resistant isolates mixed with at least 3 sensitive isolates) at various starting ratios. Inoculum composition in experiments C, D, E and F are not the same.

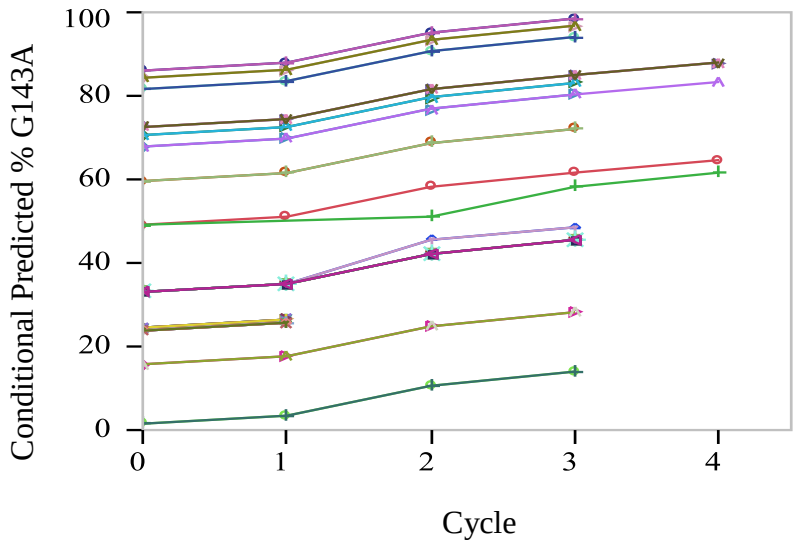


Fig. 2.4. Plot of the conditional fitted values of %G143A over cycle (0,1,2,3,4) of thirteen *Erysiphe necator* artificial populations consisting a unique mixture of QoI-resistant and sensitive single-spore isolates inoculated on fungicide-free grape plants and cycled to new plants from one to four times under laboratory conditions. Analysis was done by the Generalized Linear Mixed model, SAS v. 9.2 (P=0.03).

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C**hapter 3**

Exploring QoI Resistance Mechanisms in *Erysiphe necator*

Abstract

QoI resistance in the grapevine powdery mildew (*Erysiphe necator*) is a major practical problem in many grape-growing areas. The G143A mutation in the target molecule cytochrome *b* confers high resistance factors, and is known to be the major resistance mechanism in many pathogens. There are, however, QoI-resistant isolates that lack this mutation. To investigate the mechanism of this type of resistance, three isolates were exposed to a 'training' method, where they were cultured in parallel on azoxystrobin-treated and fungicide-free grape leaves. Using this method, one isolate out of three underwent a genetic shift only on fungicide-treated host tissue, with the G143A mutation reaching 100% after 14 culture transfers. This is evidence of the heteroplasmic nature of the mitochondria in *E. necator*. To explore an alternative oxidase pathway (AOX) as another mechanism of resistance, two AOX inhibitors were tested. Both salicylhydroxamic acid (SHAM) and propyl gallate were greatly inhibitory to spore germination at 100 µg.ml^{-1} but toxicity was reduced at 10 µg.ml^{-1}SHAM, allowing for tests on AOX inhibition at this concentration. The sensitivity to azoxystrobin of a sensitive isolate was increased four-fold in the presence of SHAM (10 µg.ml^{-1}), but no significant change in sensitivity was observed for resistant isolates that lacked G143A. On the other hand, an almost three-fold increase in EC_{50} of a resistant isolate possessing >99% G143A was observed in the presence of SHAM, but the mechanism of enhanced resistance to QoI is still not clear. These findings provide no strong support for a role of AOX in non-G143A-based resistance in *E. necator*.

Keywords: QoI resistance, azoxystrobin, *Erysiphe necator*, alternative oxidase (AOX), cytochrome *b*, SHAM, propyl gallate, heteroplasmy

3.1 Introduction

Quinone outside inhibitors (QoIs) constitute an agriculturally important fungicide class targeting a broad spectrum of plant pathogens in many crops. The mode of action is specific to inhibition of mitochondrial respiration (7). QoI molecules bind the outer quinol-oxidation site of the cytochrome bc_1 enzyme complex, preventing electron flow between cytochrome *b* and cytochrome *c1* and impairing ATP production (4). Energy-demanding processes in the pathogen such as spore germination and zoospore motility are highly affected, leading to growth inhibition, which gives QoIs an excellent protective activity (41). QoIs may have curative effects as well likely due to mycelium collapse (23) although QoIs are hardly sprayed in the field for this sole purpose.

QoI resistance has been widely attributed to a single amino acid change in the mitochondria-encoded cytochrome *b*. Because this mutation was widespread in many pathogen species from various geographical locations, the inherent resistance risk for QoI fungicides was estimated to be high (15). In fact, decline in disease control efficacy due to QoI resistance is now a problem in several pathogens of various crops (12-14, 17). Three single amino acid mutations in cytochrome *b* – G143A, F129L and G137R - are associated with naturally QoI-resistant pathogens (26). The G143A mutation is the most common in pathogens including the major grapevine powdery mildew *Erysiphe necator* (5), and is associated with high resistance factors (16). The F129L substitution confers lower levels of resistance (31, 39) in *Pyricularia grisea* (22), *Pythium aphanidermatum* (16), *Pyrenophora* sp. (36) and *Alternaria solani* (32). G137R is also associated with similar resistance level as F129L and was found at low frequency in field isolates of *P. tritici-repentis* (36).

Although target site variations are commonly associated with QoI resistance, other mechanisms may exist. Fungi may circumvent QoIs via an alternative oxidase (AOX) in the respiratory pathway. AOX acts in parallel with the bc_1 complex for oxidation of ubiquinol, and accepts electrons probably directly from ubiquinol for reduction of oxygen to water (20). In the presence of cytochrome bc_1 inhibitors, the fungus is still capable of ATP production, albeit at lower efficiency. Thus, the AOX rescues the fungus from complete respiration breakdown. To implicate a role for AOX in QoI resistance, fungal AOX inhibitors such as salicylichydroxamic acid (SHAM) and propyl gallate are commonly employed in fungicide assays, where the QoI activity may or may not be potentiated in the presence of the inhibitor (1, 2, 18, 19, 28, 34, 37). The practical significance of AOX in fungicide resistance has met some criticism because of its poor energy yield and a lack of activity *in planta* probably due to inhibition by plant flavonoids (41, 45). Nevertheless, a role for alternative oxidation in QoI resistance, even though it may sometimes be marginal, has been suggested for a few pathogens (19, 21, 30, 34, 45).

Colcol (8) surveyed QoI resistance in *E. necator* and found it to be widespread in Virginia and adjacent regions in the United States. The majority of the *E. necator* isolates in her collection were resistant to azoxystrobin (EC_{50} >1 $\mu g.ml^{-1}$). These isolates were also characterized by having a high proportion of G143A (>99%) in their mitochondrial DNA. However, a sub-set of isolates with azoxystrobin resistance lacked G143A (<1%). The objective of this study was to explore on the mechanism for this type of resistance. We demonstrated the potential for an *E. necator* isolate to undergo a genetic shift by increasing the proportion of the mutation in culture with continuous exposure to QoI selection pressure. Additionally, we

explored the utility of commonly used AOX inhibitors in demonstrating the presence of an AOX pathway in *E. necator*.

3.2. Materials and Methods

3.2.1. Isolate source and 'training' experiment. Three isolates (SUP13, GRP15, GRP18) with QoI-resistant phenotype based on leaf disc bioassay but lacking high proportions of G143A were maintained as single-spored cultures on fungicide-free grape leaves since first isolation in 2007. These isolates were first obtained in 2007 from two different locations in Virginia and were part of a 'sub-group' consisting of 14 out of 154 QoI-resistant isolates. The subgroup had $EC_{50} \geq 1$ $\mu g.ml^{-1}$ azoxystrobin and <1% G143A, which is not typical of majority of our resistant isolates (8). Only the three isolates were tested because the rest were lost during long-term culturing. The isolates were maintained on fungicide-free grape leaves for over two years. From a single culture, three sub-cultures for each isolate were started in December 2010 on fungicide-free and on Abound-treated leaves (3 $\mu g.ml^{-1}$ active ingredient, azoxystrobin). Abound was provided by Syngenta Crop Protection, Inc. (Greensboro, N.C.). Parallel culture transfers were done every 15 days from each sub-culture. Whole grape leaves were disinfested by shaking in 20% commercial bleach solution with 0.01% Tween 20 for 2 min, rinsed 3 to 4 times with sterile water then blotted dry. Fungicide solution was prepared using sterile water with 0.005% Tween 20 (STW) then sprayed on leaves using a Preval sprayer (Precision Valve Corporation) until the leaf surface was covered with uniform droplets. Sprayed leaves were immediately placed on 0.7% water agar and air-dried for at least 10 min. At least five individual spore chains were inoculated on each leaf, with three leaves per treatment per sub-culture.

3.2.2. Collection of fungal material and DNA extraction. Fungal material was collected initially after three parallel transfers then at later transfers, by scraping surface growth from three leaves into a 2-ml conical tube. The remaining fungal material was added to the tube by flushing the leaf with 1.5 ml sterile 0.005% Tween 20 water (STW) then frozen until DNA extraction. Frozen fungal material was thawed and centrifuged at 8000 x g for 10 min. Six sterile 2.5-mm glass beads (Biospec Products) were added to the resulting pellet. Spore disruption was done three times at 4 $m.s^{-1}$ for 30 s using the FastPrep-24 instrument (MP Biomedicals) with tube immersion in liquid nitrogen after the first and second bead-beating. Disrupted fungal material was re-suspended in 300 µL Buffer RLT (Qiagen) immediately after the third bead-beating. The suspension was centrifuged at 6,000 rpm for 5 min. DNA extraction was performed with the Biosprint 15 DNA Plant Kit (Qiagen) using the Plant DNA program in the Biosprint 15 workstation (Qiagen) according to manufacturer's protocol. Resulting DNA solutions were stored at -20^{o}C.

3.2.3. Quantitation of G143A by real-time PCR. Amplifications were performed in the real-time thermal iCycler iQ (Bio-RAD) using the SYBR Green I Fluorescent dye detection method (5, 8). Reactions were prepared in 25-µL volumes containing 12.5 µL of 2x SYBR Green Mix (Biorad), 5 µL template DNA, and 0.25 µL of each primer (10 µM stock). Negative controls were water in place of the DNA sample in PCR reaction mix. The forward primer for wild-type allele (G143) was PMWd (5'- TACGGGCAGATGAGCCTATGCGG-3'), and for the mutant allele (A143) was PMMt (5'- TACGGGCAGATGAGCCTATGCGG-3'). The reverse primer PMR (5'-ACCTACTTAAAGCTTTAGAAGTTTCC-3') was the same for both reactions. For each DNA sample, three reactions were prepared. The amplification parameters were as follows: pre-heating at 95^{o}C for 4 min, followed by 35 cycles at 95^{o}C for 10s, 58^{o}C for 15s and

72°C for 15s. Data collection was carried out at 72°C. The absence of non-target products was confirmed by automated melting curve analysis. The G143A frequencies were computed from C_T (threshold cycle) values for each DNA sample using the equation:

$$\%G143A = \frac{1}{1 + 2^{C_T(\text{mutant}) - C_T(\text{wildtype})}} \times 100$$

3.2.4. Testing AOX inhibitors

Testing SHAM and propyl gallate. SHAM and propyl gallate (PPG) were purchased from Sigma-Aldrich Company LLC (St. Louis, MO). Stock solutions were prepared in DMSO. The inhibitor was added to 0.7% water agar at a final concentration of 0.65 mM (in 2% DMSO). Amended agar was dispensed into 60x15mm petri dishes. All cultures used were 14 to 21 days old. A spore suspension (0.5 ml) prepared by dislodging heavy surface growth on a leaf in 15 ml sterile water was deposited on the agar. The spores were distributed by tilting the plate in all directions several times until the entire agar surface was uniformly moistened. The plates were allowed to air-dry for at least 30 min. Plates were incubated in the dark overnight at ambient temperature. The number of germinated spores per 100 spores was recorded as % germination in each of three plates per isolate. A spore was considered germinated if the germ tube length was ≥ 1/2 the spore length.

Testing SHAM and DMSO. Spore germination of five resistant isolates with 99.9% G143A was first tested on water agar amended with DMSO or with SHAM in DMSO. The final concentration of SHAM was at 0.65 mM, but DMSO was reduced to 0.25% in water agar. Spore inoculation was done as described earlier. The number of germinated spores for 100 spores was counted in three non-overlapping sections of the plate, two plates for each treatment for each isolate.

In the second test, representative isolates from each sensitivity group were included: (i) resistant (EC50>5 $\mu g.ml^{-1}$, 99.9% G143A), (ii) resistant (EC50<1) with <1% G143A, and (iii) sensitive (EC50<1) and <1%G143A. The final SHAM and DMSO concentrations were reduced to 0.325 mM and 0.125%, respectively. Spore inoculation, incubation and determination of percent germination were done as described before.

Lastly, isolates used in one or more previous tests from each sensitivity group were selected to determine percent germination at a final concentration of 0.065 mM for SHAM and 0.025% for DMSO. Spore germination was tested on water agar with or without chemical. The same procedures as described before were followed for spore inoculation and germination counts.

3.2.5. Testing SHAM with azoxystrobin. One isolate each from the sensitive and G143A-resistant groups, and two from the non-G143A resistance group were tested for the effect of SHAM with azoxystrobin (QoI). The agar concentration was increased to 1.5% to reduce available water known to be inhibitory to spore germination of *E. necator* (6), and DMSO or SHAM was added to water agar at 0.025% and 0.065 mM, respectively. DMSO-amended water agar served as the control. Azoxystrobin was added to water agar, with or without SHAM at

0.01, 1 or 10 mg.ml^{-1}. Azoxystrobin was provided by Syngenta Crop Protection, Inc. (Greensboro, NC). Spore inoculation was done as previously described. Three readings from non-overlapping sections of the plate were averaged as percent germination for that plate. Three petri dishes were employed for each treatment. Relative percent germination was calculated from the mean percent germination of the control. To estimate the EC_{50}, relative percent germination of treatments was regressed on ln-transformed fungicide concentration. From the linear section of the regression, the EC_{50} was calculated in Microsoft Excel as described by Colcol et al. (9) as follows: $EC_{50} = e^{([50\text{-}b]/m])}$, from the regression equation y = *m*.1n (concentration) + b.

3.2.6. Statistical analysis. One-way ANOVA was performed on transformed (square root) % germination values using JMP v. 9.0 (SAS Institute Inc.) to determine differences among treatments (amended and un-amended water agar) by isolate. Means were compared using Tukey-Kramer HSD test in JMP.

3.3. Results

3.3.1. Selection of G143A in single-spore isolates. Initial testing of growth on Abound-treated leaf (3 µg.ml^{-1} azoxystrobin) showed >50% relative diameter (data not included), indicating that SUP13, GRP15 and GRP18 remained QoI-resistant even with long-term subculturing in the absence of QoI selection pressure. Only SUP13 exposed to azoxystrobin by continuous transfer on Abound-treated-leaves shifted to 100% G143A (Table 3.1). The shift was observed at the 14th transfer for all three subcultures. The counterparts maintained on fungicide-free leaves remained low in A143, except for one which shifted to 100% G143A at a later transfer (T25). This may have been due to contamination from mishandling of cultures during transfer or from fungus-feeding mites that could carry spores from one culture to another, or the sample may have been mislabeled after collection of surface growth.

3.3.2. Toxicity of SHAM and propyl gallate. Seven isolates were selected to represent the three sensitivity levels in the initial testing of SHAM and propyl gallate (PPG): (i) AMP1, JRP4 and VAHP4 were highly resistant (EC50 >5 µg.ml^{-1}) with 100% G143A, (ii) SUP13 and GRP18 were highly (EC50=12.6) or moderately resistant (EC50=1.3) but lacked G143A (<1% G143A), and (iii) MVP9 and PBP1 represented the sensitive group (EC50 <1 µg.ml^{-1}) lacking G143A (8). Germination on water agar was generally below 50% for all isolates and ranged from 3 to 40% (Table 3.2). Isolates with high %G143A (AMP1, JRP4 and VAHP4) had higher percent germination on water agar than the isolates lacking G143A. In the presence of SHAM and PPG, mean percent germination was even more reduced by 61% and 100%, respectively. Inhibition was more pronounced with PPG, giving very low mean relative germination of 0 to 7%. Propyl gallate was then eliminated for AOX testing.

To determine if the solvent was contributing to the inhibition of spore germination, additional tests were done first on five highly resistant individuals. Mean relative germination on water agar amended or not amended with DMSO was statistically the same, and significantly higher than on SHAM-amended agar (P<0.001) (Table 3.3). This indicates that DMSO (at 0.25%) exerted little or no inhibition, while the addition of SHAM at 0.65 mM (equivalent to 100 µg.ml^{-1}) markedly inhibited spore germination.

To determine if isolates from different sensitivity groups are affected similarly by DMSO and SHAM, the third assay was conducted where chemicals were reduced to half the initial concentration (0.325 mM SHAM in 0.125% DMSO). The same responses as before were observed for both resistant groups (with high or low %G143A), where DMSO exerted little or no inhibition on spore germination, while SHAM reduced germination by >60% (Fig. 3.1). It is difficult to assess the extent of SHAM inhibition for the sensitive group because germination on non-amended water agar was very low (mean 7.7%). These results indicate a differential response of spore germination on water agar between QoI-resistant and QoI-sensitive isolates.

SHAM added to water agar at 0.065 mM in 0.025% DMSO did not reduce spore germination compared to that on non-amended water agar and water agar amended with 0.025% DMSO (Fig. 3.2). However, the sensitive group still exhibited poor germination, regardless of treatment.

3.3.3. Potentiation of azoxystrobin by SHAM. A four-fold decrease in EC_{50} from 0.004 to 0.001 was observed for the sensitive isolate MVP9 in the presence of SHAM. This was accompanied by a decrease in relative percent germination (RPG) at 0.01 $\mu g.ml^{-1}$ azoxystrobin with SHAM (Table 3.4). For both SUP13 and GRP18, SHAM reduced RPG at 0.01 and 1 $\mu g.ml^{-1}$ azoxystrobin, but the reduction in EC_{50} was not as pronounced as that in MVP9. In the highly resistant isolate VAHP4, a reverse effect was observed. Sensitivity of VAHP4 was decreased almost three-fold from an EC_{50} of 3.8 $\mu g.ml^{-1}$ in the absence of SHAM, to 10.6 $\mu g.ml^{-1}$ azoxystrobin in the presence of SHAM. These findings indicate that SHAM did not potentiate the effect of azoxystrobin in the two resistant isolates lacking G143A, but enhanced resistance in VAHP4.

3.4. Discussion and Conclusions

Heteroplasmic mitochondria in *E. necator*. The genetic shift in one single-spore isolate from <1% to 100% G143A after prolonged exposure to azoxystrobin points to the heteroplasmic nature of mitochondria at the level of the individual isolate, after ruling out the possibility of contamination or mixed population in culture. Heteroplasmy, a common phenomenon across eukaryotic kingdoms, is defined as the co-occurrence of more than one mtDNA sequence variant (in length or in sequence) in a single individual (3). A number of studies have pointed out the occurrence of heteroplasmy in plant pathogens. For example, in *Blumeria graminis* f. sp. *tritici*, the QoI-resistant single-spore isolates reverted to sensitive phenotype after more than 30 generations of growth in the absence of QoI selection pressure, and this was said to be due to a fitness penalty with G143A-based QoI resistance (10). Similarly, kresoxim-methyl-resistant *Venturia inaequalis* regained high levels of the wild-type mitochondria when grown for two rounds in liquid cultures devoid of the fungicide. Prolonged culturing in fungicide-free media restored high levels of the mutation which signified a lack of fitness penalty with the mutant allele (44). The degree of mitochondrial heteroplasmy was even found to be correlated with the degree of strobilurin resistance in the apple powdery mildew fungus *Podosphaera leucotricha*, and heteroplasmy was maintained for several years of cultivation in the absence of the fungicide (24). This was similar to the case of mitochondrial heteroplasmy in the insecticide-resistant mite species *Tetranychus urticae*, where the chemical bifenazate selected for resistant individuals with at least 60% mutation frequency (40). G143A-based resistance in *E. necator* did not also compromise pathogen growth in mixed populations of

QoI-resistant and sensitive isolates (33), and all our QoI-resistant isolates maintained for years on fungicide-free host tissue retained high levels of the mutation (see Chapter 2).

In the current study, it is unknown whether the mutant allele was initially present at a frequency undetected by the qPCR method used or the continuous exposure to azoxystrobin induced mutation in the mitochondrion, but clearly our finding demonstrates the selection of the mutant allele by a QoI in a single isolate. Mitochondrial DNA has a higher tendency for mutations than nuclear DNA because of the lack of repair mechanisms and histones, and its close proximity to the electron transport chain. In the presence of QoIs, mutation rates in the mitochondria could accelerate because of elevated concentrations of oxygen radicals that can oxidize nucleotides (25, 43). The mitochondrion also contains several copies of its genome; thus, it would take time for a changeover from a single mutation in a single DNA molecule to several copies of the mutation in the mitochondrial genome (41). This probably explains why it took several transfers before the shift to a high level of G143A was detected in SUP13. We do not understand yet how intracellular selection of mutant mitochondria occurs but this would significantly affect QoI resistance development at the level of individual cells. The absence of change in GRP15 and GRP18 indicates that this genetic shift could be a differential behavior in *E. necator*, with some isolates probably taking a longer time to respond than others or not at all.

AOX inhibitors and the AOX pathway. The alternative oxidase (AOX) pathway branches off from the cytochrome *bc*1 complex and does not couple electron transfer to proton translocation. Energy production by AOX is just one-third that of the regular respiratory pathway (25) but still allows the pathogen to respire in the presence of a respiratory inhibitor such as QoIs. If an AOX inhibitor is present together with a QoI, the sensitivity of the pathogen to the fungicide is expected to be enhanced, leading to a decrease in EC_{50} because the rescue impact of the AOX pathway is eliminated. We wanted to find out if an AOX is involved in QoI resistance in the three isolates with an 'atypical' form of QoI resistance. This necessitated optimizing test conditions using AOX inhibitors in spore germination assays. SHAM and PPG had strong inhibitory effects on spore germination at a high concentration of 0.65 mM (or 100 µg.ml-1 and 136 µg.ml-1, respectively), with PPG being more potent than SHAM. The toxicity of SHAM and PPG has also been observed for other pathogens (18, 35). Although AOX inhibitors can inhibit other enzymes essential for fungal processes, their adverse effect on respiration is also documented (21).

Miles et al. (30) also recorded significantly reduced spore germination of some *E. necator* isolates on agar slides amended with SHAM by 31 to 98% compared to that on non-amended agar. This prompted us to test SHAM at a much lower concentration (0.065 mM=10 $\mu g.ml^{-1}$), but even at this condition, there was still inhibition of sensitive isolates to a large extent. Interestingly, our 'group of three' (SUP13, GRP15, GRP18) behaved similarly as the sensitive isolates in their response to SHAM. Our findings indicate that, *in vitro*, QoI-resistant *E. necator* germinate better than strains lacking the A143 allele, in the absence of the fungicide, and thus, have a growth advantage. Competition assays under laboratory conditions suggest that this is not the case *in planta*. The proportion of the mutant allele tended to increase over time in mixed populations of resistant and sensitive isolates cycled on fungicide-free plants (see Chapter 2). Likewise, in the absence of competition, growth rates on host tissue did not differ between resistant and sensitive isolates.

SHAM was added to assay media to determine if in the presence of an AOX inhibitor and a cytochrome *bc1* inhibitor (QoI), the AOX pathway is eliminated, preventing the rescue effect on respiration. The inhibition of the AOX by SHAM was expected to cause significant sensitivity shifts in our atypical-resistance isolates (with low levels of or lacking G143A) but not in isolates with G143A-based resistance. The reduction in resistance level of GRP18 and SUP13, our isolates with atypical form of resistance, was not large enough to conclude SHAM inhibition of an AOX pathway. The third isolate, GRP15, was also tested; however, germination on water agar alone was very poor (<1%, data not shown) so that further comparison could not be made. Interestingly, in the resistant isolate (VAHP4) with high G143A level, resistance was increased almost three-fold in the presence of SHAM and azoxystrobin. It is unclear how resistance was enhanced. It is possible that SHAM inhibited other enzymes involved in production of mitochondrial reactive oxygen, which is otherwise harmful to the cell when in excess.

Other point mutations in cytochrome *b* (*Encytb*) have been associated with QoI resistance in other pathogens. We attempted to amplify the 'hotspot' section of *Encytb* with various degenerate primers designed from highly conserved sections of the gene gleaned from other pathogens and with published primer sequences that worked for other related species. The hotspot section contains the relevant point mutations (G143A, F129L, G137R). Our failure in these attempts might be due to the presence of a long intron within or next to this section. The cytochrome *b* gene across eukaryotic kingdoms are known to possess introns that can be larger than 1 kb (42) which can lead to an incomplete product in PCR and no bands on the gel. Other methods may be explored in the future to generate the *Encytb* sequence.

Implications of the study. A shift from low to 100% G143A within only 14 transfers in one isolate continuously grown on host tissue treated with 3 $\mu g.ml^{-1}$ azoxystrobin revealed that *E. necator* mitochondria can be heteroplasmic. The recommended rates for the commercial formulations of azoxystrobin are much higher than this; thus, in the presence of strong selection pressure, populations consisting of heteroplasmic strains can potentially undergo genetic shifts quickly under field conditions. The selection of the A143 allele has been demonstrated to be rapid in other pathogens exposed to QoI selection pressure within a growing season (11, 38). Because of the polycyclic nature of powdery mildew, sensitivity shifts in *E. necator* are expected to be rapid, too. On the other hand, withdrawal of the fungicide may possibly allow heteroplasmic strains to revert to low G143A. This is what we suspect to have happened in a vineyard that stopped QoIs in 2002 because of complete control failure, but still had both G143A-based resistance and the atypical resistance (moderate level without G143A) when surveyed in 2007. Heteroplasmy may cause the presence of G143A-based resistance to be grossly underestimated in such populations. Such underestimation due to heteroplasmy would inaccurately reflect a sensitivity shift. This can mislead the grower to put back the QoI spray in the program, which can start the cycle of disruptive selection for G143A-based resistance and lead to failure of disease control.

It was challenging to demonstrate the role of AOX *in vitro* because of the strong inhibitory effect of SHAM. However, the AOX rescue mechanism remains an interesting possibility in *E. necator* and additional tests are needed for more conclusive results. The relevance of AOX in QoI resistance may be best demonstrated *in planta*, but this will also prove to be challenging (41).

Tables and Figures

Table 3.1. Percent G143A changes in three QoI-resistant single spore populations sub-cultured successively on grape leaves treated or not treated with azoxystrobin (3 µg.ml^{-1}).

Isolate	Transfer Event[a]	% G143A					
		No azoxystrobin[b]			With azoxystrobin[b]		
		1	2	3	1	2	3
SUP13	Initial	0.17					
	T3	0	0	0.08	0	0	0.001
	T14	0	0	0	100	100	100
	T23	0.001	0	0	100	100	100
	T25	100	0.03	0	100	100	100
GRP18	Initial	0.01					
	T3	0.11	0	0.03	0	0	0.003
	T21	0	-	-	0	-	-
	T30	0	0.4	0.01	0.001	0.3	0
GRP15	Initial	0.03					
	T3	0	0	0.05	0	0	0.002
	T19	0	0	0	0	0	0

[a] Initial %G143A determined by Colcol (8); T3-T30 refer to number of transfers on host leaves
[b] Numbers 1, 2, 3 refer to independent subcultures

Table 3.2. Percent germination of *Erysiphe necator* isolates on water agar amended with AOX inhibitors SHAM and propyl gallate (at 0.65 mM).

Isolate	%G143A	Phenotype	Treatment[a]	Mean % Germination[b]±SE	Relative % Germination
AMP1	100	Resistant	Water agar	39.9±4.2	100
			SHAM	17.0±2.7	43
			Propyl gallate	0.7±0.4	2
JRP4	100	Resistant	Water agar	21.0±3.2	100
			SHAM	9.4±2.4	45
			Propyl gallate	3.1±0.9	15
VAHP4	100	Resistant	Water agar	13.4±5.0	100
			SHAM	6.2±1.4	46
			Propyl gallate	2.2±0.7	17
GRP15	<1	Resistant	Water agar	4.0±0.8	100
			SHAM	5.3±1.9	133
			Propyl gallate	2.6±0.6	64
SUP13	<1	Resistant	Water agar	2.8±0.7	100
			SHAM	1.3±0.2	48
			Propyl gallate	0.9±0.3	32
MVP9	<1	Sensitive	Water agar	16.9±5.7	100
			SHAM	6.7±2.1	39
			Propyl gallate	2.0±0.8	12
PBP1	<1	Sensitive	Water agar	4.3±1.5	100
			SHAM	2.3±1.4	54
			Propyl gallate	0	0

[a] Inhibitors SHAM (salicylhydroxamic acid) and propyl gallate added to 0.7% water agar at a final concentration of 0.65 mM. Inhibitors dissolved in DMSO which gave a final concentration of 0.25% in water agar.
[b] Mean of three counts

Table 3.3. Comparison of mean percent germination on water agar amended with DMSO (0.25%) or SHAM (0.65 mM) in DMSO for five isolates of *Erysiphe necator* with G143A-based resistance.

Treatment	n	Mean % Germination*
Water agar	12	30.7±1.3a
DMSO	12	30.2±1.3a
SHAM	12	9.3±1.0b

* Differences tested by Tukey's HSD test ($P<0.0001$) on transformed values; means with the same letters are not significantly different at $\propto=0.05$.

Table 3.4. Relative percent spore germination (RPSG) and sensitivity shifts (EC_{50}) in *Erysiphe necator* isolates based on relative percent spore germination on azoxystrobin-water agar amended or non-amended with SHAM (0.065 mM).

Isolate	Group	RPSG without SHAM*				RPSG with SHAM*			
		Azo 0.01	Azo 1	Azo 10	EC_{50}	Azo 0.01	Azo 1	Azo 10	EC_{50}
MVP9	S, <1%G143A	44.9±0.9	6.3±1.3	0	0.004	36.4±4.2	9.7±2.4	0	0.001
SUP13	R, <1%G143A	94.8±6.3	11.5±2.6	0	0.12	85.4±1.2	5.2±1.6	0	0.08
GRP18	R, <1%G143A	94.2±1.8	54.2±1.0	28.0±1.1	1.18	81.9±2.4	47.5±1.3	34.4±1.6	0.90
VAHP4	R, 99.9% G143A	74.9±2.7	73.9±0.5	33.7±2.1	3.81	60.7±1.3	64.0±2.5	43.9±3.6	10.61

* Azoxsytrobin added at concentrations of 0, 0.01, 1 and 10 $\mu g.ml^{-1}$. RPSG was calculated based on % germination on water agar without azoxystrobin (0 $\mu g.ml^{-1}$)

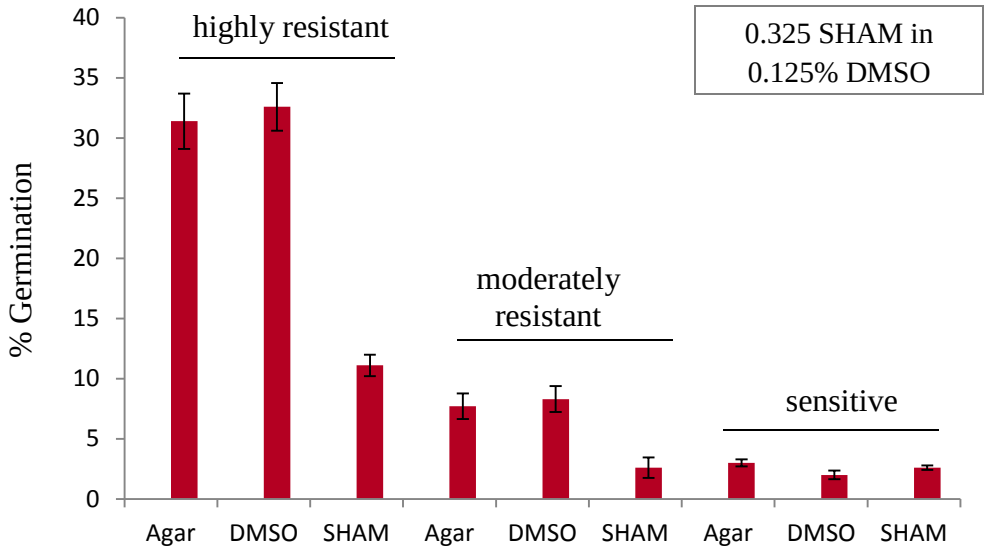

Fig. 3.1. Mean percent spore germination of *Erysiphe necator* of different QoI sensitivity profile on 0.7% water agar with or without salicylhydroxamic acid (SHAM, 0.325 mM) or dimethylsulfoxide (DMSO, 0.125%). Highly resistant isolates with >99% G143A, moderate resistant and sensitive isolates with <1% G143A.

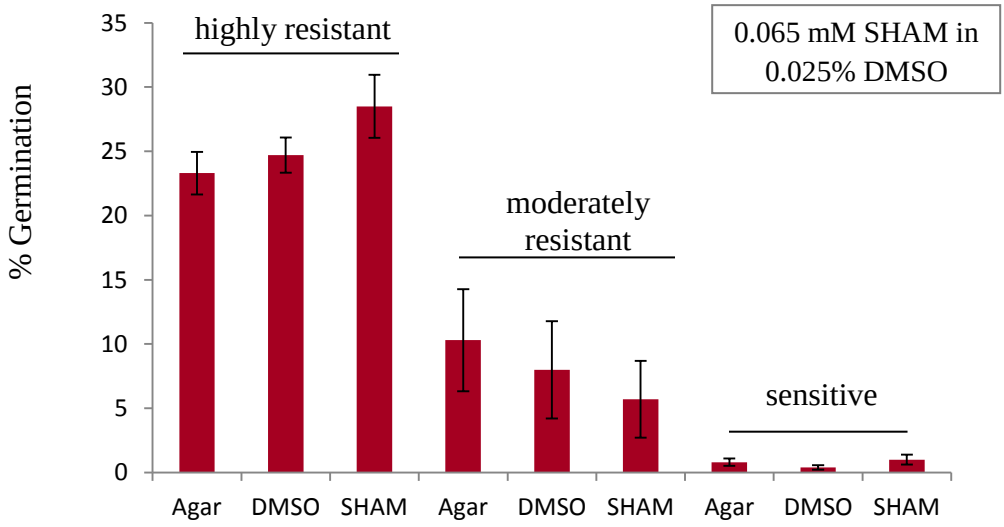

Fig. 3.2. Mean percent spore germination of *Erysiphe necator* of different QoI sensitivity profile on 0.7% water agar with or without salicylhydroxamic acid (SHAM, 0.065 mM) or dimethylsulfoxide (DMSO, 0.025%). Highly resistant isolates with >99% G143A, moderate resistant and sensitive isolates with <1% G143A.

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Chapter 4

Mechanisms of DMI Resistance in *Erysiphe necator*

Abstract

Demethylation inhibitors (DMIs) constitute an integral part of the disease control program against grapevine powdery mildew caused by *Erysiphe necator*. Long-term, intensive use of DMIs has resulted in the proliferation of populations with various degrees of resistance. To increase our understanding of DMI resistance development in *E. necator*, we investigated the sequence of the target gene *cyp51* and conducted gene expression assays. CYP51 is highly conserved in *E. necator* and only one polymorphism at the 136th amino acid was associated with DMI resistance. Two *cyp51* alleles based on the 136th codon corresponded to three genotypes: wildtype, TAT and two mutant genotypes, TTT and TWT. The TWT genotype suggested the presence of both wildtype and mutant alleles. The mutant genotypes resulted in the Y136F change, which only partially explained resistance to tebuconazole, myclobutanil and fenarimol. *Cyp51* over-expression was associated with Y136F, with relative copies of the target gene ranging from 1.4- to 19-fold more in mutant than in wildtype isolates. Our findings support at least two mechanisms of DMI resistance in *E. necator* - the Y136F mutation which may be working in conjunction with *cyp51* overexpression for enhanced resistance, possibly due to multiple gene copies.

Keywords: DMI, CYP51, Y136F, resistance mechanism, overexpression, *cyp51* alleles, tebuconazole, myclobutanil, fenarimol

4.1. Introduction

Commercial varieties of the European grape species *Vitis vinifera* are susceptible to powdery mildew caused by *Erysiphe necator* [syn. *Uncinula necator* (Schw.) Burr.] (21, 22). Disease control relies heavily on the use of protective and systemic fungicides in conjunction with aggressive cultural practices. Among the widely employed fungicides against *E. necator* is the class of demethylation inhibitors (DMIs). DMIs inhibit the cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$), encoded by the *cyp51* gene (26). This demethylase acts as a key enzyme for fungal sterol biosynthesis (27).

The first DMI labeled for use with grapes was triadimefon, first introduced in 1982 (30). Other DMIs such as fenarimol, myclobutanil, flumizole and tebuconazole followed suit. In 2009 and 2010, tetraconazole and difenoconazole were registered for use in grapes, respectively. The DMIs now constitute the largest group of fungicides registered for grapevine powdery mildew control in the United States. However, the intensive use of DMIs has led to outbreaks of pathogen resistance in Northern America. Early reports were made for *E. necator* populations in California (29, 41, 57), New York (24, 55), and Ontario (44). Recently, DMI resistance was reported in Virginia and neighboring states (12). DMI resistance and sensitivity shifts have also been observed in *E. necator* populations in Europe and other continents (23, 48, 52, 53). However, complete loss of efficacy has not yet been reported. Possible reasons are the association of a fitness cost with DMI resistance (11) and the quantitative nature of resistance (8). To alleviate the selection pressure towards fungicide resistance and to improve disease control, DMIs are often used as rotational or mixture partners with non-cross-resistant chemistries (34).

Resistance to DMIs has been correlated with mutations that generate amino acid changes in the target molecule (38). The *Encyp51* has been sequenced, and the protein inferred to be 524 amino acids in length is encoded by the 1.572 kb coding region of the 1.683 kb gene with six highly conserved amino acid domains (CR1 to 6) that were highly typical of cytochrome P450 and CYP51s (18). An amino acid change from tyrosine to phenylalanine at position 136 (Y136F) was found in triadimenol-resistant isolates. Y136F was further correlated with moderate to high resistance to triadimenol in European isolates (19), and to various DMIs in two isolates from the northeastern United States (12). DMI resistance in other organisms has also been attributed to (i) other CYP51 mutations in combination or not with Y136F (1, 6, 9, 10, 17, 25, 36, 43, 56); (ii) increased *cyp51* expression (31, 37, 39, 40, 50); and (iii) increased efflux pump activity or over-expression of ABC transporters that could confer multi-drug resistance (32, 46, 49, 58). A combination of mechanisms could also be responsible for DMI resistance in certain fungi, such as target site mutations and over-expression in *Mycosphaerella graminicola* (14).

Cross-resistance to fungicides with the same mode of action can occur. However, different mechanisms may allow for differential selection of resistant strains. For example, the I381V mutation in CYP51 of *M. graminicola* was selected by tebuconazole and difenoconazole, but less aggressively or not at all by other triazoles (25), possibly because of a different mechanism. Interestingly, a group of isolates in our collection having high resistance to tebuconazole and myclobutanil have variable sensitivity to the other DMIs such as triadimefon and fenarimol. This may be due to the inherent activity of the fungicides but may also be due to molecule-specific mechanisms. To expound on the mechanisms of DMI resistance in *E. necator*,

we further investigated the *cyp51* gene by sequence analysis and determined the over-expression of *cyp51* in isolates with *cyp51* nucleotide polymorphism. We particularly wanted to find out whether or not the variation in DMI sensitivity is related to different mechanisms of resistance or is largely controlled by the single point mutation (Y136F) as shown previously in European strains (19) and in some of our resistant isolates (12). Our findings revealed that increased copies of the *cyp51* gene may be responsible for elevated expressions and higher resistance levels to DMIs in *E. necator*. We also report an easy and quick method using Taqman chemistry for a high-throughput evaluation of isolates for resistance based on the Y136F mutation.

4.2. Materials and Methods

4.2.1. **Culture preparation and DNA extraction**. The isolates were grown on young grape leaves for 14 days or until profuse sporulation (up to 24 days). The spores were harvested from three leaves by scraping the surface growth into a 2-ml conical tube. The remaining fungal material was added to the tube by flushing the leaf with 1.5 ml sterile 0.005% Tween 20 water (STW) then frozen until DNA extraction.

To extract DNA, frozen fungal material was thawed and centrifuged at 10,000 rpm for 10 min. After drawing the liquid out, six sterile 2.5 mm-glass beads (Biospec Products) were added to each tube. Spore disruption was done three times at 4 m s^{-1} for 30 s using the FastPrep-24 instrument (MP Biomedicals) with tube immersion in liquid nitrogen after the first and second bead-beating. The disrupted spores were re-suspended in 300 µL Buffer RLT (Qiagen) immediately after the third bead-beating. The suspension was centrifuged at 6,000 rpm for 5 min. DNA extraction was performed with the Biosprint 15 DNA Plant Kit (Qiagen) and using the Plant DNA program in the Biosprint 15 workstation (Qiagen) according to manufacturer's protocol. The resulting DNA solutions were stored at -20^{o}C.

4.2.2. Fungicide sensitivity assay. The powdery mildew isolates selected for sequence analysis had been maintained on fungicide-free grape leaves for over two years. Their sensitivity profile to myclobutanil, tebuconazole and fenarimol was determined by leaf bioassays before samples were obtained for DNA and RNA extraction. Stock solutions of tebuconazole (Bayer Crop Science LP, Research Triangle Park, NC), myclobutanil (Dow Agrosciences LLC, Indianapolis, IN) and fenarimol (Gowan Company, Yuma, AZ) were prepared in acetone from technical grade fungicide and stored at -10 to 12^{o}C. Three-fold serial dilutions were performed with STW as diluent from 0.001 to 1 µg.ml-1 or from 0.01 to 30 µg.ml-1, depending on the isolate. Leaves were slowly shaken in fungicide solution for 1 hr, blotted dry and plated on 0.7% water agar. Two leaves were used per concentration and leaves soaked in STW were included as control. Individual spore chains (average of 6) from each culture were inoculated on different parts of the leaf. Plates were incubated at 25^{o}C under a 12-hr light regime for 6 to 8 days. The diameters of colonies that developed on the leaves were measured with an ocular micrometer at 17x total magnification under a dissecting microscope. Relative growth was calculated from the mean of the diameters on the two leaves as percentage of the mean diameter of the control. To estimate EC_{50}, the relative diameter of treatments was regressed on natural log-transformed fungicide concentration. From the linear section of the regression, the EC_{50} was calculated in Microsoft Excel as described by Colcol, Rallos, and Baudoin (12): $EC_{50} = e^{([50\text{-}b]/m])}$, from the regression equation $y = m.1n\ (concentration) + b$. The resistance factor (RF) for each fungicide was calculated as EC_{50} of the isolate divided by the mean EC_{50} of sensitive isolates.

4.2.3. *Cyp51* sequence analysis. Three primer sets were designed using the Primer 3 software (47) based on the *Encyp51* sequence with GenBank accession no. U72657 (18) to generate three overlapping sequences from twenty-seven isolates with different DMI resistance profiles. The primers are summarized in Table 4.1. The 20-µl PCR reaction consisted of 0.2 µM for each primer, 1x iProof HF Master Mix (Biorad) and 10 to 50 ng of template DNA. The PCR reaction was performed in the Mastercycler (Eppendorf) with the following cycling conditions: initial denaturation of 98^{o}C for 30 s, then 35 cycles of denaturation at 98^{o}C for 10 s, annealing at 59^{o}C (primers F1/R900), 52^{o}C (primers F502/R1288) or 55^{o}C (primers F1300/R1300) for 30 s, and extension at 72^{o}C for 1 min. The final extension was set at 72^{o}C for 5 min. The fourth primer pair L419/R419 was used for verifying the sequence encompassing the 136th position for some of the isolates and for three additional isolates collected from localities that least likely received DMI sprays. The PCR conditions were the same except for the annealing temperature set at 53^{o}C. Five microliters of each PCR product were cleaned enzymatically using the following reaction: 1 µl of 1 unit µl^{-1} shrimp alkaline phosphatase (USB), 0.1 µl of 10 units µl^{-1} Exonuclease I (USB) and 5 µl water. The reaction was incubated at 37^{o}C for 30 min, then at 65^{o}C for 20 min. Cleaned products were submitted to the University of Chicago Sequencing Facility. Both forward and reverse sequences were assembled using the SeqMan Pro ver. 8.1.3 (Lasergene, DNASTAR, Inc.). Consensus sequences were translated into amino acids with EditSeq ver. 8.1.3 (Lasergene, DNASRAR, Inc.) and compared with the predicted amino acid sequence for CYP51 (UniProtKB/Swiss-Prot Accession no. O14442) (18). The consensus sequence harboring Y136F was designated the mutant sequence, while the sequence lacking the mutation was considered the wildtype.

4.2.4. Single nucleotide polymorphism (SNP) genotyping. The *EnCyp51*-specific forward and reverse primers and two allele-specific TaqMan MGB probes (Applied Biosystems), each labeled with a different reporter dye at the 5′-end (Probes 1 and 2, Table 4.1) were designed from the *cyp51* consensus sequences obtained in this study using the Custom Taqman Design Tool (Life Technologies). The VIC-labeled probe detects the mutation at nt 495 of the *cyp51* gene, while the FAM-labeled probe detects the wildtype. Each 20 µL reaction consisted of 1x Taqman Universal PCR Master Mix (Applied Biosystems), 1x Taqman CYP51 Genotyping Assay mix containing the primers and probes (Applied Biosystems) and DNA template (5 to 8 ng µl^{-1}). Reactions were carried out in the StepOne Plus instrument (Applied Biosystems). The fluorescence of the reporter dyes was normalized to the fluorescence of the passive reference dye (ROX). The StepOne Plus Real-time PCR software v. 2.1. (Applied Biosystems) plots the normalized intensities of the reporter dyes in each sample well on the Allelic Discrimination Plot. In the plot, the reporter dye intensities for the probe can be differentiated. A clustering algorithm assigns a genotype call according to the position on the plot. Samples were assigned one of the following genotypes: pure wildtype (‘homozygous allele1/allele1’), pure mutant (‘homozygous allele2/allele2’) or mixed (‘heterozygous allele1/allele2’). All isolates for sequencing were subjected to the SNP genotyping protocol, and additional isolates collected from plants sprayed with Elite (active ingredient: tebuconazole) were also genotyped. The genotyping reaction was done at least two times for each isolate.

4.2.5. *Cyp51* overexpression. Isolates from each genotype group --BLP4, MVP9, PBP1, SCCP4) for wildtype; BXPIA, MDMRP5, MDMRP7, AMP1, JRP4, VAHP4, VAHP1, IVP4-- were grown on ten grape leaves. RNA was extracted by scraping the surface growth with a sterile spatula into a Lysing Matrix C tube (MP Biomedicals). Tubes were frozen at -50^{o}C

until RNA extraction. Buffer RLC (450 µL, Qiagen) was added to the tube immediately after it was taken out of the freezer. The fungal tissue was disrupted by bead-beating at 4 m s^{-1} for 50 s in the FastPrep-24 instrument (MP Biomedicals) for two consecutive times. RNA extraction was completed using the RNEasy Plant Mini Kit (Qiagen) following manufacturer's protocol for filamentous fungi. The resulting RNA sample was diluted to 30 ng mL^{-1} and reverse-transcribed using the High Capacity cDNA Kit (Applied Biosystems). The cDNA levels of *cyp51* were quantified by the comparative C_T method ($\Delta\Delta C_T$) in the StepOne Plus instrument (Applied Biosystems). Forward and reverse primers for *Encyp51* and *Entub* are indicated in Table 4.1. *Cyp51* expression was normalized to the expression of the β-tubulin gene (*Entub*) of *E. necator*. Probes labeled with the fluorescent dye FAM at the 5′-end and a non-fluorescent quencher with a minor groove binder (MGB) at the 3′-end (Table 4.1) were utilized to enable detection of the specific PCR product as it accumulated during amplification. All primers and probes were designed in the Custom TaqMan Assay Design Tool of Life Technologies (www.lifetechnologies.com) using our consensus sequence for *Encyp51* and Genbank Accession no. AY074934 for *Entub* (2). The primers were customized into a CYP51 and a β-TUB Gene Expression Assay mix by Applied Biosystems. Reactions were performed in a total volume of 20 µl containing 1x Taqman Universal PCR Mix (Applied Biosystems), 1x Taqman Gene Expression Assay mix (Applied Biosystems), and 2 µl of the cDNA template. Separate reaction wells were designated for the target and the reference genes, each of which was amplified in triplicate reactions with the following thermocycling program: a pre-amplification step at 50°C for 2 min, an initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Reverse transcription and gene expression quantitation were done twice for each RNA sample. The relative expression (relative quantification, RQ) was calculated by the StepOnePlus software v.2.1 (Applied Biosystems) as $2^{-\Delta\Delta C_T}$. Correlation analysis was done by Pearson's pairwise comparison of RQ and RF. Isolates were grouped into genotype and mean RQ for genotype group was compared by ANOVA. All statistical analyses were done in JMP v. 9 (SAS Institute Inc.).

4.3. Results

4.3.1. Conserved nature of *EnCyp51* and *Cyp51* genotypes. The primers L1/F900, L502/R1000 and L1300/R1300 generated an approximately 1.8kb-fragment from which a 1.7kb-section was extracted for comparison with existing sequences of *cyp51*. Two consensus DNA sequences were generated from the 24 isolates (Fig. 4.1). BLAST search revealed 99% similarity with the *Encyp51* sequences from Europe (GenBank accession no. AF042067, UNU83840, UNU72657) (18-20) and Australia (GenBank accession no. EF649776, EF649777), indicating not only successful amplification of the *Encyp51* for the US isolates but also the highly conserved nature of this gene in *E. necator*. The consensus sequences were translated into 524 amino acids which were 99.4% identical with the predicted sequence of Delye, Laigret, and Corio-Costet (18) (Fig. 4.1). The consensus sequence that possessed a tyrosine at amino acid 136 with a corresponding codon of TAT was designated wildtype. The other sequence with a phenylalanine at the same position, encoded by TTT, was designated mutant. Two amino acids in the US sequences differed from the European CYP51. Valine at position 37 replaced glycine, and threonine replaced isoleucine at position 156.

Five out of 24 isolates were generally DMI sensitive, with resistance factor (RF) ≤2 for tebuconazole, RF≤5 for myclobutanil and RF≤1.3 for fenarimol, and possessed the wildtype

codon TAT; hence, tyrosine (Table 4.2). Isolates with higher resistance levels (16/24) to the three DMIs possessed phenylalanine, encoded by TTT at the same position. Two isolates (GRP15, IVP3), with RF within the range of the wildtype group for one or more of the DMIs tested, still possessed the mutation, while one isolate (VAHP6) with moderate resistance factors to the three DMIs was wildtype (TAT) at codon 136. Four isolates with high resistance factors revealed a TWT at codon 136, which indicates the presence of nucleotides A and T in the position of W. *Cyp51* sequencing thus revealed three genotypes at the 136th codon for the 19 mutant isolates – (i) TTT genotype (11), (ii) TWT genotype (4/19) and (iii) TAT genotype (4/19). The sequence chromatograms showed that the TTT produced a single peak for each nucleotide while the TWT and TAT genotypes of isolates with high resistant factors produced double peaks at nucleotide 495 (Fig. 4.2). The peaks for nucleotides A and T in the TWT-genotype were of the same intensity, but the resistant TAT genotype had a double peak with the A peak distinctly taller than the T peak. VAHP6 with a wildtype genotype had a single A peak in the chromatogram (Fig. 4.2). A new primer set (L419/R419, Table 4.1) was designed to resolve the ambiguity of the TAT genotype/double peak pattern and the case of VAHP6. The isolates with the TAT-genotype/double peak combination (AMP1, IVP4, JRP3) turned out to be TWT-genotype with the same signal intensity for A and T (Table 4.2). Three more isolates (SNP1, SNP3, FH9-1), sequenced only with the L419/R419 primer pair, possessed a wildtype genotype (TAT, single peak). These isolates were bioassayed for resistance only to tebuconazole and RFs supported their classification into the wildtype group. Isolates with either high RF (>100) or low RF (<5) to one or more DMIs could also have either the TTT or TWT genotype. Overall, the single nucleotide change at nt 495, leading to a codon change from TAT to TTT at the 136th codon, and resulting in the Y136F mutation in CYP51, was the only mutation associated with resistance in the isolates tested. However, it does not completely account for DMI resistance in *E. necator*.

Other nucleotide polymorphisms were found in individual sequences but none of these were associated with DMI resistance and none altered the amino acid in their respective codons (codons 326 and 343). The third nucleotide (nt 1089) in codon 323 could be a C or A while that for codon 343 could be a G or A (Table 4.2). Interestingly, a double peak ('R') registered for this nucleotide position in the same isolates having the TWT genotype at codon 136. These same isolates also possessed a C at nt1089, while the rest of the mutant isolates (TTT genotype) as well as wildtype isolates had an A. VAHP6, with the TAT/single peak combination, possessed a G for codon 326 and an A for codon 343 (nt 1137), which makes it a class of its own.

4.3.2. Verification of SNP genotypes. All isolates lacking Y136F based on sequencing were assigned a pure wildtype genotype (TAT) with SNP genotyping (Table 4.2). Additional isolates (SNP1, SNP3, FH9-1) with a wildtype genotype based on sequencing with L419/R419 primer set were also assigned a pure 'wildtype' (TAT) by the SNP genotyping method. The isolates with a TTT genotype were designated 'pure' mutants, whereas the TWT genotype was given a "mix" call (TAT/TTT). VAHP6 turned out to be a unique isolate because two independent samples tested by SNP genotyping gave different calls. One was a mix and the other was pure mutant.

To further test the ability of the SNP genotyping method to detect the TWT and TTT genotypes, new isolates were collected from spray trials in 2010 in three different locations (AF,

SC, RO) and subjected to the method. AF and RO are located in Virginia and SC in North Carolina. These locations are more than a hundred kilometers apart from each other. The isolates were initially taken from infected potted grape plants sprayed weekly with Elite at 10 mg L^{-1} (active ingredient: tebuconazole), then transferred to tebuconazole-treated leaves. The isolates were able to grow well on leaves treated with 10 mg L^{-1} tebuconazole on the first culture transfer, indicating a resistant phenotype. The SNP genotyping method assigned either a mix or a pure mutant genotype to these isolates (Table 4.3). The eight isolates from two locations consisted of either only the mix genotype (AFs) or the pure mutant genotype (ROs). All isolates from SC consisted of mix and pure genotypes. Altogether, SNP genotyping results support the existence of the TTT and TWT genotypes in field isolates of *E. necator* and show its good ability in detecting these genotypes. The method was also tested with DNA from grape leaves and from fungal contaminants isolated from grape leaves. No signals were generated from these samples (data not shown), indicating the specificity of the method for *E. necator*.

4.3.3. Over-expression of *cyp51*. A standard curve analysis with a 10-fold dilution series of cDNA was performed on the calibrator isolate (BLP4). PCR efficiency and slopes of *Entub* and *Encyp51* were nearly equal with values of 105% and -3.2 (R^2=0.99), and 106% and -3.18 (R^2=0.99), respectively (Fig. 4.3*)*. The expression of *cyp51* was found to be higher in all isolates possessing Y136F than isolates lacking the mutation. However, expression levels (RQ) were significantly different only in seven out of nine isolates possessing Y136F (P<0.0001) (Fig. 4.4). The highest RQ was given by JRP4, having a TWT-genotype, with a 19-fold increased mRNA level than the reference isolate (BLP4). The lowest expression level was obtained for IVP3, which did not significantly differ from the three wildtype isolates with TAT-genotype. As a group, the TWT-genotype resulted in a slightly higher expression level but this was not statistically different from that of the TTT-genotype (Table 4.4). A significant correlation was also found between cyp51 expression level and resistance factor to the three fungicides (Fig. 4.5). A strong correlation was obtained for myclobutanil (r=0.80, P<0.0001) and tebuconazole (r=0.74, P<0.0001), but slightly lower correlation for fenarimol (r=0.64, P<0.0001). This indicates a tendency toward increased up-regulation of cyp51 with increased resistance level to the DMI.

4.4. Discussion and Conclusions

Conserved nature of *En*CYP51 and mutations. A high degree of similarity (>99%) in the *cyp51* nucleotide sequence was found among European, Australian and US isolates, which reflects the highly conserved nature of *cyp51* in this species. Alignment of one predicted amino acid sequence of *Encyp51* from Europe and from the United States showed two amino acid differences at position 37 (G→V) and 156 (I→T). Amino acid 37 is part of the putative trans-membrane domain, while amino acid 156 is located in the second conserved region (CR2) of CYP51 (18). The trans-membrane portion on the N-terminal side of the protein may serve as the anchor of *En*CYP51 to the endoplasmic reticulum (19). Glycine residues in CYP51 may be important to the functional flexibility of the enzyme since this amino acid can fit into hydrophobic and hydrophilic environments with its simple side chain (35). Valine is a non-polar amino acid and its substitution for glycine may increase the hydrophobicity of this portion. The 156th amino acid change from isoleucine to threonine is interesting because it can also decrease hydrophobicity; however, this amino acid is several positions away from the conserved section of the CR2 domain which is a putative substrate-recognition site (4). Mutations in the active

sites and other sections of CYP51can influence substrate interactions as shown in the yeast *Candida albicans* (5). The differences in *E. necator* may have evolved as a result of environmental pressures that the population has been exposed to or may simply be due to genetic drift. Protein modeling studies may reveal if these amino acid changes impact the function of the respective domain. In general, amino acid sequences of the putative substrate-binding regions of CYP51 (CR1 to 6 in *E. necator*, Fig. 4.1) are highly conserved across biological kingdoms (3). Lepesheva and Waterman (35) further proposed that conserved sequences in two substrate-binding regions (1 and 4) serve as CYP51 signature. In our *E. necator* sequence, we located these conserved amino acid sequences from positions 121 to 139 (CR2 domain) and positions 306 to 315 (CR4 domain).

CR2 contains the amino acid change Y136F (Fig. 4.1) which has been associated with loss of sensitivity to triadimenol in *E. necator* (19), and in *Mycospharella graminicola* due to the corresponding mutation Y137F (42). Protein structure modeling of CYP51 in *M. graminicola* provided an explanation how this mutation could bring about increased resistance. Azoles dock on heme-binding region (found in CR6 in *E. necator*). The 137th amino acid in *M. graminicola* resides on the access channel end of the binding pocket and increases the heme cavity volume substantially. This pushes the residue F137 into a position that occludes triadimenol-binding. The slight loss of resistance to other azoles, such as tebuconazole, epoxiconazole and prochloraz in *M. graminicola*, could not be due to this conformational change based on the model but could be due to the loss of the hydroxyl group in phenylalanine and the movement of a Y459 residue away from interactions with these azoles. Such findings indicate that the mutation at amino acid 136 of *E. necator* may also exert different impacts on different DMI molecules. In general, the occurrence of the Y136F mutation in isolates with high RFs and its absence in isolates with low RFs strengthen its association with DMI resistance (12, 18); however, Y136F did not explain resistance completely among isolates tested because a few isolates possessed the mutation but had a sensitivity level similar to that of the wildtype to one or more DMIs. This indicates that other mechanisms likely exist in *E. necator*.

Three genotypes of *cyp51* based on the 136th codon. The SNP genotyping supported the existence of two alleles of *cyp51* relevant to DMI resistance in *E. necator*. This further led to the discovery of three possible genotypes - TAT, TTT and TWT – for the 136th codon. The TAT-allele was present only in isolates collected from locations that had probably received little or no exposure to DMIs at the time of sampling (wild grapevines, or a new vineyard not near existing commercial vineyards). The TTT and the TWT genotypes were detected in isolates with low to high resistance to tebuconazole, myclobutanil and fenarimol. Although there was one isolate that could not be resolved by the SNP genotyping, the method still proved reliable in *cyp51*-allele detection most of the time. In fact, it was able to clarify ambiguities in results for isolates showing double peaks with TAT genotype but turned out to have TWT genotype in repeat sequencing (e.g. AMP1, IVP4, JWP1, Table 4.2). Additional newer field isolates tested with the SNP genotyping protocol corroborated the existence of both 'pure'/TTT and 'mix'/TWT genotypes. SNP genotyping may be a quicker alternative to sequencing in detecting *cyp51* genotypes based on the 136th codon. Because it is a Taqman-based chemistry and it measures fluorescence signal relative to the amount of the target fragment, it can be optimized further to quantify the proportion of the mutation in the population. Such quantitative methods can be applied in population studies. For example, a study done in France showed the Y136F proportion to be low at the national level but increasing in two succeeding years (23). With large

variability in percentages among locations, it was suggested that the differences were probably due to local spray programs. Our SNP genotyping method has a high degree of specificity because it utilizes *En*-specific probes. We have seen the possibility of a certain genotype dominating the population at a local scale – the mix genotype in the AF vineyard and the pure mutant genotype in the RO vineyard. Finding out the impact of different DMI fungicides on the evolution of genotypes in field populations might be useful in resistance management.

Cyp51* is over-expressed in mutant *E. necator. Since *E. necator* is a haploid fungus, our detection of the TWT genotype raised the possibility of the presence of at least two copies of the gene, one carrying the wildtype codon and the other carrying the mutant codon at position 136. The TAT and TTT genotypes may or may not also represent duplicate or multiple copies. This led to the hypothesis that increased copy number is related to overall gene expression. Therefore, we expected isolates carrying the mutant genotype, either TTT or TWT, to have elevated gene expression. We demonstrated an increased expression of *cyp51* in mutant *E. necator* using the comparative $\Delta\Delta C_T$ Taqman-based method with the *Entub* gene (2) as the endogenous reference. Significant expression levels of *cyp51* in this present study ranged from 6- to 19-fold more in the genomes of mutant isolates than in those lacking Y136F. We have further shown that isolates with increased resistance to DMIs tended to have increased *cyp51* constitutive expression, indicating that over-expresssion of *cyp51* may explain the quantitative nature of DMI resistance in *E. necator*. In *Cercospora beticola*, this was also observed, where the highest expression levels of *cyp51* were obtained for highly resistant isolates, while lower expression levels were exhibited by moderately resistant isolates (43).

The expression levels that we obtained in this study were similar to those observed for epoxiconazole-resistant *Puccinia triticina* (51), fluconazole-resistant *Candida albicans* (40), DMI-resistant *M. graminicola* (13), myclobutanil-resistant *Venturia inaequalis* (50), propiconazole-resistant *Monilinia fructicola* (37) and fenbuconazole-resistant *Blumeria jaapii* (39). Expression levels of *cyp51* can be increased by more than a hundred fold in triflumizole-resistant *Penicillium digitatum* (31) and DMI-resistant *C. beticola* (7, 43). In most cases, increased expression was due to an insertion in the promoter region of *cyp51*. It is unlikely that insertions or repeats are responsible for increased expression in our *E. necator* isolates since preliminary PCR reactions using the C14/C14R primers of Delye, Laigret, and Corio-Costet (18), which covered almost the entire 5′-UTR to 3′-UTR of *cyp51*, generated the same band size for our mutant and wildtype isolates on agarose gel (data not shown).

Another explanation for increased expression is increased gene copy number. In *C. albicans*, the entire chromosome carrying the *cyp51* gene was duplicated resulting in overexpression. Increased itraconazole resistance was also conferred by extra copies of the P-450 14∝-demethylase gene, *pdmA* in *A. fumigatus* and *A. nidulans* (45). The resistance level was increased up to 36 times that of the wildtype controls. A correlation of increased copy number of the target gene *ESPS* was also found with glyphosate-resistance in populations of the weed *Amaranthus palmeri* (28). Duplicate copies may serve to compensate for each other functionally as demonstrated in knock-out experiments of *cyp51* genes in the opportunistic human pathogen *A. fumigatus* (Mellado et al. 2001). This is congruent with the findings of Wapinski et al. (54), that duplicated genes in fungi rarely diverge in their biochemical function but diversify at the level of gene regulation, resulting in a 'partial division of labor' or sub-functionalization between the two paralogous proteins. Gene copy number variation may be an

adaptive mechanism for certain environmental or pathological conditions. Not only is there more than one copy of *cyp51/erg11* in fungi but many genes involved in the ergosterol pathway are duplicated as well. As many as three copies of *erg11* exist in *Aspergillus oryzae* and *Magnaporthe grisea*, and two copies occur in *Fusarium graminearum* (16), indicating the possibly widespread phenomenon of increased copy number among fungi.

The different expression levels among the isolates tested raised the possibility of copy number variation within the species. Copy number variation within a species is not uncommon, and has been documented for 18S rDNA in *A. fumigatus* (33) and for three ribosomal genes and the *Bip* gene (a chaperone gene) in the arbuscular mycorrhizal fungus *Glomus intraradices* (15). In the case of our mutant isolates IVP3 and VAHP1, which were either sensitive or slightly resistant to DMIs and expressed *cyp51* at wildtype level, sensitivity shifts might have occurred after prolonged culturing on fungicide-free host tissues. These shifts could have been accompanied by loss of some copies of the gene, resulting in low expression of *cyp51*. There is evidence of reversion to a sensitive state in *C. glabrata* due to a gradual loss of duplicated chromosomes carrying the *cyp51* gene (40). To determine whether *cyp51* copies are distributed in more than one chromosome, or whole chromosomes duplicated in *E. necator* can be a future research goal. We also found other neutral mutations specific to the TWT genotype. These may have utility as additional molecular markers for monitoring this group in field populations.

In summary, we found two *cyp51* alleles based on the 136th codon corresponding to three genotypes: wildtype (TAT) genotype and two mutant genotypes (TTT and TWT). The mutant genotypes resulted in the Y136F change. However, Y136F only partially explained resistance to tebuconazole, myclobutanil, and fenarimol. *Cyp51* over-expression was associated with the presence of Y136F. It was higher for the TWT genotype, and strongly correlated with resistance to DMIs. Our findings indicate that the mutation is necessary for resistance, and may work in conjunction with copy number variation to enhance resistance in *E. necator*.

Tables and Figures

Table 4.1. Primer information for various applications in the study of DMI resistance mechanisms in *Erysiphe necator*.

Primer Pair	Sequence (5'→3')	Application
L1	TTGTCGACCCCCAAGACTAC	amplification of first 1.5kb section of *cyp51*
R900	GACTTGACGCTCCTGTGCTA	
L502	CGCCGAAGAGATTTACACTA	amplification of nt 405-1238
R1000	GATCCCATTTGAGAGGGTCT	
L1300	CATGGAAGAGTTGTATGAGGAACA	amplification of nt 1097-1800
R1300	CAATTCTTCTAACCCTAACACCTG	
L419	CAGTCTATCTGGGACTTCAAGG	amplification of shorter fragment of *cyp51* to verify 136th codon
R419	AACAGTTCTTTGGGCATGAT	
FS*cyp51*	ACTAATTTAACAACTCCGGTCTTTGGA	SNP genotyping
RS*cyp51*	ACTCGACCATTTACGGACCTTTTT	
Probe 1	VIC-TTGGACAATCAAATACAAC	probe for mutation at nt495 in SNP genotyping
Probe 2	FAM-TTTGGACAATCATATACAAC	probe for wildtype sequence in SNP genotyping
FG*cyp51*	CATGCGCGAGATCGTTCAC	*cyp51*-forward primer for expression
RG*cyp51*	CAGAAATGGTTTGCCGAAAGCA	*cyp51*-reverse primer for expression
FG*tub*	TGATTGTCCAAATTCCAAACTCATGGA	*tub*-forward primer for expression
RG*tub*	AGGAATGGAACGCTTCAATGGT	*tub*-reverse primer for expression
P*cyp51*	FAM-AAGAGCCGTTTTCATAAACTTT	probe for *Encyp51* in expression
P*tub*	FAM-CCAATGCGGAAATCAA	probe for *Entub* in expression

Table 4.2. DMI resistance phenotype and *cyp51* sequence information for *Erysiphe necator* isolates with various DMI sensitivities.

Isolate	RF[a] Teb	RF[a] Myc	RF[a] Fen	136[th] codon based on sequencing[b]	Peak at nt495 in chromatogram[b]	Repeat sequencing of 136[th] codon[c]	Codon 326[e]	Codon 343[f]	SNP genotyping[d] of *cyp51*
BLP1	1.5	0.6	0.4	TAT	single	-	A	G	Wildtype
BLP4	1.0	3.4	0.7	TAT	single	TAT	A	G	Wildtype
MVP9	0.6	1.2	0.6	TAT	single	-	A	A	Wildtype
PBP1	1.0	0.7	1.0	TAT	single	-	A	A	Wildtype
SCCP4	1.8	0.08	2.0	TAT	single	TAT	A	A	Wildtype
SNP1	1.1	n/a	n/a	-	single	TAT	-	-	Wildtype
SNP3	0.6	n/a	n/a	-	single	TAT	-	-	Wildtype
FH9-1	0.4	n/a	n/a	-	single	TAT	-	-	-
VAHP6	22.2	62.9	>19	TAT	single	TAT	C	G	Mix or WT
BXP1A	6.2	12.1	6.5	TTT	single	TTT	A	A	pure mutant
GRP15	16.9	0.04	17.2	TTT	single	-	A	A	pure mutant
GRP18	16.6	112	10.5	TTT	single	TTT	A	A	pure mutant
IVP3	1.5	0.4	2.0	TTT	single	-	A	A	pure mutant
IVP11	65.2	-	15.2	TTT	single	-	A	A	pure mutant
MDMRP5	20.5	65.7	6.5	TTT	single	-	A	A	pure mutant
MDMRP7	23.8	129.5	23.7	TTT	single	TTT	A	A	pure mutant
PRP7	43.4	197.5	15.0	TTT	single	-	A	A	pure mutant
ROP14	25.8	100.0	11.2	TTT	single	TTT	A	A	pure mutant
SUP13-2	10.3	65.2	4.9	TTT	single	-	A	A	pure mutant
VAHP1	9.2	10.5	4.5	TTT	single	-	A	A	pure mutant
AMP1	60.9	43.3	58.2	TAT	double	TWT	C	R	Mix
IVP4	21.3	101.1	28.6	TAT	double	TWT	C	R	mix
JRP3	27.6	128.2	43.8	TAT	double	TWT	C	R	Mix
VAHP4	23.6	51.0	31.9	TWT	double	TWT	C	R	mix
JRP1	30.4	164.3	8.1	TWT	double	-	C	R	Mix
JRP4	35.5	121.9	26.4	TWT	double	TWT	C	R	Mix

JWP1	288	58.8	78.9	TWT	double	-	C	R	Mix

[a] Resistance factor from EC50 mean of three assays per culture; '-' isolate lost before repeat assay ; n/a – not tested
[b] Corresponding to codon 136; TAT = tyrosine (Y) and TTT = phenylalanine (F)
[c] Repeat sequencing with primer pair L419/R419
[d] Genotyping at the 136th codon: wildtype=TAT; pure mutant=TTT; mix= TTT/TAT or TWT
[e] Nucleotide 1086 resulting in synonymous mutation at codon 326 (Ala-GCC/A; M=A/C)
[f] Nucleotide 1137 resulting in a synonymous mutation at codon 343 (Gly=GGG/A; R=A/G)

Table 4.3. SNP genotyping of *Erysiphe necator* isolates isolated from field plants on grape leaves treated with 10 mg L^{-1} tebuconazole.

Isolate	SNP genotyping*
AF10-1	mix
AF10-12	mix
AF10-18	mix
AF10-19	mix
AF10-20	mix
AF10-21	mix
AF10-22	mix
AF10-23	mix
SC10-20	mix
SC10-13	mix
SC10-14	mix
SC10-21	mix
SC10-15	pure mutant
SC10-19	mix
SC10-23	pure mutant
SC10-30	pure mutant
RO10-7	pure mutant
RO10-17	pure mutant
RO10-20	pure mutant
RO10-25	pure mutant
RO10-27	pure mutant
RO10-28	pure mutant
RO10-30	pure mutant
RO10-34	pure mutant

* By a Taqman-based SNP genotyping protocol done in the StepOne Plus (Applied Biosystems). Mix genotype indicates presence of both wildtype (TAT) and mutant (TTT) alleles representing the 136th codon of *Encyp51*; pure mutant refers only to TTT.

Table 4.4. Comparison of *cyp51* expression levels (mean RQ) of *Erysiphe necator* isolates with different genotypes (TWT, TTT, TAT) based on the 136th codon of *cyp51*.

Genotype	n	Mean RQ* ±SE
TWT	10	10.65±1.4a
TTT	8	7.87±1.6a
TAT	6	1.12±1.8b

* Determined by the $C_T\Delta\Delta$ method Means with the same letter are not significantly different at $\propto$=0.05, P<0.0001

Putative trans-membrane region

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#O14442   1   MYIADILSDLLTQQTTRYGWIFMVTSIAFSIILLAVGLNVLSQLLFRRPYEPPVVFHWFP   60
R-USA     1   MYIADILSDLLTQQTTRYGWIFMVTSIAFSIILLAVVLNVLSQLLFRRPYEPPVVFHWFP   60
W-USA     1   MYIADILSDLLTQQTTRYGWIFMVTSIAFSIILLAVVLNVLSQLLFRRP EPPVVFHWFP   60

                                CR1              37
#O14442   61  IIGSTISYGIDPYKFYFDCRAKYGDIFTFILLGKKVTVYLGLQGNNFILNGKLKDVNAEE  120
R-USA     61  IIGSTISYGIDPYKFYFDCRAKYGDIFTFILLGKKVTVYLGLQGNNFILNGKLKDVNAEE  120
W-USA     61  IIGSTISYGIDPYKFYFDCRAKYGDIFTFILLGKKVTVYLGLQGNNFILNGKLKDVNAEE  120

                    CR2
#O14442   121 IYTNLTTPVFGRDVVYDCPNSKLMEQKKFMKTALTIEAFHSYVTIIQNEVEAYINNCVSF  180
R-USA     121 IYTNLTTPVFGRDVVFDCPNSKLMEQKKFMKTALTTEAFHSYVTIIQNEVEAYINNCVSF  180
W-USA     121 IYTNLTTPVFGRDVVYDCPNSKLMEQKKFMKTALTTEAFHSYVTIIQNEVEAYINNCVSF  180

                          136                 156      CR3
#O14442   181 QGESGTVNISKVMAEITIYTASHALQGEEVRENFDSSFAALYHDLDMGFTPINFTFYWAP  240
R-USA     181 QGESGTVNISKVMAEITIYTASHALQGEEVRENFDSSFAALYHDLDMGFTPINFTFYWAP  240
W-USA     181 QGESGTVNISKVMAEITIYTASHALQGEEVRENFDSSFAALYHDLDMGFTPINFTFYWAP  240

#O14442   241 LPWNRARDHAQRTVARTYMNIIQARREEKRSGENKHDIMWELMRSTYKDGTPVPDREIAH  300
R-USA     241 LPWNRARDHAQRTVARTYMNIIQARREEKRSGENKHDIMWELMRSTYKDGTPVPDREIAH  300
W-USA     241 LPWNRARDHAQRTVARTYMNIIQARREEKRSGEXKHDIMWELMRSTYKDGTPVPDREIAH  300

          CR4
#O14442   301 MMIALLMAGQHSSSSTSSWIMLWLAARPDIMEELYEEQLRIFGSEKPFPPLQYEDLSKLQ  360
R-USA     301 MMIALLMAGQHSSSSTSSWIMLWLAARPDIMEELYEEQLRIFGSEKPFPPLQYEDLSKLQ  360
W-USA     301 MMIALLMAGQHSSSSTSSWIMLWLAARPDIMEELYEEQLRIFGSEKPFPPLQYEDLSKLQ  360

               CR5
#O14442   361 LHQNVLKEVLRLHAPIHSIMRKVKNPMIVPGTKYVIPTSHVLISSPGCTSQDATFFPDPL  420
R-USA     361 LHQNVLKEVLRLHAPIHSIMRKVKNPMIVPGTKYVIPTSHVLISSPGCTSQDATFFPDPL  420
W-USA     361 LHQNVLKEVLRLHAPIHSIMRKVKNPMIVPGTKYVIPTSHVLISSPGCTSQDATFFPDPL  420

                                                CR6 [heme-binding]
#O14442   421 KWDPHRWDIGSGKVLGNDAVDEKYDYGYGLTSTGASSPYLPFGAGRHRCIGEQFATLQLV  480
R-USA     421 KWDPHRWDIGSGKVLGNDAVDEKYDYGYGLTSTGASSPYLPFGAGRHRCIGEQFATLQLV  480
W-USA     421 KWDPHRWDIGSGKVLGNDAVDEKYDYGYGLTSTGASSPYLPFGAGRHRCIGEQFATLQLV  480
```

#O14442	481	TIMATMVRFFRFRNIDGKQGVVKTDYSSLFSMPLAPALIGWEKR	524
R-USA	481	TIMATMVRFFRFRNIDGKQGVVKTDYSSLFSMPLAPALIGWEKR	524
W-USA	481	TIMATMVRFFRFRNIDGKQGVVKTDYSSLFSMPLAPALIGWEKR	524

Fig. 4.1. Alignment of the consensus amino acid sequence of CYP51 of *Erysiphe necator* isolates from the United States, with Y136F (R-USA) and without Y136F (W-USA) with the CYP51 (UniProtKB/Swiss-Prot Accession no. O14442) of *E. necator* isolate from Europe. Conserved domains are underlined in the European sequence as proposed by Delye et al. (1). Amino acids in red indicate a variation from at least one other sequence.

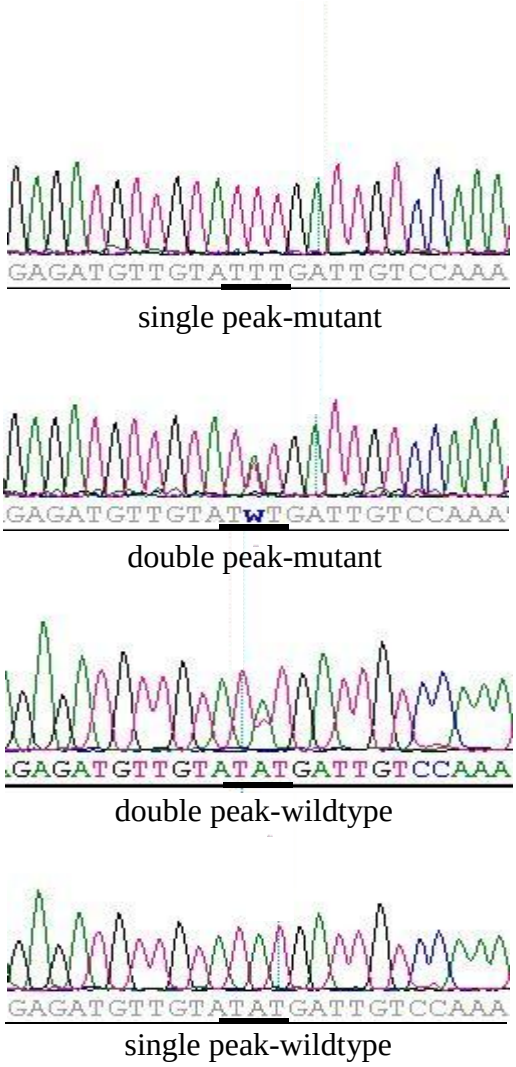

Fig. 4.2. Partial sequence chromatograms of a partial section of the *Erysiphe necator cyp51* gene showing the three genotypes at codon 136 – TAT, TTT, TWT (underlined with dark solid line). Description of peaks is shown below each graph.

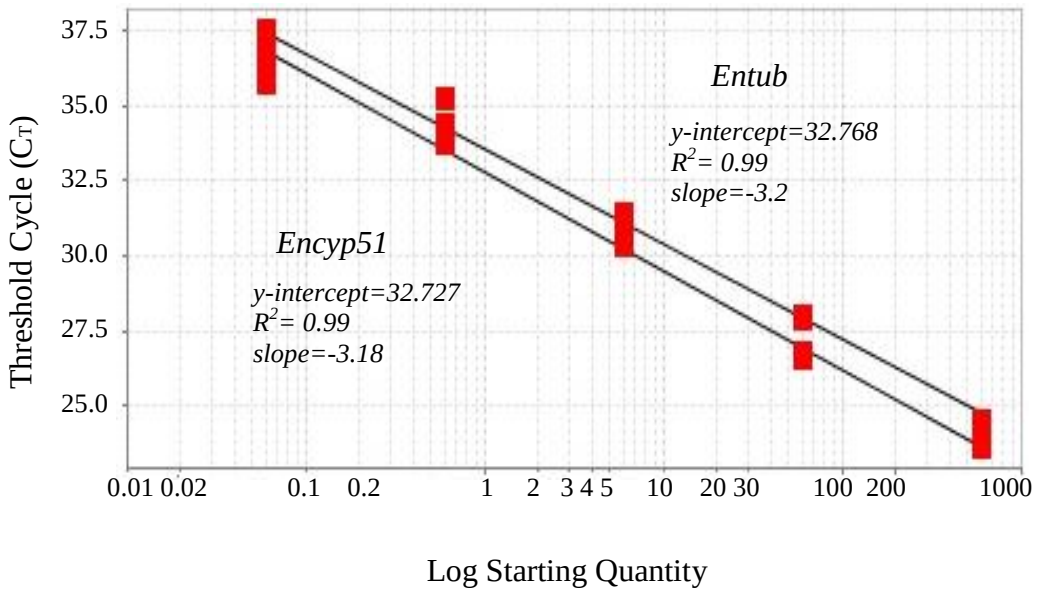

Fig. 4.3. Real-time PCR standard curve for *Erysiphe necator beta-tubulin* (upper line) and *cyp51* cDNA (lower line) generated in the StepOne Plus instrument (Applied Biosystems).

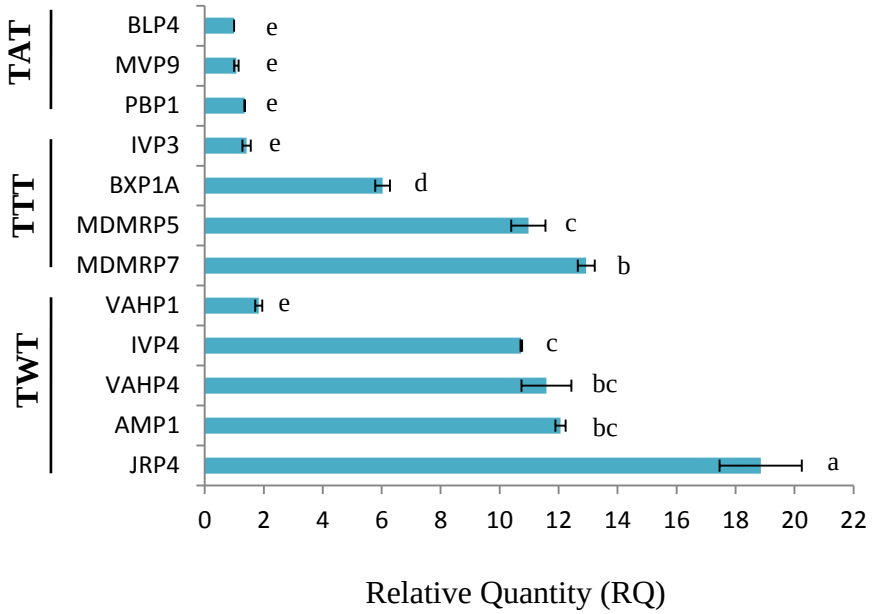

Fig. 4.4. Relative quantity of *cyp51* in *Erysiphe necator* isolates with different genotypes at the 136th codon. TAT- wildtype, TTT-pure mutant and TWT-mix mutant. Relative expression was determined using the $\Delta\Delta C_T$ method with the *En* β-tubulin gene as endogenous reference and BLP4 as the calibrator.

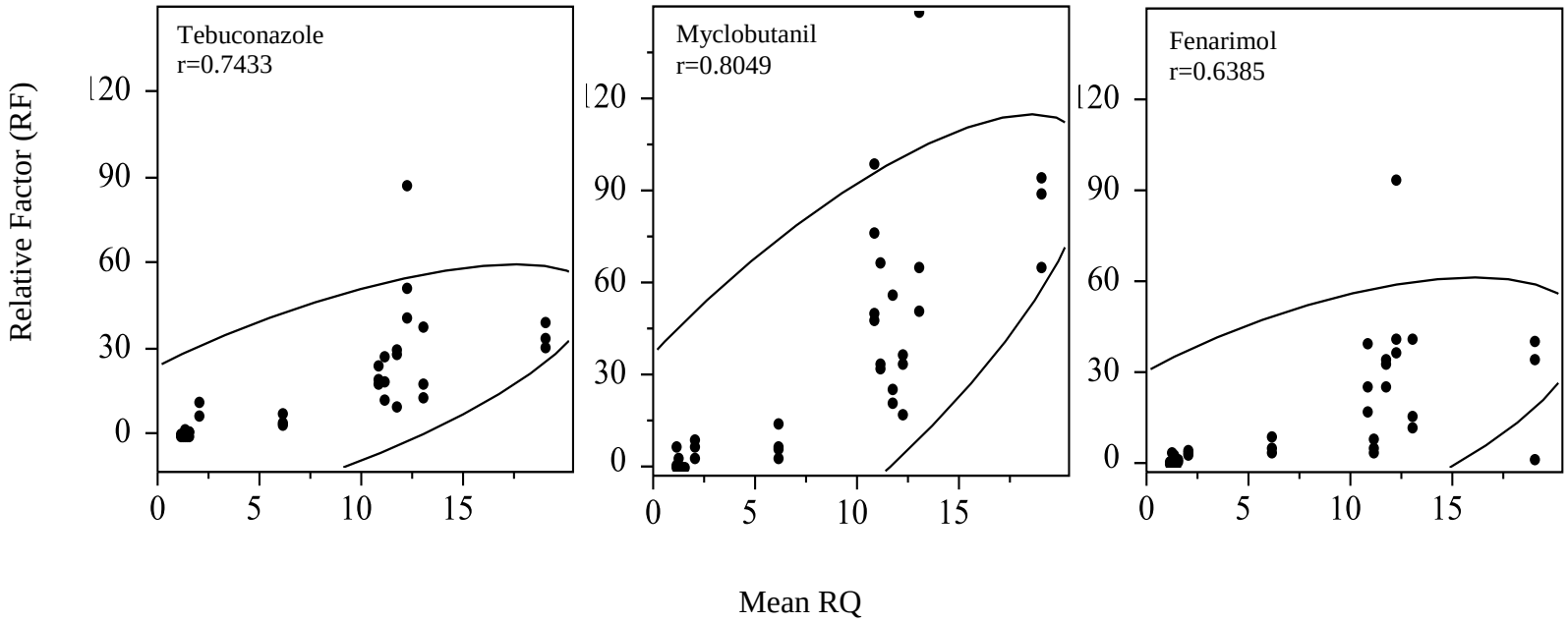

Fig. 4.5. Correlation of the relative quantity of *cyp51,* RQ (compared to a reference sensitive isolate without the Y136F as the internal calibrator, and the *En*β-tubulin gene as the normalizer) in *Erysiphe necator* isolates and their sensitivity (Resistance Factor=RF) to tebuconazole ($P<0.0001$), myclobutanil ($P<0.0001$) and fenarimol ($P<0.0001$). Each RF value is a mean of at least three independent assays and each RQ value is the mean of two independent reverse transcription and gene expression reactions from the same RNA source.

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Chapter 5

Stability of DMI Resistance in *Erysiphe necator*

Abstract

The stability of DMI resistance in *Erysiphe necator* was determined for isolates maintained under short and long-term subculturing on fungicide-free host tissue. Sensitivity shifts for tebuconazole, myclobutanil and fenarimol occurred in several highly and moderately resistant isolates maintained several years on fungicide-free host leaves. The decline in resistance did not always occur for all three DMIs in an isolate, and the magnitude of decline differed among the fungicides tested, indicating the variation in response of *E. necator* to different DMIs. Sensitivity shifts were not evident after a few transfers in the absence of tebuconazole. Similarly, a rapid decline under laboratory conditions (within two or three culture transfers on fungicide-free host tissue) was rarely observed for isolates previously exposed to myclobutanil in the field, and not at all for difenoconazole-exposed isolates. Isolates continuously maintained on fungicide-free host leaves expressed *cyp51* 17 to 21 times more than a sensitive isolate, indicating that *cyp51* is constitutively expressed. Exposure to tebuconazole at 3 $\mu g.ml^{-1}$ slightly enhanced *cyp51* expression in two out of four isolates tested. Overall, our results demonstrated the phenotypic instability of DMI-resistant *E. necator* cultured under laboratory conditions.

Keywords: DMI resistance, instability, tebuconazole, myclobutanil, fenarimol, difenoconazole, *cyp51* expression

5.1. Introduction

Fungicides are an integral component of many fungal disease management programs. A major setback with continued use of fungicides is the emergence of resistant strains. This can lead to practical resistance where the sensitivity of the pathogen population is greatly reduced to levels that cannot be controlled effectively by the chemical. The decline in fungicide efficacy can translate into serious economic consequences such as crop loss, and result in the loss of valuable tools for disease control. The risk of resistance development is a function of several variables that relate to the biological characteristics of the pathogen, the nature of the fungicide, and the spray regimen (4). The selection for resistance is expected to be more rapid with more frequent fungicide applications on target pathogens having shorter generation times and abundant asexual spore production (18).

Erysiphe necator is a major fungal pathogen of grapevines. To alleviate disease pressure, a combination of cultural practices and chemical spray applications is necessary. Demethylation inhibitors (DMIs) constitute one of the largest fungicide classes used for grapevine powdery mildew control. The risk of resistance development to DMIs in *E. necator* is moderate (12) but resistance has already emerged in pathogen populations in a number of grape-growing areas in the United States (6, 11, 23, 29-31). Nevertheless, complete loss of efficacy of DMIs has not been reported so far. This may mean slow resistance development in the pathogen, as it could take several steps or mechanisms in the population before practical resistance is achieved, or the presence of fitness penalties. DMI resistance in *E. necator* is quantitative, i.e., it follows a continuous distribution of sensitivities (6, 11). This type of resistance is often assumed to be polygenic in nature, although resistance in *E. necator* has been attributed so far only to the Y136F mutation in the target protein CYP51 (6, 10). In another study, we have observed the correlation of *cyp51* overexpression with DMI resistance in mutant isolates (see Chapter 4), suggesting the possible involvement of other genes in the regulation of expression.

One question of practical importance is whether resistance is stable after the selection pressure is removed. The persistence of resistance in a fungicide-free environment (such as when the grower stops applying the fungicide) is dependent on the fitness of individuals. If there is significant fitness cost, the sensitive strains, either those that escaped fungicide effects throughout the spraying season or those that migrated from nearby unexposed sources, will out-compete the resistant fraction leading to a sensitivity shift in the population. DMI stability can be tested *in vitro*, with results revealing different responses for different fungal pathogens (7, 19, 20, 22, 32). A decline in DMI resistance under laboratory conditions may reflect the potential for shifts in field conditions when DMI selection pressure is reduced.

In our work with single-spored isolates that had been maintained on detached host leaves for many generations, we gained the impression that DMI resistance levels sometimes declined with time. The main objective of this study was to test this possibility, and to determine the stability of DMI resistance in *E. necator* isolates maintained on fungicide-free host tissues under prolonged and short-term culturing.

5.2. Materials and Methods

5.2.1. Testing stability of resistance after long-term culturing. Stock solutions of tebuconazole (Bayer Crop Science LP, Research Triangle Park, NC), myclobutanil (DOW Agrosciences LLC, Indianapolis, IN) and fenarimol (Gowan Company, Yuma, AZ) were prepared in acetone from the technical grade fungicide and stored at -10^{o}C. The nineteen isolates assayed for the three DMIs are part of our collection obtained from various locations in Virginia and nearby states from 2005 to 2008. The fungicide sensitivities were initially determined by Colcol (5) and used as the reference point (first assay in 2008) for comparison in the current study. From first isolation, the isolates were maintained on fungicide-free grape leaves as single-spored cultures and transferred every 15 to 25 days. To determine sensitivity changes, fungicide assays were done in 2009 and 2010 for tebuconazole and fenarimol using the settling tower method described by Colcol, Rallos, and Baudoin (6), and in 2011 for tebuconazole and myclobutanil using a spore-inoculation method. For the spore-inoculation method, fungicide solutions and leaf treatments were done as described in Colcol, Rallos, and Baudoin (6) but leaves were point-inoculated with five to six spore chains.

To prepare leaf treatments, three-fold serial dilutions of the fungicide were prepared using sterile water with 0.005% Tween 20 (STW) as diluent. Three-fold dilutions were done starting at 0.001 to 1 µg.ml^{-1} or at 0.01 to 30 µg ml $^{-1}$, depending on the isolate. Leaves were soaked in fungicide solution for 1 hr, blotted dry and plated on 0.7% water agar. Since the DMIs used in the experiment has a property of translamilar movement, the a.i., should move into the tissues after 1 hr of soaking. Two leaves were used per concentration and leaves soaked in STW were included as control. Individual spore chains (average of 6) from each culture were inoculated on different parts of the leaf. Plates were incubated at 25^{o}C under a 12-hr light regime for 6 to 8 days.

The diameter of colonies that developed was measured with an ocular micrometer at 17x total magnification under a dissecting microscope. Relative growth was calculated from the mean of the diameters on the two leaves as percentage of the mean diameter of the no-fungicide-treatment control. For each isolate, there were three replications (i.e., three leaves incubated independently) per experimental run (i.e., transfer). To estimate EC_{50}, the relative diameter of treatments was regressed on natural-log-transformed fungicide concentration. From the linear section of the regression, the EC_{50} was calculated in Microsoft Excel using the formula adopted by Colcol, Rallos, and Baudoin (6) as follows: $EC_{50} = e^{([50\text{-}b]/m])}$, from the regression equation y = *m*.1n (concentration) + b. The resistance factor (RF) for each fungicide was calculated as EC_{50} of the isolate (mean from at least three assays) divided by the mean EC_{50} of sensitive isolates assayed in the same period. A minimum of two-fold difference in resistance factor was considered a sensitivity shift. Isolates were categorized into sensitivity groups based on their initial RF calculated in 2008 by Colcol (5). Isolates with RF≥100 were grouped as highly resistant, while isolates with 100<RF≥10 were moderately resistant (Table 5.1). Low resistance was characterized as having 2>RF<10; only one isolate fell within this group for fenarimol. Isolates representing different patterns of sensitivity shifts were selected and the RF values were plotted over the assay period to show the decline over time.

5.2.2. Testing rapid decline in tebuconazole resistance in 2011 isolates. Field isolates were collected in August 2011. AF populations were taken from a field where vines were sprayed biweekly with Elite 45WP at 4 oz/A (active ingredient tebuconazole) for a total of four

sprays. MR populations were obtained from a field where vines were sprayed weekly with Rally (active ingredient 10 µg.ml^{-1} myclobutanil) for nine weeks. Both AF and MR vineyards are located in Virginia, and are approximately 250 km apart. Infected leaves from AF were placed in Ziploc bags and transported to the laboratory in a Styrofoam chest, after which a series of culture transfers were made. Samples from MR were mailed in a cardboard box to the laboratory.

The schematic diagram for the culture transfers is shown in Fig. 5.1. Disease-free leaves from greenhouse grown plants were first disinfested by shaking for 2 min in 20% commercial bleach with 0.01% Tween 20, rinsed three to four times with sterile water, then shaken in 3 µg.ml^{-1} tebuconazole for 1 hr. Treated leaves were blotted dry and placed on 0.7% water agar, with the abaxial side on the agar surface. An infected leaf from the field (AF and MR samples) was gently pressed on the disinfested leaf. This culture transfer was called a "rub". From one colony that developed from this rub, a single chain of spores was transferred to a tebuconazole (teb)-treated leaf (T1) in scheme A. A second transfer (T2) was made on another teb-treated leaf, after which cultures were established by parallel transfers on water-treated (T3-W) and teb-treated leaves (T3-Teb). This scheme was followed for MR11-B2, MR11-B3 and MR11-B10. MR11-B3 was further cultured in parallel successively until the eighth transfer (T8). In scheme B, isolates AF11-2, AF11-3, AF11-4 and MR11-B1 were cultured in parallel on water-treated and teb-treated leaves directly from the rub to generate T1 subcultures. Only the AF11 isolates in scheme B were transferred in succession up to T7. In both schemes A and B, the cultures were parallel-transferred six times. Fungicide assays for tebuconazole resistance were done as described in the previous section at the first single-spore transfer (T1 or T2), and at the beginning (T2 or T3) and end (T7 or T8) of parallel transfers. The indices used to measure growth were colony diameter, relative diameter, number of colonies that developed, and success rate (number of colonies that developed out of the number of colonies inoculated).

5.2.3. Testing recovery of tebuconazole resistance by a "training" experiment. The subcultures in the previous section (5.2.2) at T7 or T8 that were continuously grown on water-treated leaves were subjected to a "training" experiment whereby conidia were serially transferred while increasing concentrations of tebuconazole. The training was initiated on fungicide-treated leaves at 0.01 µg.ml^{-1} tebuconazole then at increasing concentrations as follows: one transfer each on 0.03, 0.1 and 0.3 µg.ml^{-1}, three transfers on 1 µg.ml^{-1}, then two on 3 µg.ml^{-1} and two on 5 µg.ml^{-1} tebuconazole. The final assay was conducted on T17 (AF11-2 and AF11-4) or T18 (MR11-B3). Only MR11-B3, AF11-2 and AF11-4 were retained at 5 µg.ml^{-1} tebuconazole. All bioassays were done by the spore-inoculation method. Subcultures were assayed at least three times, using two leaves per treatment. The indices used to measure growth were colony diameter, relative diameter, number of colonies that developed, and success rate (number of colonies that developed out of the number of colonies inoculated).

5.2.4. Testing rapid decline for myclobutanil and difenoconazole in 2012 isolates. A vineyard in Franklin County, Virginia was selected as source of DMI-resistant isolates. Three plots were assigned randomly for each spray treatment. Each plot consisted of two parallel rows, with four vines of Cabernet Sauvignon per row. A total of 24 vines for each treatment were used. Plots were separated by a buffer zone (unsprayed) of one or two rows of vines. Treatments consisted of: (i) Elite WP (tebuconazole at 4 oz/acre), (ii) Rally 40 WSP (myclobutanil at 5 oz/acre), (iii) Inspire Super, (cyprodinil and difenoconazole at 16 fl oz/acre).

Presidio (Valent Canada Inc., Ontario, Canada) was added to all treatments at 4 oz/acre to control downy mildew. Plots were sprayed three times at two-week-intervals starting on May 24, 2012. Infected leaf samples were collected from each plot before the second and third spray, on June 22 and July 6, because infection was already visible. Samples from each plot were considered as one population. From each population, an attempt was made to collect strains with high resistance by initially rubbing the infected leaf on leaves treated with 10, 30, 60 and 100 $\mu g.ml^{-1}$ for myclobutanil and tebuconazole, or 0.1, 0.3, 1 and 3 $\mu g.ml^{-1}$ difenoconazole. From a colony that developed on the leaves, a single-spore culture (T1) was initiated for each population on fungicide-treated leaves and non-treated leaves. The fungicides used for T1 were 10 $\mu g.ml^{-1}$ tebuconazole, 10 and 30 $\mu g.ml^{-1}$ myclobutanil and 0.01 and 0.1 $\mu g.ml^{-1}$ difenoconazole. Conidia were transferred to water-treated leaves to initiate T2 and T3 for some isolates. Other isolates from T1 were maintained on fungicide-treated leaves (10 $\mu g.ml^{-1}$ myclobutanil, or 0.1$\mu g.ml^{-1}$ difenoconazole) all the way to T3. Diameter was measured at T2 and T3 on growth on leaves treated with discriminatory doses of the fungicide (1 and 10 $\mu g.ml^{-1}$ myclobutanil, or 0.01and 0.1 $\mu g.ml^{-1}$ difenoconazole, or 3 and 10 $\mu g.ml^{-1}$ tebuconazole). Relative percent diameter was calculated from the mean diameter on fungicide-treated leaves divided by the mean diameter of the same isolate on water-treated leaves. Another transfer was made on fungicide-free host leaves (T2). To determine a decline in resistance, relative growth was determined based on diameters of colonies on leaves treated with the same discriminatory dose.

5.2.5. *Cyp51* expression analysis. Surface growth consisting of spores and hyphal fragments from the 2011 isolates were collected from both parallel transfers at T7 (AF11s) or T8 (MR11-B3) and subjected to gene expression analysis. Four sample sets (four isolates each with a sample from the control and from the tebuconazole-treated leaves), and two sets per isolate were obtained. Fungal tissues were collected by scraping the infected leaf surface with a sterile spatula, then deposited into a Lysing Matrix C tube (MP Biomedicals). Tubes were frozen at -50^{o}C until RNA extraction. Buffer RLC (450 µL, Qiagen) was added to the tube immediately after it was taken out of the freezer. Fungal tissue was disrupted by bead-beating two consecutive times at 4 $m.s^{-1}$ for 50 s in the FastPrep-24 instrument (MP Biomedicals). RNA extraction was completed using the RNEasy Plant Mini Kit (Qiagen) following manufacturer's protocol for filamentous fungi. The resulting RNA sample was diluted to 15 $ng.\mu l^{-1}$ and reverse-transcribed using the High Capacity cDNA Kit (Applied Biosystems). The cDNA levels of *cyp51* were quantified by the comparative C_T method ($\Delta\Delta C_T$) in the StepOne Plus instrument (Applied Biosystems). *Cyp51* expression was normalized to the expression of the β-tubulin (*Entub*) gene of *E. necator*. Probes labeled with the fluorescent dye FAM at the 5′-end and a non-fluorescent quencher with a minor groove binder (MGB) at the 3′-end were utilized to enable detection of the specific PCR product as it accumulated during amplification. All primers and probes were designed in the Custom TaqMan Assay Design Tool of Life Technologies (www.lifetechnologies.com) using our consensus sequence for *Encyp51* and Genbank Accession no. AY074934 for *Enβ-tub* (see Chapter 4 for primer information) (1). The primers were customized for a CYP51 and a β-TUB Gene Expression Assay mix by Applied Biosystems. Reactions were performed in a total volume of 20 µl containing 1x Taqman Universal PCR Mix (Applied Biosystems), 1x Taqman Gene Expression Assay mix (Applied Biosystems), and 2 µl of the cDNA template. Separate reaction wells were designated for the target and the reference genes, each of which was amplified in triplicate reactions with the following thermocycling program: a pre-amplification step at 50°C for 2 min, an initial denaturation at 95^{o}C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Reverse transcription and gene expression

quantitation were done twice for each RNA sample. The relative expression (RQ) was calculated by the StepOnePlus software v.2.1 (Applied Biosystems) as $2^{-\Delta\Delta C_T}$. A sensitive isolate (BLP4) was used as reference.

5.2.6. Statistical analysis. To determine a decline in tebuconazole resistance, the effects of transfer source and bioassay concentration of tebuconazole on different indices of growth of *E. necator* were analyzed by comparing slopes and intercepts of regression lines by group (grown with tebuconazole-treatment/Teb or with water-treatment/W) and by vineyard source (MR11 or AF11) using analysis of covariance in JMP 10 Pro (SAS Institute, Cay, NC), with $P \leq 0.05$ as the significant level. Four variables of growth were measured in order to visualize the effect of vineyard source, leaf treatment, and transfer source on the response of *E. necator* isolates to increasing concentrations of tebuconazole. Actual diameter and relative diameter were measurements of growth, while number of colonies and isolation success rate were indices of colony establishment. Slopes and intercepts were calculated from the parameter estimates of the AnCOVA output as follows: intercept = estimate of intercept + estimate of transfer source [T#], and slope = estimate of concentration + estimate of [transfer source [T#] x concentration]. To determine if mean relative diameters of the first and second transfers for isolates in the myclobutanil and difenoconazole decline experiments are the same, a *t*-test assuming unequal variances was done in JMP Pro v.10.0 (SAS Institute Inc.). To determine differences in gene expression between treatments (tebuconazole and water), mean relative quantity (RQ) was analyzed by isolate using one-way ANOVA in JMP, with experimental trials treated as block.

5.3 Results

5.3.1. DMI decline during long-term subculturing. A decline in EC_{50} was observed for a number of isolates maintained in the laboratory for several years on fungicide-free host tissue. However, the decline did not always occur for all three DMIs tested. For example, a slight decline was observed for AMP1 with tebuconazole, but was absent for myclobutanil and fenarimol (Fig. 5.2). In the case of GRP18 and JRP4, the decline was obvious for fenarimol and tebuconazole, but not for myclobutanil. The decline was evident only in the first two assay intervals as shown for myclobutanil in SUP13 and VAHP4, and for fenarimol in VAHP4.

In general, sensitivity shifts were observed for tebuconazole, myclobutanil and fenarimol in highly and moderately resistant isolates (Table 5.1). Observations on shifts in the low-resistance group could not be made because of the lack of isolates. Within the first and second assay periods (2008 to 2009), the highest frequency of shifts was observed for fenarimol where all nine highly resistant isolates experienced a two-fold decline in RF (Table 5.1). Changes were still observed up to 2010 but at lower frequencies (<50%) for both sensitivity groups. By 2010, RF values declined 4 to 9 times for moderate resistance (from an initial RF=25-88 to final RF=6-10), and 22 to 23 times for high resistance (from initial RF=112-391 to final RF=5-17).

Only one of the eight highly resistant isolates to tebuconazole underwent a shift within a year; on the other hand, higher frequencies (>50%) for both the moderate- and high-resistance groups were observed during the 2009 to 2011 interval (Table 5.1). By 2011, the decline in RF values was 3 to 13 times in the moderate-resistance group, from an initial RF=26-99 to a final RF=2-25, and 2 to102 times for the high resistance group, from an initial RF≥112-514 to a final RF=6-120.

A high frequency (8/15) of isolates in the high-resistance group shifted in sensitivity to myclobutanil from 2008 to 2009 (Table 5.1). None of the moderately resistant isolates underwent a shift within a year, but most of the isolates continued to decline after the 2009 assay period in both sensitivity groups. By 2010, RF values had declined to 0.03-7 from an initial RF=39-96 for the moderate resistance group, and to 0.2-80 from RF=101-1707 for the high-resistance group. The shifts for myclobutanil were greatest among the three fungicides, because resistance declined by as much as 3200 times.

The findings indicate that sensitivity shifts occurred in many of our DMI-resistant *E. necator* isolates during long-term subculturing on fungicide-free host tissue. It should be noted that some isolates have declined to RF≤2 which was within the range of the sensitivity of sensitive isolates (data not shown).

5.3.2. DMI decline during short-term subculturing. There was an overall declining trend in colony diameter, relative diameter, number of colonies, and success rate (Fig. 5.3). Aside from the leaf treatment (tebuconazole or water), it appeared that there was also an effect by vineyard source and source of transfers. In Fig. 5.3, an example of this declining trend is shown for isolation success rate, plotted by vineyard source and transfer source, with concentration of tebuconazole as the independent variable. When the effects of vineyard source, source of transfer (T#), and concentration of tebuconazole were examined, significant results were found with colony diameter, relative diameter, number of colonies, and success rate (P<0.001 for each). The effect of vineyard source was then analyzed for each source of transfer over tebuconazole concentration using analysis of covariance to find out if responses are different between the two vineyard sources, and among sources of transfer. From the analysis, the intercept and slope for growth over increasing tebuconazole concentrations were examined by vineyard source and by source of transfer.

The intercepts and slopes by vineyard source, leaf treatment, and transfer source are presented in Table 5.2. Both intercept and slope from the late transfer (T7 for AF11 and T8 for MR11) were compared with other sources of transfer (T1, T2 or T3). With tebuconazole treatment, intercepts of the late transfer did not significantly differ from the early transfer, except for number of colonies which produced significantly higher values in the early transfers than in the late transfers. In both vineyard sources, water treatment resulted in intercepts for relative diameter that were significantly lower at T7 or T8 than at T2 and/or T3. However, intercepts were significantly higher for number of colonies and isolation success rate. These findings indicate that as the source of transfer (and hence, the number of transfers) increased, the reduction in colony number observed among isolates continuously exposed to tebuconazole was not accompanied by a change in colony size. However, when colony size was expressed as relative diameter, a significant reduction was observed, while colony establishment (number of colonies and isolation success rate) was significantly increased.

For AF11 isolates continuously exposed to tebuconazole, the analysis resulted in significantly less negative slope (i.e, became less sensitive to higher concentration of tebuconazole) at late transfer (Table 5.2). However, for AF11 isolates transferred to water-treated leaves, a more negative (or steeper) slope was obtained for colony size (actual and relative diameters) but not for indices of establishment (number of colonies and success rate). For MR11 isolates continuously grown on tebuconazole, no significant changes in slope were observed. On the other hand, MR11 isolates grown on water-treated leaves responded in the

same way as the AF11 isolates in the water treatment group. Slopes for actual and relative diameters were significantly steeper (or more negative) at T8, while slopes for number of colonies and success rate were significantly less steep (or less negative) at T8. The above findings revealed different responses of AF11 and MR11 isolates. AF11 isolates continuously exposed to tebuconazole tended to become less sensitive to higher concentrations of the fungicide, while the MR11 isolates remained unchanged over a series of transfers. For isolates from both vineyard sources transferred to water-treated leaves, growth measurements tended to be smaller, i.e., isolates have become more sensitive to higher tebuconazole concentrations, while measurements of colony establishment tended to be larger. Therefore, there was a tendency for better colony establishment at T8 than at T2. In addition, since intercepts were not significantly different, colony sizes at 0 $\mu g.ml^{-1}$ tebuconazole were not different between early and late transfers. However, the differences in slope indicated that colony sizes at higher tebuconazole concentration tended to be smaller at late transfer than at early transfer.

5.3.3. Training to increase DMI resistance. All three subcultures of AF11-3 failed to grow during "training" at 0.3 $\mu g.ml^{-1}$; hence, no further tests were conducted on this isolate. Out of the remaining nine subcultures subjected to training, subculture 1 of AF11-2 was not recovered at 3 $\mu g.ml^{-1}$, while subculture 2 of AF11-4 was not recovered at 5 $\mu g.ml^{-1}$ (Table 5.3).

Subcultures responded differently from each other. Only two out of three subcultures of MR11-B3 increased in EC_{50} more than two-fold after exposure up to 5 $\mu g.ml^{-1}$ tebuconazole (Table 5.3). EC_{50} increased by more than two-fold after training up to 3 $\mu g.ml^{-1}$ tebuconazole in some subcultures, but did not increase any further (MR11-B3 subculture 2, AF11-2 subculture 2) or declined (AF11-4 subcultures 1), when transferred to 5 $\mu g.ml^{-1}$ tebuconazole. A slight increase in EC_{50} may occur such as in AF11-2 subculture 3 and AF11-4 subculture 3. In summary, two out of eight subcultures had increasing EC_{50} with training up to 5 $\mu g.ml^{-1}$, while two increased then retained that resistance level. One subculture increased, then reverted to the initial resistance level.

5.3.4. Decline in myclobutanil and difenoconazole resistance under short-term subculturing. Powdery mildew developed on rubbed leaves from diseased leaves obtained from tebuconazole-sprayed plots. However, no growth was recovered from the "rubbed" culture when single spores were transferred to tebuconazole-treated host leaves to initiate T1. This indicates that shifts towards tebuconazole sensitivity may occur rapidly from the field to the laboratory.

Among six isolates tested for a decline in resistance to myclobutanil, only one was found to change significantly. Isolate M6 was reduced in diameter at both discriminatory doses of 1 ($P<t=0.003$) and 10 $\mu g.ml^{-1}$ ($P<t=0.01$) (Table 5.4). The seven isolates maintained for two transfers on myclobutanil-treated grape leaves did not undergo a sensitivity shift except for M5. Growth of M5 was reduced from a 100% relative diameter at T2 to 19% at T3, but this occurred only at the lower discriminatory dose of 1 $\mu g.ml^{-1}$ myclobutanil ($P<t=0.03$). No significant decline resistance to difenoconazole of single-spored isolates from the difenoconazole-sprayed plots were found after one transfer to fungicide-free host tissue (Table 5.5).

5.3.5. Enhancement of *cyp51* expression by tebuconazole. *Cyp51* expression of isolates maintained in either the presence or absence of tebuconazole was compared to that of BLP4, a DMI-sensitive isolate (see Chapter 4). *Cyp51* expression levels (RQ) for the resistant isolates were 17 to 21 times higher than the reference sensitive isolate (BLP4) in the absence of

tebuconazole, and 17 to 27 times higher in the presence of tebuconazole (Table 5.6). Mean RQ was significantly higher with tebuconazole exposure than with water treatment for AF11-3 and AF11-4, although only one trial was reported for the latter because of poor RNA quality in the second extraction. Mean RQ for AF11-2 and MR11-B3 was the same with and without exposure to tebuconazole.

5.4. Discussion and Conclusions

Decline after prolonged subculturing. Sensitivity shifts for tebuconazole, myclobutanil and fenarimol occurred in several highly and moderately resistant *E. necator* isolates maintained in culture for several years in the absence of DMI selection pressure. The decline in resistance in a particular isolate did not always occur for all three materials, indicating the variation in response of *E. necator* to different DMIs. Initial bioassays in 2008-2009 that reflected changes in EC_{50} prompted us to suspect a loss of potency of the fungicide stock. The activity of the old stocks was compared to new stocks obtained in 2010 or 2011 using either powdery mildew or a facultative fungus as test organisms in bioassays (data not included). It appeared that a reduction in potency in the old stock occurred only for tebuconazole. Further testing of the new tebuconazole stock in 2010 revealed that the material retained its activity for at least 6 months; thus, the large magnitude of decline observed among isolates from 2008 to 2011 was not likely due to loss of potency of the fungicide but an actual response to subculturing in the absence of the fungicide.

The instability of DMI resistance has been tested in other fungal pathogens cultured by repeated transfer on fungicide-free media (7, 21) or stored under various conditions for some length of time (20, 32). Although no decline in resistance was observed during growth on DMI-free media for *Venturia inaequalis* (20) and *in vivo* and *in vitro* in *Cercospora beticola* (19), changes in resistance occurred upon cold storage. Storage under various conditions also brought about a decline in propiconazole resistance in *Monilinia fructicola (32)*. Our *E. necator* isolates, being obligate parasites, were continuously maintained on the host tissue under laboratory conditions. Attempts were made to store conidia frozen using the procedure of Stummer et al. (28), but these were largely unsuccessful (A. Baudoin, personal communication). The observed decline suggests the potential for such changes to occur in the field, although, other factors such as fitness of individuals would have a greater impact on their persistence in field populations. The magnitude of change was also higher for highly resistant than for moderately resistant isolates, indicating that sensitivity shifts are dependent on the initial resistance level. This difference between sensitivity groups was also observed in *C. beticola* (19) and *V. inaequalis* (20). However, in *V. inaequalis*, highly resistant isolates were more stable, while moderately resistant isolates underwent greater changes. The greatest sensitivity shifts in our *E. necator* isolates were recorded for myclobutanil; thus, the magnitude of change also differed from one DMI to another. Overall, our findings revealed the instability of DMI resistance in *E. necator* under prolonged subculturing on fungicide-free host tissue.

Decline after short-term subculturing. We tested the hypothesis that there would be a rapid decline in resistance, within a few transfers from field samples to the laboratory, for tebuconazole, myclobutanil and difenoconazole. The first two are DMI fungicides that have been used against powdery mildew for several years. Difenoconazole was registered for grapes in the USA in 2009, and is sold as a mix with cyprodinil (Inspire Super) or mandipropamid

(Revus Top). The second ingredients have probably slight or no activity against powdery mildew, and good control has been observed with difenoconazole so far in field trials (3).

In testing the decline in tebuconazole resistance, growth of *E. necator* was reduced significantly when isolates from both vineyard sources (MR11 and AF11) were transferred seven or eight consecutive times on fungicide-free host leaves. This indicates that the isolates became more sensitive to the fungicide. In contrast, isolates continuously challenged with tebuconazole for seven or eight transfers did not decline in resistance. Interestingly, AF11 and MR11 isolates grown with continuous exposure to tebuconazole had different responses. AF11 isolates became more resistant to tebuconazole, while MR11 isolates retained the initial resistance level. This could be due to different adaptive responses impacted by the nature of previous fungicide exposure of the populations or may be due to different resistance mechanisms. It appears then that in *E. necator,* a change to a tebuconazole-free environment can result in rapid sensitivity shifts. On the other hand, no immediate shifts were observed for most or all of the isolates tested for myclobutanil or difenoconazole upon one to two transfers on fungicide-free grape leaves. These results revealed that *E. necator* isolates respond at different rates to different DMIs and imply that under field conditions, sensitivity changes in the pathogen population may not become apparent right after the selection pressure is withdrawn. The lack of decline for difenoconazole may be because powdery mildew has not yet developed high levels of resistance to this fungicide, and thus, populations are essentially still sensitive. Our initial bioassays on isolates maintained for several years in the laboratory revealed that some isolates resistant to other DMIs were as sensitive to difenoconazole as sensitive isolates (data not shown), although higher EC_{50} values were also obtained for highly DMI resistant isolates. For myclobutanil, drastic shifts were not observed for the M isolates grown on fungicide-free host leaves, but decline might become apparent with prolonged subculturing as revealed in isolates that we maintained for several years in the laboratory.

Training for recovery of resistance. The isolates used in the training experiment also possessed the Y136F mutation (data not shown) that we previously determined to be associated with DMI resistance (see Chapter 4 and reference #4), indicating that these isolates are not wildtype. Our training experiment revealed the capacity for *E. necator* to gain resistance upon step-wise exposure to tebuconazole, although not all isolates could be trained (i.e. some failed to grow at some point during the training). Furthermore, growing the isolate continuously at the same dose of tebuconazole allowed the fungus to maintain its EC_{50}. We observed this for isolates continuously grown up to the eighth transfer in the presence of tebuconazole (parallel transfers in the DMI decline experiment). We do not know yet if fungicide resistance can also be induced by this method in isolates that lack Y136F (sensitive), since we did not train any from our collection.

The training experiment indicates that shifts towards resistance may also occur in field populations of *E. necator* upon exposure to frequent and/or increasing doses of DMI sprays. This method, sometimes referred to as "forced selection" by repeated exposure of individual isolates to sub-lethal concentrations of the fungicide, might also indicate a practical resistance risk (4). In general, the ability to develop tolerance to a fungicide upon prolonged exposure is a common feature of fungi. However, results in other training experiments did not always yield stable resistance or useful strains for further experimentation (13, 14, 27). The effect of fungicide dose is known to occur in the field, and has been demonstrated for barley powdery mildew

populations, where higher doses of fenpropimorph not only caused faster and greater increases in fungicide resistance, but also a rapid decrease in population diversity (24). This was also observed for field populations of *Phytophthora infestans*, where directional selection toward metalaxyl resistance and a reduced genetic diversity were obtained when plots were sprayed at the highest recommended rate every 6 to11 days in one growing season (15).

Induction of *cyp51* expression by tebuconazole. Isolates obtained from DMI-exposed populations and subcultured for seven to eight transfers both in the presence and absence of tebuconazole expressed *cyp51* from 15 to 29 times more than a reference sensitive isolate, supporting our earlier finding that *cyp51* expression is associated with DMI resistance (see Chapter 4). Tebuconazole, at low concentration (3 $\mu g.ml^{-1}$), slightly enhanced gene expression in two isolates but not in the other two tested. Other mechanisms may also be responsible for the sensitivity shifts in isolates such as an MR11 isolate which underwent sensitivity changes with and without continuous exposure to tebuconazole, and yet whose *cyp51* expression was not induced by tebuconazole. Efflux pumps could be involved, as proposed to be responsible for the rapid, transient fluconazole resistance in *Candida albicans* (21) and as demonstrated in *Mycosphaerella graminicola* (33) and *Pyrenophora tritici-repentis* (25). Reimann and Deising (25) further demonstrated the potential for fungicide treatment to induce efflux transporters under laboratory and field conditions. We have attempted to detect efflux pump activity in *E. necator* by employing common inhibitors (verapamil and CCCP) of the ABC and MFS transporters, two groups of efflux pumps known to be involved in DMI resistance (2, 8, 9, 16, 17, 25, 26). The inhibitor is expected to render resistant strains susceptible, when combined with the fungicide, because the fungus loses the ability to pump out the toxic material from the cell. Our preliminary trials showed a lack of inhibition by these chemicals (data not shown), suggesting the need for optimizing experimental conditions and testing of other inhibitors.

It is not certain if DMIs other than tebuconazole can induce gene expression in *E. necator* since we have not tested other materials. Cross-resistance is known among fungicides with the same mode of action, and good correlations of gene expression with resistance were found for myclobutanil and fenarimol as well (see Chapter 4), thus, a similar response is expected for other DMIs. Overall, our results suggest that DMI resistance is unstable in *E. necator*. This instability may have practical significance in the field. If DMI selection pressure is introduced during the growing season, the frequency of resistant phenotypes could increase, leading to practical resistance and erosion of disease control. Complete loss of efficacy of DMI fungicides against powdery mildew has not been reported to date. This could be due to a gradual development of resistance in field populations and/or a result of the use of partner fungicides that have remained effective over years of use. On the other hand, withdrawal of DMI sprays may also cause the population to gradually shift towards sensitive levels. When the sensitive fraction of the population becomes dominant, good control of the population by the same fungicide is expected.

Tables and Figures

Table 5.1. Shifts in resistance factor (RF) and frequencies of *Erysiphe necator* isolates with at least two-fold RF changes upon successive subculturing on fungicide-free grape leaves for several years under laboratory conditions.

Group*	**Fenarimol**					
	Initial RF	No. Tested	Frequency with ≥2-fold decrease in RF			Final RF
			2008-2009	2009-2010	-	
Highly R	112-391	9	9	4		5-17
Mod R	25-88	6	3	2		6-10
Low R	<10	1	0	0		-
Group	**Tebuconazole**					
	Initial RF	No. Tested	2008-2009	2009-2010	2010-2011	Final RF
Highly R	>112-514	8	1	5	6	6-120
Mod R	26-99	11	0	8	8	2-25
Low R	<10	0				
Group	**Myclobutanil**					
	Initial RF	No. Tested	2008-2009	-	2009-2011	Final RF
Highly R	101-1707	15	8		12	0.2-80
Mod R	39-96	4	0		2	0.03-7
Low R	<10	0				

* Highly resistant (High R) isolates with RF≥100; moderately resistant (Mod R) with RF between 10 and 99; weakly resistant isolates (Low R) with RF < 10.

Table 5.2. Comparison of growth (based on colony diameter, relative colony diameter, number of colonies and success rate) between late transfer and early transfers of *Erysiphe necator* isolates from two vineyard sources (AF11 and MR11) grown continuously on tebuconazole-treated or water-treated grape cv. Chardonnay leaves under laboratory conditions.

Vineyard Source	Leaf Treatment	Transfer Source	Colony Diameter (mm)		Relative Colony Diameter (%)		Number of Colonies		Mean Success Rate	
			Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
AF11	Tebuconazole	T1	2.68	- 0.71**	76.44	-20.68**	3.61**	-0.81**	0.91	-0.14**
		T2	2.42**	-0.29*	65.93**	-10.63	2.47**	-0.61	0.69**	-0.16
		T7	2.83	-0.51	75.93	-14.46	3.00	-0.56	0.84	-0.12
AF11	Water	T2	3.47	-1.03**	102.08**	-32.27	3.80	-0.43**	0.87**	-0.27**
		T7	3.41	-1.41	95.15	-38.33	3.69	-0.32	0.96	-0.14
MR11	Tebuconazole	T1	3.89	-0.82	89.02	-17.28	4.51**	-0.56	1.00	-0.10
		T2	4.03**	-0.71	98.36**	-17.33	3.60	-0.71	0.91	-0.16
		T3	2.66**	-0.99*	67.64**	-27.09**	2.72**	-0.78*	0.78**	-0.19*
		T8	3.74	-0.82	90.99	-20.46	3.70	-0.63	0.94	-0.12
MR11	Water	T2	3.98	-1.16**	95.81*	-36.19*	2.53**	-1.08	0.69	-0.36**
		T3	5.05**	-1.03**	106.80**	-23.44**	3.95**	-0.66*	0.96**	-0.10
		T8	4.12	-2.32	43.25	-44.86	3.48**	-1.02	0.91	-0.13

[a] Treatment either on tebuconazole-treated (3 µg.ml^{-1}) or water-treated leaves; cultures transferred successively on treated leaves from T1 for tebuconazole group and from T2 for water group.

[b] Comparison against the last transfer (T7 for AF11 or T8 for MR11). Asterisk (*) significance level of 0.05; (**) significance level of 0.01.

Table 5.3. EC_{50} changes in *Erysiphe necator* isolates 'trained' on increasing concentrations of tebuconazole after several cycles of growth on fungicide-free host tissue.

Isolate	Subculture[a]	Initial EC_{50}[b]	EC_{50} at Teb 3[c]	EC_{50} at Teb 5[c]
MR11-B3	1		0.53±0.07	2.41±1.21
	2	0.42±0.01	1.07±0.45	0.74±0.25
	3		0.34±0.05	2.06±0.13
AF11-2	1		Lost	-
	2	0.48±0.08	1.67±0.26	1.87±0.32
	3		0.94±0.22	0.80±0.11
AF11-4	1		3.53±0.70	0.92±0.04
	2	0.89±0.06	0.55±0.08	-
	3		1.37±0.53	0.66±0.06

[a] Single-spored cultures previously grown for 7 (AF11 isolates) or 8 (MR11-B3) transfers on fungicide-free grape leaves

[b] Mean of three assays conducted on T7 or T8 growth on fungicide-free grape leaves; except for AF11-4 which was based on only two assays

[c] Measured on T15 (AF11s) or T16 (MR11) growth serially transferred on tebuconazole increased by three-fold increments from 0.01 to 5 $\mu g.ml^{-1}$

Table 5.4. Sensitivity shifts within two to three transfers (T2, T3) of *Erysiphe necator* field isolates based on percent relative diameter of colonies on grape leaves treated with a low and high discriminatory dose of myclobutanil.

Isolate**	% Relative Diameter			
	1 µg.ml^{-1} Dose		10 µg.ml^{-1} Dose	
	T2	T3	T2	T3
M1	26.7±15.4	90.7±22.8	0	5.3± 1.7
M2	84.5±15.2	72.3± 7.7	9.3± 5.4	13.0± 5.2
M6	192.0± 3.5	77.7± 6.9*	23.0± 1.8	4.0± 2.3*
M12	68.3±22.5	89.7±17.5	17.0± 9.8	11.3± 6.5
M14	78.7± 5.3	108.7±19.5	37.3± 2.6	42.3±12.2
M16	70.0	115.0± 7.2	49.3±10.5	15.3± 5.5
M5	109.0±15.1	18.7± 8.1*	7.2± 4.1	5.7± 1.6
M7	86.5±17.3	83.7± 3.6	16.3± 6.3	4.0± 2.3
M9	81.7± 6.5	90.0± 3.4	32.3± 3.3	32.7± 4.2
M10	93.0± 9.2	132.0±35.2	52.7±16.0	13.3± 6.3
M11	36.0±20.0	66.3±13.3	0	0
M13	97.0± 5.9	98.7±20.7	34.3± 5.5	57.7±28.3
M15	91.0± 8.3	88.0±11.9	65.7±11.1	70.0± 7.9

* Significant difference between T2 and T3, analysis by T-test of unequal variances; M6 $P<t=0.003$ for 1 µg.ml^{-1} dose and $P<t=0.01$ for 10 µg.ml^{-1} dose; M5 $P<t=0.03$ for 1 µg.ml^{-1} dose

** Isolates M1 to M16 were transferred to fungicide-free grape leaves (T2 and T3) after one growth cycle on myclobutanil-treated leaves (T1); isolates below the line (M5 to M15) were maintained on 10 µg.ml^{-1} myclobutanil-treated leaves for three growth cycles (T1 to T3)

Table 5.5. Sensitivity shifts within two to three transfers (T1, T2) of *Erysiphe necator* field isolates based on percent relative diameter of colonies on grape leaves treated with a low and high discriminatory dose of difenoconazole.

Isolate	% Relative Diameter			
	0.01 µg.ml^{-1} Dose		0.1 µg.ml^{-1} Dose	
	T1	T2	T1	T2
D1	84.0± 7.2	88.7± 7.6	17.3±5.5	3.0± 1.8
D2	59.3± 7.3	130.0±12.7	17.0±5.1	3.0± 1.8
D3	87.7± 7.3	98.7± 5.0	-	-
D5	104.0	108.7±10.0	64.0	27.3±15.7
D6	89.3±24.3	92.0± 6.3	7.3±4.1	40.7± 2.6
D7	55.3± 6.4	74.7±14.1	6.3±2.0	10.0± 5.9
D8	105.7±13.8	85.7± 2.5	0	36.0± 7.6

Table 5.6. Comparison of means of relative quantity (RQ) of *cyp51* cDNA from *Eryisphe necator* isolates subcultured continuously in the presence or absence of tebuconazole, with BLP4 as reference isolate.

Isolate	No of Trials	Mean RQ[a]	
		Water	Tebuconazole
AF11-2	2	20.5±0.93	21.4±0.99
AF11-3	2	21.0±0.48	26.8±0.42**
AF11-4	1	19.5±0.45	21.2±0.52*
MR11-B3	2	16.9±0.44	16.5±0.70
BLP4[b]	2	1.0±0.02	-

[a] Mean with * is significant at $\propto$=0.05; with ** is significant at $\propto$=0.01
[b] Reference isolate, DMI-sensitive

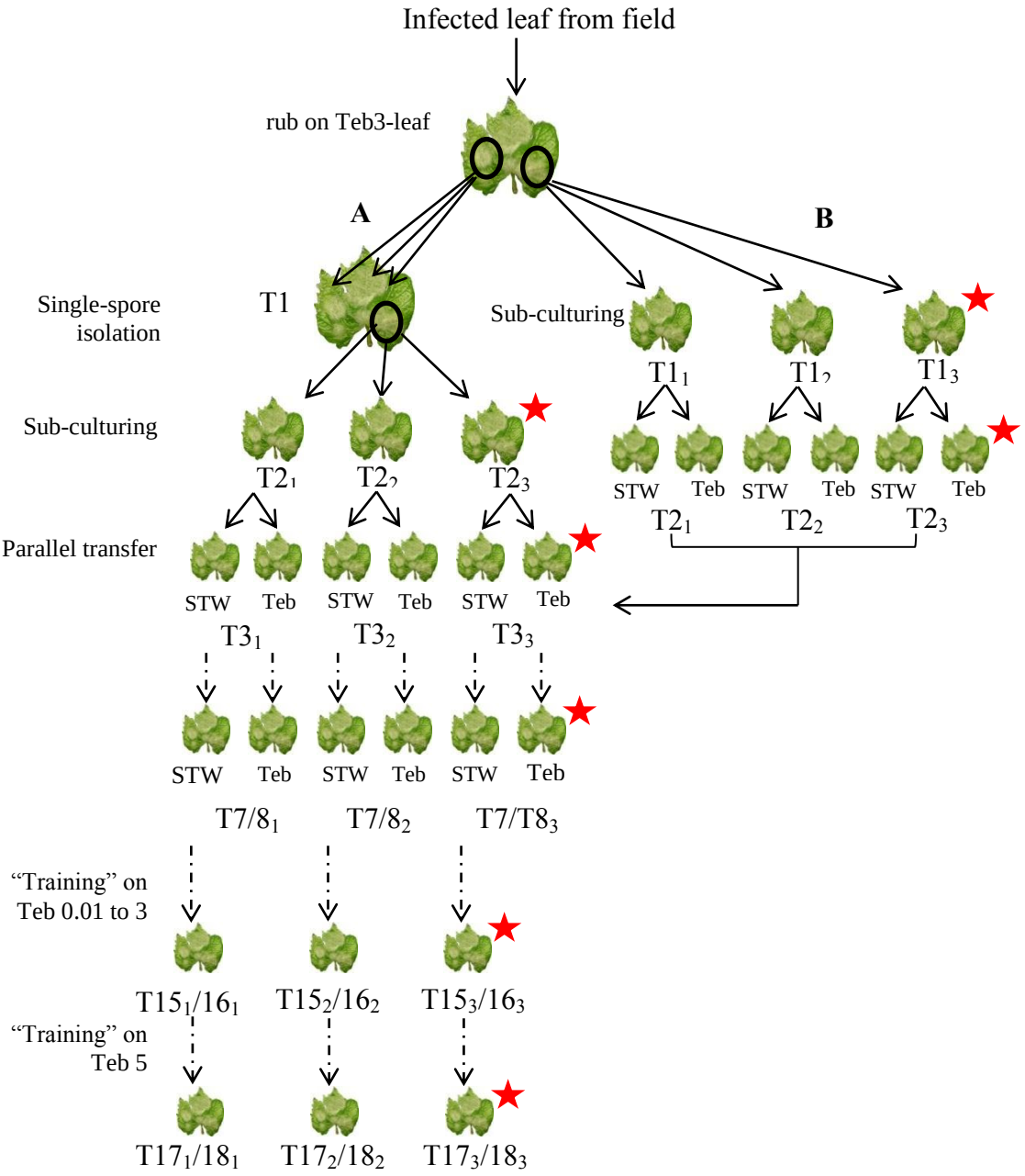

Fig. 5.1. Schematic diagram of testing the decline of DMI resistance in *Erysiphe necator* isolates obtained from field populations initially rubbed on 3 µg.ml^{-1} tebuconazole (Teb3)-treated grape (cv. Chardonnay) leaves, followed by single spore isolation on Teb3-treated leaves, and successive subculturing in parallel on sterile water with Tween 20 (STW) and Teb3-treated leaves (from T2 or T3 to T2 to T7 or T8). For the training experiment, cultures were grown back from T7 or T8 (dotted line) to T18 or T19 on tebuconazole by serial transfers on Teb-treated leaves from 0.01 to 5 µg.ml^{-1}. (A) Transfer scheme for MR11-B2/3/10; (B) Transfer for AF11-2/3/4 and MR11-B1. Red mark indicates cultures in that transfer were bioassayed for sensitivity to tebuconazole.

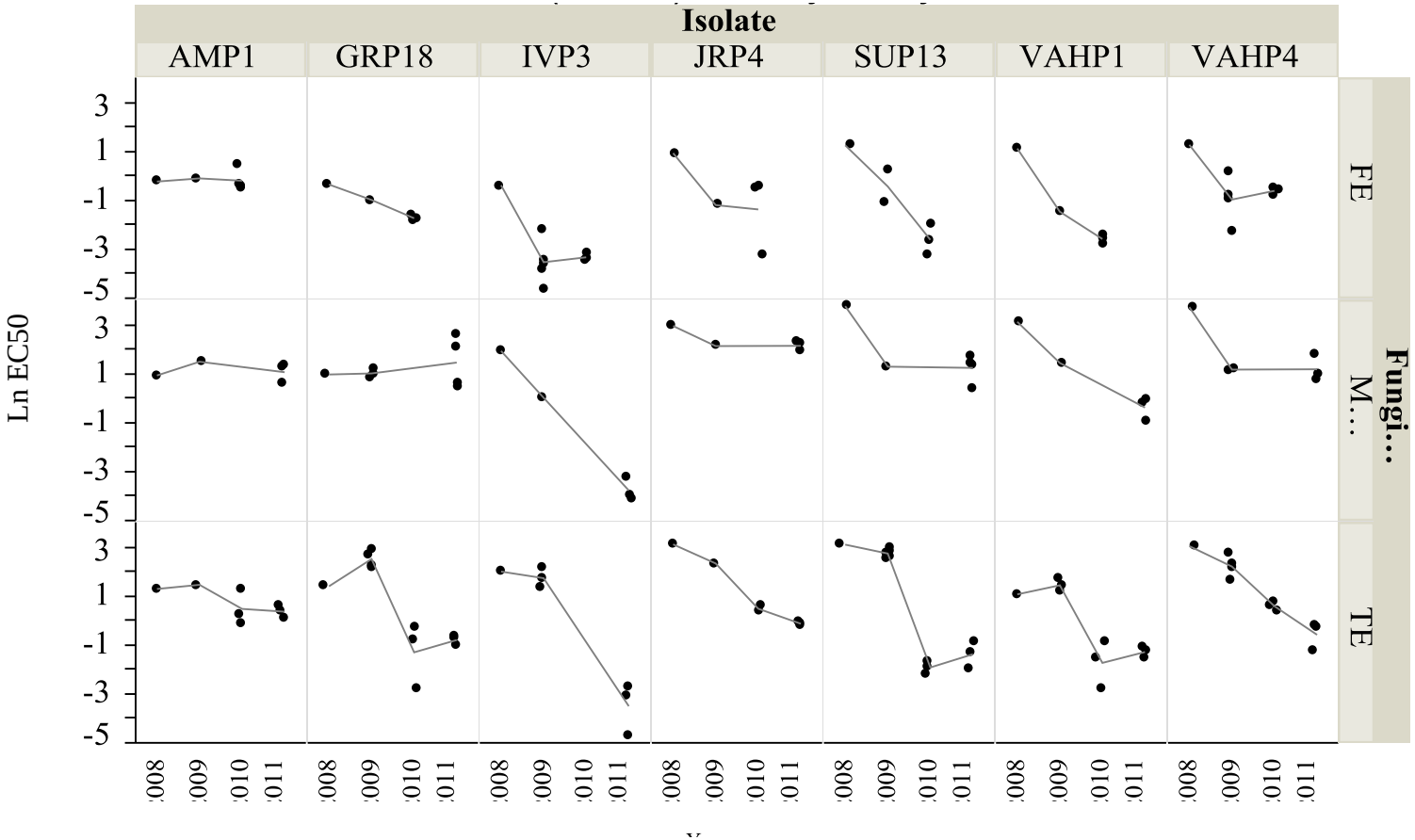

Fig. 5.2. Decline in DMI resistance in some *Erysiphe necator* isolates maintained for several years on fungicide-free grape leaves under laboratory conditions. Fen=fenarimol, Myc=myclobutanil, Teb=tebuconazole.

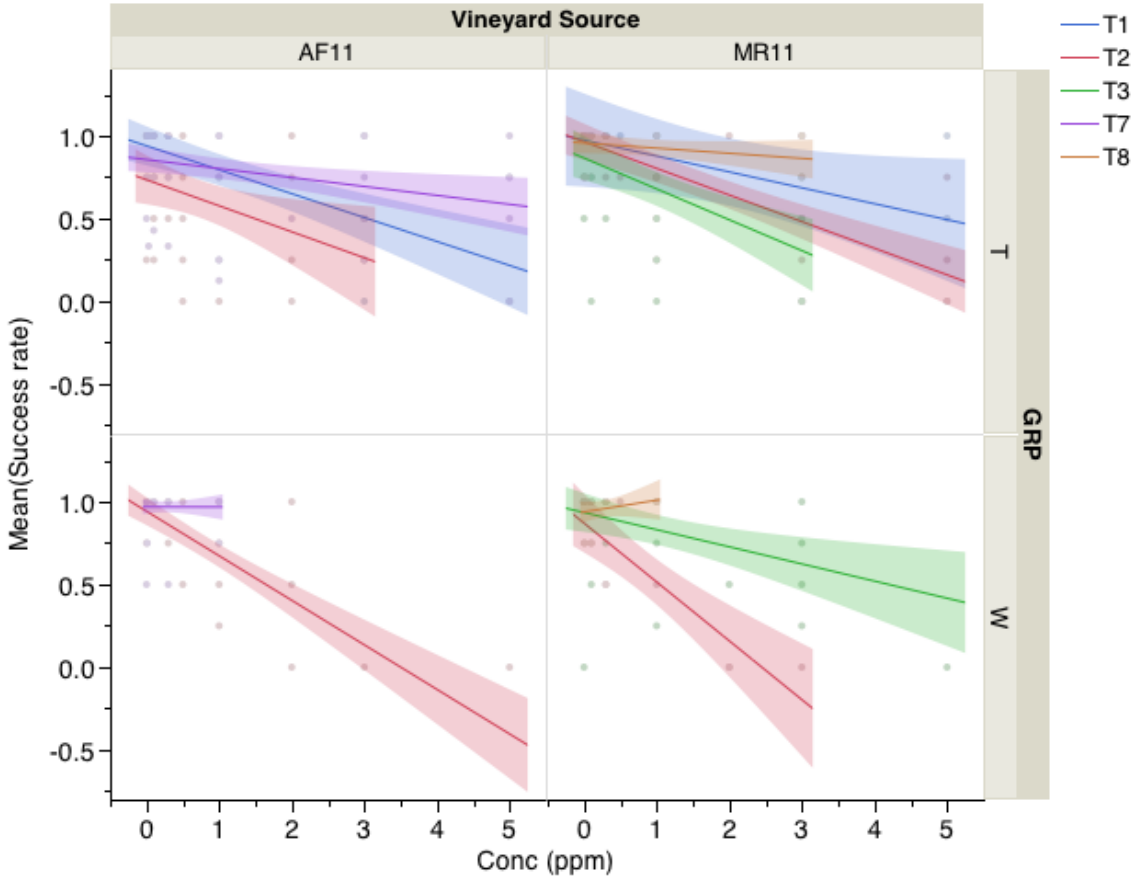

Fig. 5.3. Effects of culture condition (T = tebuconazole-treated leaf, W = water-treated leaf) and vineyard source (AF11 and MR11) on the changes in DMI resistance measured as success rate over tebuconazole concentration (ppm) for isolates transferred successively (T1 to T7 for MR11, T2 to T8 for AF11) on treated grape host leaves (T or W). Each line represents source of transfers, and light-shaded bands show 95% confidence interval.

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Chapter 6

Future Directions

QoI Resistance

We still have not completely resolved the mechanism underlying QoI resistance in two of three isolates with "moderate" resistance but lacking G143A. A mechanism of primary interest involves target site mutations in the cytochrome *b* gene such as F129L and G137R. PCR amplification of *cyt b* has proved difficult, probably due to the presence of large introns. We have partial sequence information that can be used to optimize other PCR-based methods such as RACE-PCR. Transcriptome sequencing may also provide additional information useful in the study of molecular mechanisms of resistance. The heteroplasmic nature of *E. necator* mitochondria is a new finding; thus, its relevance to QoI resistance is still unclear. An important question pertaining to heteroplasmy is whether it occurs, and how long it persists in vineyards that have withdrawn QoI fungicides. In Chapter 3, we have pointed out this possibility for a vineyard where both G143A-based resistance and atypical resistance (moderate level but without G143A) co-existed years after use of QoI fungicide was stopped. Thus, vineyards that have withdrawn QoIs may be revisited for detection of non-G143A-based resistance. Other questions we are interested in pertain to cellular mechanisms of heteroplasmy. For instance, how many mutated mitochondria are needed to confer resistance to a cell? Or mutated cells to confer resistance to an individual/isolate? Such investigations will require isolation of mitochondria and quantitation of mutation at the cellular and mitochondrial level.

DMI Resistance

The Y136F mutation in *cyp51* was associated with DMI resistance. In most mutant isolates tested, there was also good correlation between *cyp51* expression and DMI resistance level in *E. necator*. However, there were two isolates that possessed Y136F and yet expressed *cyp51* only at baseline levels (no significant difference with sensitive isolates), indicating that the mutation had no observable impact on gene expression. There are two issues that can be pursued from these findings. First, is there another mechanism operating in these two isolates? A possible mechanism is efflux pump activity, which allows the pathogen to survive in the presence of the fungicide by expelling the chemical from the cell. This may also explain the sensitivity shift observed in isolates that exhibited a decline in resistance over prolonged subculturing on fungicide-free leaves. Detection of efflux pump activity requires testing of a number of pump inhibitors, since studies showed that there are compatibility issues between transporter and inhibitor. The second question pertains to gene copy number. Are there fewer copies of *cyp51* in the two mutant isolates that expressed *cyp51* at baseline level and more in other mutant isolates with much more enhanced *cyp51* expression? How many copies of wildtype and/or mutant *cyp51* are needed to cause some resistance level in the isolate? This will involve testing the hypothesis that copy number variation (CNV) of the *cyp51* gene is responsible for the phenotypic difference among isolates and between sensitive and resistant strains. CNV can be studied using genome-wide sequencing, comparative genomic

hybridization methods, SNP genotyping arrays and third generation PCR such as droplet digital PCR.

We also found two *cyp51* genotypes - TTT and TWT - in resistant *E. necator*, and have seen the possibility of one genotype dominating a population, although mixed genotypes may abound. We want to know the contribution of Y136F and/or genotypes to the development of DMI resistance in the field. Questions arising from this finding are: (i) will different DMI materials (including newly registered) select for a particular genotype and (ii) will an increase in the proportion of mutants accompany an increase in resistance level? These will involve monitoring changes in population structures in the presence and absence of fungicide application.

The practical importance of the observed decline in DMI resistance in laboratory isolates may be tested under field conditions. We have attempted to determine sensitivity shifts in field populations exposed and not exposed to DMI fungicides, but very little powdery mildew infection developed during one trial, and very few samples from sprayed plots were generated during another trial. It is very time consuming and laborious to collect powdery mildew samples and maintain them in the laboratory for further experimentation. However, the SNP genotyping method that we developed in this research could be optimized for population studies using pooled samples instead of single-spored isolates. Also, DNA extraction directly from diseased leaves from the field may be developed to reduce processing time and obtain data that can accurately reflect the field situation.

Resistance Monitoring

We still have not seen unusual resistance levels in *E. necator* for relatively 'newer' fungicides (meaning, newly registered for grapes in the USA) such as boscalid (registered in 2003), quinoxyfen (2003). These belong to different fungicide classes and have modes of action different from DMIs and QoIs. Monitoring of resistance development to these fungicides continues. Newly registered fungicides are also out in the market and these include difenoconazole (registered in 2009), a DMI sold as a pre-mix with cyprodinil (Inspire Super) or mandipropamid (Revus Top). Other pre-mixed products such as Adament (tebuconazole + trifloxystrobin) and Pristine (pyraclostrobin + boscalid) should be carefully monitored because these contain ingredients (QoI and/or DMI) for which resistance is already causing problems in disease control.

Appendices for Chapter 2

Appendix Table 2.1. Primers for the microsatellite analysis of *Erysiphe necator*.

Locus	Forward(5′→3′)	Reverse (5′→3′)	Allele	
			Sizes (bp)	No.
EnMS1	TCACGACCTTTCCAAAATCC	TGTCCGTTTTGAACTCCAGA	236-269	11
EnMS3	TGTGTCGATGCCACGTTATT	AAATTGGATCCCCACCTCTC	222-243	8
EnMS7	AGGATGCCAACAAGAGCCTA	TTTGCCCCTCGATTATCAAC	187-211	9

Appendix Table 2.2 CYP51-SNP genotyping of leaf populations sampled from 2009-2011 in Afton, Virginia, based on a clustering algorithm used by the StepOne Plus program (Applied Biosystems) to generate genotype calls (pure wildtype= WT; pure mutant Mt; mix= WT/Mt) based on the normalized fluorescence signal (ΔRn) of the wildtype allele (WT) and mutant allele (Mt) in a DNA sample.

Sample	Mt ΔRn	Wt ΔRn	Genotype Call for Y136F	%G143A
BLP4-control	0.252	2.830	WT	0
BLP4-control	0.215	2.823	WT	
BLP4-control	0.242	2.843	WT	
BXPIA-control	2.555	0.549	Mt	99.9
BXPIA-control	2.651	0.559	Mt	
BXPIA-control	2.649	0.530	Mt	
AM11-2-1	0.909	1.904	Mt/WT	16.2
AM11-2-1	0.857	1.766	Mt/WT	
AM11-2-1	0.858	1.873	Mt/WT	
AM11-2-2	0.478	0.555	Mt/WT	100
AM11-2-2	0.462	0.560	Mt/WT	
AM11-2-2	0.562	0.653	Mt/WT	
AM11-2-4	0.458	0.629	Mt/WT	65
AM11-2-4	0.438	0.652	Mt/WT	
AM11-2-4	0.474	0.664	Mt/WT	
AM11-2-5	0.457	1.034	Mt/WT	43
AM11-2-5	0.391	1.025	Mt/WT	
AM11-2-5	0.426	1.011	Mt/WT	
AM11-2-7	0.135	1.113	WT	0
AM11-2-7	0.131	1.028	WT	
AM11-2-7	0.133	1.012	WT	
AM11-2-3	0.207	0.524	Undetermined	7
AM11-2-3	0.208	0.525	Undetermined	
AM11-2-3	0.208	0.467	Undetermined	
AM11-2-3-2	0.488	0.658	Mt/WT	68
AM11-2-3-2	0.522	0.653	Mt/WT	
AM11-2-3-2	0.518	0.632	Mt/WT	
AM11-2-4-2	0.938	2.087	Mt/WT	23

AM11-2-4-2	0.946	2.087	Mt/WT	
AM11-2-4-2	0.935	1.989	Mt/WT	
AM11-2-5-2	0.228	2.808	WT	2
AM11-2-5-2	0.262	2.609	WT	
AM11-2-5-2	0.251	2.620	WT	
AM11-2-6-2	0.562	1.863	Mt/WT	10
AM11-2-6-2	0.676	1.711	Mt/WT	
AM11-2-6-2	0.625	1.816	Mt/WT	
AM11-2-7-2	0.217	2.553	WT	
AM11-2-7-2	0.190	2.518	WT	0
AM11-2-7-2	0.206	2.448	WT	
AM11-2-8-2	0.165	1.928	WT	0
AM11-2-8-2	0.166	1.895	WT	
AM11-2-8-2	0.160	1.855	WT	
AM11-2-9-2	0.107	0.923	WT	0
AM11-2-9-2	0.143	1.129	WT	
AM11-2-9-2	0.133	1.082	WT	
AM11-2-10-2	0.184	2.130	WT	0
AM11-2-10-2	0.189	2.078	WT	
AM11-2-10-2	0.192	2.103	WT	
AM11-2-5-2	0.223	1.177	WT	
AM11-2-5-2	0.215	1.165	WT	
AM11-2-5-2	0.218	1.154	WT	
AM11-2-8	0.172	1.103	WT	0
AM11-2-8	0.170	1.121	WT	
AM11-2-8	0.181	1.097	WT	
AM11-2-8-3	0.171	1.059	WT	0
AM11-2-8-3	0.161	1.058	WT	
AM11-2-8-3	0.169	1.036	WT	
AM11-2-9-2	0.231	2.611	WT	
AM11-2-9-2	0.291	2.713	WT	
AM11-2-9-2	0.282	2.719	WT	
AM11-2-10-2	0.213	2.081	WT	0
AM11-2-10-2	0.202	2.136	WT	
AM11-2-10-2	0.197	2.009	WT	
AM11-2-10	0.241	2.543	WT	0
AM11-2-10	0.224	2.512	WT	
AM11-2-10	0.243	2.385	WT	
AM11-1A	0.139	0.195	Mut/WT	22
AM11-1A	0.154	0.208	Mut/WT	
AM11-1A	0.115	0.233	Mut/WT	
AM11-1B	0.12	0.15	Mut/WT	100
AM11-1B	0.13	0.16	Mut/WT	
AM11-1B	0.11	0.14	Mut/WT	
AM11-1C	1.43	2.25	Mut/WT	59
AM11-1C	1.36	2.02	Mut/WT	

AM11-1C	1.40	2.05	Mut/WT	
AM11-1D	0.11	0.14	Mut/WT	65
AM11-1D	0.13	0.14	Mut/WT	
AM11-1D	0.12	0.13	Mut/WT	
AM11-1E	0.169	1.094	WT	0.01
AM11-1E	0.175	1.096	WT	
AM11-1E	0.171	1.069	WT	
AM11-1G	0.159	1.009	WT	0
AM11-1G	0.176	1.006	WT	
AM11-1G	0.161	0.865	Undetermined	
Leaf 1A-2010	1.12	2.05	Mut/WT	43
Leaf 1A-2010	1.13	1.95	Mut/WT	
Leaf 1A-2010	1.09	1.92	Mut/WT	
Leaf 2A-2010	1.58	2.08	Mut/WT	100
Leaf 2A-2010	1.54	2.04	Mut/WT	
Leaf 2A-2010	1.53	2.01	Mut/WT	
Leaf 1B-2010	1.11	1.70	Mut/WT	91
Leaf 1B-2010	1.17	1.64	Mut/WT	
Leaf 1B-2010	1.14	1.63	Mut/WT	
Leaf 3B-2010	0.94	1.39	Mut/WT	100
Leaf 3B-2010	0.87	1.52	Mut/WT	
Leaf 3B-2010	0.87	1.47	Mut/WT	
Leaf 4A-2010	1.26	2.16	Mut/WT	100
Leaf 4A-2010	1.23	2.18	Mut/WT	
Leaf 4A-2010	1.26	2.12	Mut/WT	
Leaf 4B-2010	0.83	0.98	Mut/WT	27
Leaf 4B-2010	0.51	1.21	Mut/WT	
Leaf 4B-2010	0.82	0.96	Mut/WT	
Leaf 6A-2010	0.39	2.03	WT	1.4
Leaf 6A-2010	0.34	1.88	WT	
Leaf 6A-2010	0.33	2.18	WT	
Leaf 6B-2010	0.64	1.94	WT	5
Leaf 6B-2010	0.73	1.91	WT	
Leaf 6B-2010	0.70	1.97	WT	
Leaf 7A-2010	1.05	2.10	Mut/WT	39
Leaf 7A-2010	1.01	2.11	Mut/WT	
Leaf 7A-2010	0.88	1.60	Mut/WT	
Leaf 3A-2010	0.97	1.84	Mut/WT	100
Leaf 3A-2010	0.98	1.76	Mut/WT	
Leaf 3A-2010	1.00	1.81	Mut/WT	
Berry 2A-2010	1.04	1.86	Mut/WT	99
Berry 2A-2010	1.12	1.98	Mut/WT	
Berry 2A-2010	1.14	1.99	Mut/WT	
Berry 2B-2010	1.18	2.13	Mut/WT	100
Berry 2B-2010	1.26	2.22	Mut/WT	
Berry 2B-2010	1.28	2.11	Mut/WT	

AM1-09	0.11	0.13	Mut/WT	42
AM1-09	0.10	0.13	Mut/WT	
AM1-09	0.09	0.11	Mut/WT	
AM2-09	0.91	1.57	Mut/WT	21
AM2-09	0.89	1.64	Mut/WT	
AM2-09	0.87	1.60	Mut/WT	
AM3-09	0.23	2.31	WT	0.1
AM3-09	0.26	2.23	WT	
AM3-09	0.25	2.30	WT	
AM4-09	0.69	2.11	WT	5
AM4-09	0.65	2.17	WT	
AM4-09	0.63	2.21	WT	
AM6-09	1.26	1.52	Mut/WT	99
AM6-09	1.22	1.55	Mut/WT	
AM6-09	1.22	1.62	Mut/WT	
AM7-09	1.03	1.62	Mut/WT	55
AM7-09	1.05	1.63	Mut/WT	
AM7-09	1.08	1.68	Mut/WT	
AM8-09	1.59	1.94	Mut/WT	95
AM8-09	1.62	2.00	Mut/WT	
AM8-09	1.61	1.93	Mut/WT	

Appendix Table 2.3. Summary of independent experiments for the competition assays employing mixed populations of QoI-resistant and sensitive isolates cycled on fungicide-free grape plants grown under laboratory conditions.

Experiment	Inoculation	No. isolates	Plants/trmt[a]	Cycles[b]	%G143A at time 0
A	paired	4 resistant 4 sensitive	2	4	various
B	paired	5 resistant 5 sensitive	2	3	70-80
C	bulk	5 resistant 5 sensitive	3	3	70-80
D	bulk	8 resistant 8 sensitive	2-3	3	various
E	bulk	2 resistant 3 sensitive	3	1	5 and 40
F	bulk	3 resistant 3 sensitive	3	1	5 and 40

[a] Number of plants used per inoculation treatment

[b] One cycle is equal to 14 days

Appendix Table 2.4. Allele sizes (bp) of three microsatellite loci in various isolates of *Erysiphe necator* used in the competition assays.

	MS1	MS3	MS7	Assigned Grouping
AMP1	252	236	187	1
AVP7	251	236	190	2
HUP1	251	236	193	3
LIP3	252	228	193	4
VAHP4	266	228	196	5
AW9-1	251	236	196	6
BP-P1	251	233	193	7
IVP11	266	233	193	8
JRP4	239	233	187	9
MDMRP4	239	228	193	10
PRP4	266	236	208	11
ROP6	250	242	190	12
GRP20	236	233	196	13
WI9-1	257	236	196	14
FH9-1	269	243	202	15
SCCP4	252	231	187	16
BLP11	236	222	202	17
BLP4	239	222	208	18
BLP6	239	222	208	18
CB9-1	239	222	208	18
CL9-3	239	236	198	19
GRP1	251	236	196	6
LI9-1	269	239	196	20
MVP9	239	233	193	21
PBP1	245	236	199	22
CMP1	266	236	193	23
SHP6	239	233	193	22
SHP7	236	233	196	13
SNP1	254	222	211	24
VIP6	266	236	193	22

Appendix Table 2.5. Growth rate (diameter/day) for QoI-resistant (R) and sensitive (S) *Erysiphe necator* isolates grown on fungicide-free grape leaves.

Isolate	Phe	Subj	Growth period (day)	Growth rate-mm/day
VAHP4	R	1	6-7	1.28
VAHP4	R	2	6-7	1.42
VAHP4	R	3	6-7	1.20
VAHP4	R	4	6-7	1.10
ROP6	R	5	6-7	1.09
ROP6	R	6	6-7	1.24
ROP6	R	7	6-7	1.15
ROP6	R	8	6-7	
AVP7	R	9	6-7	0.54
AVP7	R	10	6-7	0.56
AVP7	R	11	6-7	0.83
AVP7	R	12	6-7	0.66
GRP20	R	13	6-7	1.26
GRP20	R	14	6-7	0.92
GRP20	R	15	6-7	1.05
GRP20	R	16	6-7	
AMP1	R	17	6-7	1.35
AMP1	R	18	6-7	0.98
AMP1	R	19	6-7	1.02
AMP1	R	20	6-7	1.03
HCGP4	S	21	6-7	1.00
HCGP4	S	22	6-7	1.33
HCGP4	S	23	6-7	1.22
HCGP4	S	24	6-7	1.09
MVP9	S	25	6-7	0.94
MVP9	S	26	6-7	1.31
MVP9	S	27	6-7	1.38
MVP9	S	28	6-7	1.25
SCCP4	S	29	6-7	1.16
SCCP4	S	30	6-7	1.23
SCCP4	S	31	6-7	1.17
SCCP4	S	32	6-7	
PBP1	S	33	6-7	1.67
PBP1	S	34	6-7	1.46
PBP1	S	35	6-7	1.31
PBP1	S	36	6-7	1.31
BLP4	S	37	6-7	1.80
BLP4	S	38	6-7	0.97
BLP4	S	39	6-7	1.25
BLP4	S	40	6-7	0.86
BLP11	S	41	6-7	1.07

BLP11	S	42	6-7	1.09
BLP11	S	43	6-7	1.02
BLP11	S	44	6-7	
CMP1	S	45	6-7	0.68
CMP1	S	46	6-7	1.31
CMP1	S	47	6-7	
CMP1	S	48	6-7	
VAHP4	R	1	7-8	0.71
VAHP4	R	2	7-8	0.73
VAHP4	R	3	7-8	0.90
VAHP4	R	4	7-8	1.28
ROP6	R	5	7-8	0.92
ROP6	R	6	7-8	0.82
ROP6	R	7	7-8	1.09
ROP6	R	8	7-8	
AVP7	R	9	7-8	0.48
AVP7	R	10	7-8	0.53
AVP7	R	11	7-8	0.66
AVP7	R	12	7-8	0.55
GRP20	R	13	7-8	0.90
GRP20	R	14	7-8	0.98
GRP20	R	15	7-8	1.20
GRP20	R	16	7-8	
AMP1	R	17	7-8	0.67
AMP1	R	18	7-8	1.06
AMP1	R	19	7-8	1.02
AMP1	R	20	7-8	0.91
HCGP4	S	21	7-8	0.95
HCGP4	S	22	7-8	0.75
HCGP4	S	23	7-8	0.94
HCGP4	S	24	7-8	
MVP9	S	25	7-8	0.14
MVP9	S	26	7-8	0.59
MVP9	S	27	7-8	0.75
MVP9	S	28	7-8	0.79
SCCP4	S	29	7-8	1.01
SCCP4	S	30	7-8	0.91
SCCP4	S	31	7-8	0.70
SCCP4	S	32	7-8	
PBP1	S	33	7-8	0.82
PBP1	S	34	7-8	0.77
PBP1	S	35	7-8	0.49
PBP1	S	36	7-8	0.12
BLP4	S	37	7-8	0.73
BLP4	S	38	7-8	1.00
BLP4	S	39	7-8	0.97

BLP4	S	40	7-8	0.75
BLP11	S	41	7-8	1.20
BLP11	S	42	7-8	1.10
BLP11	S	43	7-8	1.06
BLP11	S	44	7-8	
CMP1	S	45	7-8	0.70
CMP1	S	46	7-8	0.53
CMP1	S	47	7-8	0.90
CMP1	S	48	7-8	

Blank cells= no measurement made

Appendix Table 2.6. Two-way ANOVA for phenotype and growth rate at two consecutive rating dates for QoI-resistance and sensitive *Erysiphe necator* isolates.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	3	2.5181356	0.839379	13.3796	<.0001
Error	80	5.0188565	0.062736		
C. Total	83	7.5369921			

Appendix Table 2.7. Parameter estimates for the two-way ANOVA for growth rate and phenotype of QoI-resistance and sensitive *Erysiphe necator* isolates.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.9686458333	0.0276118289	35.08	<.0001
Phe[R]	-0.021979167	0.0276118289	-0.80	0.4284
GR Group[1]	0.1520625	0.0276118289	5.51	<.0001
Phe[R]*GR Group[1]	-0.0610625	0.0276118289	-2.21	**0.0299**

Appendix Table 2.8. Changes in %G143A in mixed populations of *E.necator* cycled on fungicide-free grape plants.

	Replication/	% G143A				
Paired-Trial A	Treatment	Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4
AMP1/MVP9	Plant 1	24.8	51.7	44.8	41.4	97.2
	Plant 1	44.8		39.8	66.67	88.2
	Plant 1	43.1	61.9	46.5	46.5	80.0
	Plant 1	44.8			55.2	
	Plant 2	31.8	50.0	n/a	73.9	
	Plant 2	33.3	63.5	n/a	77.7	
	Plant 2	33.3	81.1	n/a	55.2	

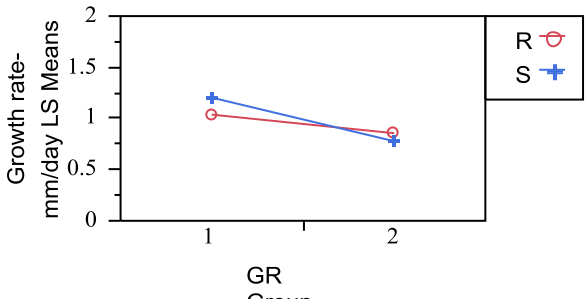
	Plant 2	34.9			58.2	
IVP11/BLP4	Plant 1	23.5	17.9	24.8	20.0	
	Plant 1	13.3	15.0	21.1	22.3	
	Plant 1	24.8	20.0	28.9	22.3	
	Plant 1	13.3				
	Plant 2	8.7	18.9	41.4	2.3	
	Plant 2	13.3	11.1	39.8	10.4	
	Plant 2	8.1				
	Plant 2	6.3				
JRP4/SJHP7	Plant 1	2.8	3.0	7.6	0.5	
	Plant 1	5.9	1.7	7.6	1.1	
	Plant 1	3.0	2.5	8.1	1.3	
	Plant 1	3.2	2.0			
	Plant 2	4.5	2.8	4.5	8.7	
	Plant 2	5.2	2.0	3.5	8.7	
	Plant 2	5.9		1.7	5.5	
	Plant 2	4.8			6.3	
PBP1/ROP6	Plant 1	63.5	n/a	87.4	75.5	
	Plant 1	76.5	n/a	86.7	77.7	
	Plant 1	53.5	n/a	90.8	81.1	
	Plant 1	75.5			83.1	
	Plant 2	69.7	81.1	n/a	55.2	94.1
	Plant 2	60.3	85.0	n/a		91.4
	Plant 2	71.1	91.9	n/a		92.9
	Plant 2	63.5				
Paired-Trial B						
AVP7/GRP1	Plant 1	68.2	99.8	99.7	99.6	
	Plant 1	66.7	99.9	99.9	98.8	
	Plant 1		99.9	99.9	98.5	
	Plant 2	83.1	99.9	99.9	99.9	
	Plant 2	90.8	99.9	0	99.9	
	Plant 2	88.2	99.9	99.9	99.9	
SUP9/PRP4	Plant 1	69.7	87.4	99.8	98.2	
	Plant 1	71.1	85.8	99.9	99.1	
	Plant 1		83.1	99.8	98.8	
	Plant 2	96.8	92.8	99.4	97.8	
	Plant 2	95.8	91.3	98.2	96.8	
	Plant 2		93.3	99.8	95.2	
MdMRP4/ViP6	Plant 1	55.2	13.3	34.9	68.2	
	Plant 1	53.5	21.1	41.4	68.2	
	Plant 1			41.4		
	Plant 2	98.7	76.5	77.7	92.9	
	Plant 2	96.0	83.1	72.5	95.5	

	Plant 2		83.1	78.9	94.8	
VAHP4/SCCP4	Plant 1	61.9	60.3	85.8	95.8	
	Plant 1		71.1	88.9	94.1	
	Plant 1		58.6		92.9	
	Plant 2	81.1	48.3	95.5	90.2	
	Plant 2	75.9	56.9	94.8	93.7	
	Plant 2	73.9		93.7	88.2	
LiP3/SHP6	Plant 1	53.5		99.3	99.9	
	Plant 1	51.7		99.3	99.9	
	Plant 1			99.8	99.9	
	Plant 2	87.4	96.5	99.9	99.9	
	Plant 2	81.1	97.0	96.3	99.4	
	Plant 2		97.4	100	99.9	
Bulk-Trial C	subsample 1	76.5		82.1		95.8
	subsample 1	71.1		76.5		92.4
	subsample 2	75.2		78.9		90.2
	subsample 2	77.7		80.0		91.9
	subsample 3	84.1		72.5		84.5
	subsample 3	78.9		82.1		84.1
Bulk –Trial D	10R:90S-Plant1	9.1	12.6	15.9		
	10R:90S-Plant2	9.8	9.0	11.7		
	10R:90S-Plant3	11.5	19.1	31.7	34.1	
	20R:80S-Plant1	23.4	44.2	84.5	81.9	
	50R:50S-Plant1	56	75.7	63.4	55.8	
	50R:50S-Plant2	56	85.1	91.6	98.2	
Bulk-Trial E	5R:95S-Plant1	5.5	2.5	3.3	5.5	
	5R:95S-Plant2	5.6	9.5	13.4	14.6	
	30R:70S-Plant1		30.1	23.3	40.7	
	40:60-Plant1		41.3	45.1	42.4	
	80R:20S-Plant1		80.1	91.6	98.2	
Bulk-Trial F	5R:95S-Plant 1	8.7	2.4			
	5R:95S-Plant 2	6.7	3.4			
	5R:95S-Plant 3	11.1	17.9			
	40R:60S-Plant 1	57.4	31.4			
	40R:60S-Plant 2	52.3	2.4			
	40R:60S-Plant 3	68.0	29.9			
Bulk- Trial G	5R:95S-Plant 1	2.7	2.9			
	5R:95S-Plant 2	4.4	4.2			
	5R:95S-Plant 3	4.7	6.8			
	40R:60S-Plant 1	43.2	37.1			
	40R:60S-Plant 2	49.4	40.9			
	40R:60S-Plant 3	36.6	49.4			

n/a –software output; no quantitiation

Appendix Table 2.9. Sensitivity shifts in *Erysiphe necator* mixed populations consisting of QoI-resistant and sensitive isolates on fungicide-free grape plants.

	Mean % G143A*				
Sample	Control	Sensitive	Resistant	R/S-1	R/S-2
Inoculum		0.77±0.41 (n=3)	96.63±0.71 (n=3)	4.70±0.70 (n=3)	
July 2011	0.36±0.37 (n=11)	0.04±0.01 (n=8)	13.91±4.14 (n=11)	14.30±5.78 (n=15)	0.10±0.03 (n=9)
Sept 2011	0.09±0.07 (n=5)	0.15±0.01 (n=14)	11.3±11.76 (n=9)	0.10±0.05 (n=9)	4.26±2.28 (n=15)



Appendix Fig. 2.1. Interaction plot for the factor 1 (phenotype: resistant/R or sensitive/S) and factor 2 (growth rate/GR group). The GR group 1 include growth rate measurements done on 6 to 7 days after inoculation; group 2, on 7 to 8 days after inoculation. Interaction (phenotype x GR group) was significant at 0.05 level of significance (P=0.03).

Appendices for Chapter 3

Appendix Table 3.1. ANOVA for testing effect of SHAM and DMSO on % germination of seven QoI-resistant *E. necator* isolates on water agar.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Trtmt	2	60.27641	30.1382	13.6512	<0.0001
Error	33	72.85523	2.2077		
C. Total	35	133.13163			

Appendix Table 3.2. Percent germination of five QoI-resistant *Erysiphe necator* isolates on chemical-amended agar*.

Isolate	No. Assays	Mean % Germination				
		Water Agar	DMSO*-WA	Relative % Germ'n	SHAM in DMSO*	Relative % Germ'n
JRP4	2	49.0	52.0	108	22.0	41
MDMRP7	2	44.5	39.0	88	5.5	12
GRP20	2	37.0	37.5	105	20.5	49
MDMRP4	2	19.5	21.5	110	3.5	18
JRP3	4	17.0	15.5	95	2.3	13
Mean	12	30.7	30.2	100.2	9.3	24
Max	12	59.0	59.0	150	36.0	76
Min	12	12.0	10.0	57	1.0	7

*DMSO –dimethylsulfoxide solvent at 0.25%; SHAM – salicylhydroxamic acid, 0.65 mM in DMSO (0.25%)

Appendix Table 3.3. Percent germination of *Erysiphe necator* isolates of different sensitivity levels to QoI on chemical-amended agar*.

	% Germination								
	Highly resistant, 100% G143A			Moderately resistant, 0% G143A			Sensitive, 0% G143A		
	Agar	DMSO	SHAM	Agar	DMSO	SHAM	Agar	DMSO	SHAM
Mean	31.4	32.6	11.1	7.7	8.3	2.6	3.0	2.0	2.6
Range	16-67	3-21	2-25	2-20	3-21	0-13	1-8	0-7	0-4
Mean Relative		92	35		127	18		45	46
Total Isolates (#Assays)	8(8)	8(8)	8(8)	3(6)	3(6)	3(6)	8(8)	8(8)	8(8)

*DMSO –dimethylsulfoxide solvent at 0.125%; SHAM – salicylhydroxamic acid, 0.325 mM in DMSO

Appendix Table 3.4. Percent germination on chemical-amended agar* of *Erysiphe necator* isolates of different sensitivity levels to QoI.

	% Germination								
	Highly resistant, 100% G143A			Moderately resistant, 0% G143A			Sensitive, 0% G143A		
	Agar	DMSO	SHAM	Agar	DMSO	SHAM	Agar	DMSO	SHAM
Mean	23.3	24.7	28.5	10.3	8.0	5.7	0.8	0.4	1.0
Range	12-42	11-36	10-50	2-24	0-21	0-2	0-1	0-2	0-4
Mean Relative		90	101		79	39		45	46
Total Isolates (#Assays)	6(6)	6(6)	6(6)	3(3)	3(3)	3(3)	3(3)	3(3)	3(3)

*DMSO –dimethylsulfoxide solvent added to agar at 0.025%; SHAM – salicylhydroxamic acid, added to agar at 0.065 mM in DMSO

Appendix Table 3.5. Raw data to test the effect of SHAM with azoxystrobin on water agar amended with inhibitors on spore germination of three isolates with different sensitivity to QoI.

Isolate	Treatment	% Germination			Average	Relative %Germ'n	Slope	Intercept	EC50
MVP9	WA-DMSO	19	23	16	19				
MVP9	WA-DMSO	19	27	14	20				
MVP9	WA-DMSO	19	16	23	19	**20**			
MVP9	SHAM-DMSO	17	19	20	19	**95**			
MVP9	SHAM-DMSO	15	25	13	18	**90**			
MVP9	SHAM-DMSO	22	21	11	18	**92**			
MVP9	Azo 0.01	10	11	7	9	**48**	-6.77	11.85	0.0036
MVP9	Azo 0.01	11	6	8	8	**43**			
MVP9	Azo 0.01	10	9	7	9	**44**			
MVP9	Azo 1	3	0	2	2	**9**			
MVP9	Azo 1	2	2	1	2	**9**			
MVP9	Azo 1	1	0	0	0	**2**			
MVP9	Azo 10	0	0	0	0	**0**			
MVP9	Azo 10	0	0	0	0	**0**			
MVP9	Azo 10	0	0	0	0	**0**			
MVP9	Azo 0.01+SHAM	8	9	7	8	**41**	-5.34	11.24	0.0007
MVP9	Azo 0.01+ SHAM	8	7	12	9	**46**			
MVP9	Azo 0.01+SHAM	5	2	6	4	**22**			
MVP9	Azo 1+SHAM	1	0	0	0	**2**			
MVP9	Azo 1+SHAM	4	2	3	3	**15**			
MVP9	Azo 1+SHAM	1	3	3	2	**12**			
MVP9	Azo 10+SHAM	0	0	0	0	**0**			
MVP9	Azo 10+SHAM	0	0	0	0	**0**			
MVP9	Azo 10+SHAM	0	0	0	0	**0**			
VAHP4	WA-DMSO	47	40	35	41				
VAHP4	WA-DMSO	31	31	29	30				
VAHP4	WA-DMSO	31	31	28	30	**34**			

VAHP4	SHAM-DMSO	20	27	17	21	**63**			
VAHP4	SHAM-DMSO	25	23	23	24	**70**			
VAHP4	SHAM-DMSO	27	28	27	27	**81**			
VAHP4	Azo 0.01	27	23	21	24	**70**	-5.15	56.88	3.81
VAHP4	Azo 0.01	24	27	20	24	**70**			
VAHP4	Azo 0.01	25	28	32	28	**84**			
VAHP4	Azo 1	23	33	19	25	**74**			
VAHP4	Azo 1	29	24	20	24	**72**			
VAHP4	Azo 1	20	23	33	25	**75**			
VAHP4	Azo 10	5	8	16	10	**29**			
VAHP4	Azo 10	16	10	15	14	**41**			
VAHP4	Azo 10	17	7	8	11	**32**			
VAHP4	Azo 0.01+SHAM	15	25	19	20	**58**	-1.99	54.69	10.61
VAHP4	Azo 0.01+ SHAM	20	21	25	22	**65**			
VAHP4	Azo 0.01+SHAM	22	22	15	20	**58**			
VAHP4	Azo 1+SHAM	21	19	16	19	**55**			
VAHP4	Azo 1+SHAM	23	24	22	23	**68**			
VAHP4	Azo 1+SHAM	18	25	26	23	**68**			
VAHP4	Azo 10+SHAM	18	20	14	17	**51**			
VAHP4	Azo 10+SHAM	21	6	22	16	**49**			
VAHP4	Azo 10+SHAM	10	9	13	11	**32**			
SUP13	WA-DMSO	7	17	7	10				
SUP13	WA-DMSO	11	10	12	11				
SUP13	WA-DMSO	14	12	6	11	**11**			
SUP13	SHAM-DMSO	9	9	10	9	**28**			
SUP13	SHAM-DMSO	9	16	13	13	**38**			
SUP13	SHAM-DMSO	8	6	9	8	**23**			
SUP13	Azo 0.01	17	6	13	12	**113**	-18.10	11.46	0.12
SUP13	Azo 0.01	9	10	5	8	**75**			
SUP13	Azo 0.01	15	7	9	10	**97**			
SUP13	Azo 1	2	1	3	2	**19**			

SUP13	Azo 1	1	3	0	1	**13**			
SUP13	Azo 1	0	1	0	0	**3**			
SUP13	Azo 10	0	0	0	0	**0**			
SUP13	Azo 10	0			0	**0**			
SUP13	Azo 10	0			0	**0**			
SUP13	Azo 0.01+SHAM	9	11	6	9	**81**	-17.42	5.21	0.08
SUP13	Azo 0.01+ SHAM	12	6	10	9	**88**			
SUP13	Azo 0.01+SHAM	14	6	8	9	**88**			
SUP13	Azo 1+SHAM	3	0	0	1	**9**			
SUP13	Azo 1+SHAM	0	0	2	1	**6**			
SUP13	Azo 1+SHAM	0	0	0	0	**0**			
SUP13	Azo 10+SHAM	0	0	0	0	**0**			
SUP13	Azo 10+SHAM	0	0	0	0	**0**			
SUP13	Azo 10+SHAM	0	0	0	0	**0**			
GRP18	WA-DMSO	58	58	62	59				
GRP18	WA-DMSO	59	60	59	59				
GRP18	WA-DMSO	61	60	58	60	**59**			
GRP18	SHAM-DMSO	47	47	51	48	**144**			
GRP18	SHAM-DMSO	42	57	55	51	**152**			
GRP18	SHAM-DMSO	56	36	38	43	**129**			
GRP18	Ab 0.01	58	64	50	57	**96**	-9.45	51.56	1.18
GRP18	Ab 0.01	64	54	57	58	**98**			
GRP18	Ab 0.01	48	53	56	52	**88**			
GRP18	Ab 1	39	29	33	34	**57**			
GRP18	Ab 1	34	26	38	33	**55**			
GRP18	Ab 1	31	38	22	30	**51**			
GRP18	Ab 10	20	11	15	15	**26**			
GRP18	Ab 10	19	22	16	19	**32**			
GRP18	Ab 10	14	14	19	16	**26**			
GRP18	Ab 0.01+sham	51	48	50	50	**84**	-6.96	49.24	0.90

GRP18	Ab 0.01+sham	54	49	54	52	**88**
GRP18	Ab 0.01+sham	39	45	48	44	**74**
GRP18	Ab 1+SHAM	36	27	29	31	**52**
GRP18	Ab 1+SHAM	28	26	30	28	**47**
GRP18	Ab 1+SHAM	24	24	30	26	**44**
GRP18	Ab 10+SHAM	26	22	20	23	**38**
GRP18	Ab 10+SHAM	16	16	20	17	**29**
GRP18	Ab 10+SHAM	20	19	25	21	**36**

Appendices for Chapter 4

Appendix Table 4.1. ANOVA for gene expression of *cyp51* in *E. necator* mutant and wildtype isolates.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample	11	2443.5723	222.143	278.7950	<0.0001
Error	59	47.0110	0.797		
C. Total	70	2490.5833			

Appendix Table 4.2. Comparison of mean relative quantitation of gene expression (RQ) means by Tukey's test (JMP v. 9, SAS Institute, Inc.).

Isolate	N	Mean RQ
JRP4	6	18.6±0.91a
MDMRP7	6	12.9±0.37b
AMP1	5	12.0±0.13bc
VAHP4	6	11.6±0.60bc
MDMRP5	6	11.0±0.39c
IVP4	6	10.7±0.16c
BXPIA	6	6.0±0.19d
VAHP1	6	1.8±0.08e
IVP3	6	1.4±0.10e
PBP1	6	1.4±0.01e
MVP9	6	1.0±0.05e
BLP4	6	1.0±0.02e

Means with same letters are not significantly different at $\propto$=0.05, P<0.001

Appendix Table 4.3. ANOVA for gene expression of *cyp51* of *Erysiphe necator* based on genotype (TWT, TTT, TAT) of isolates.

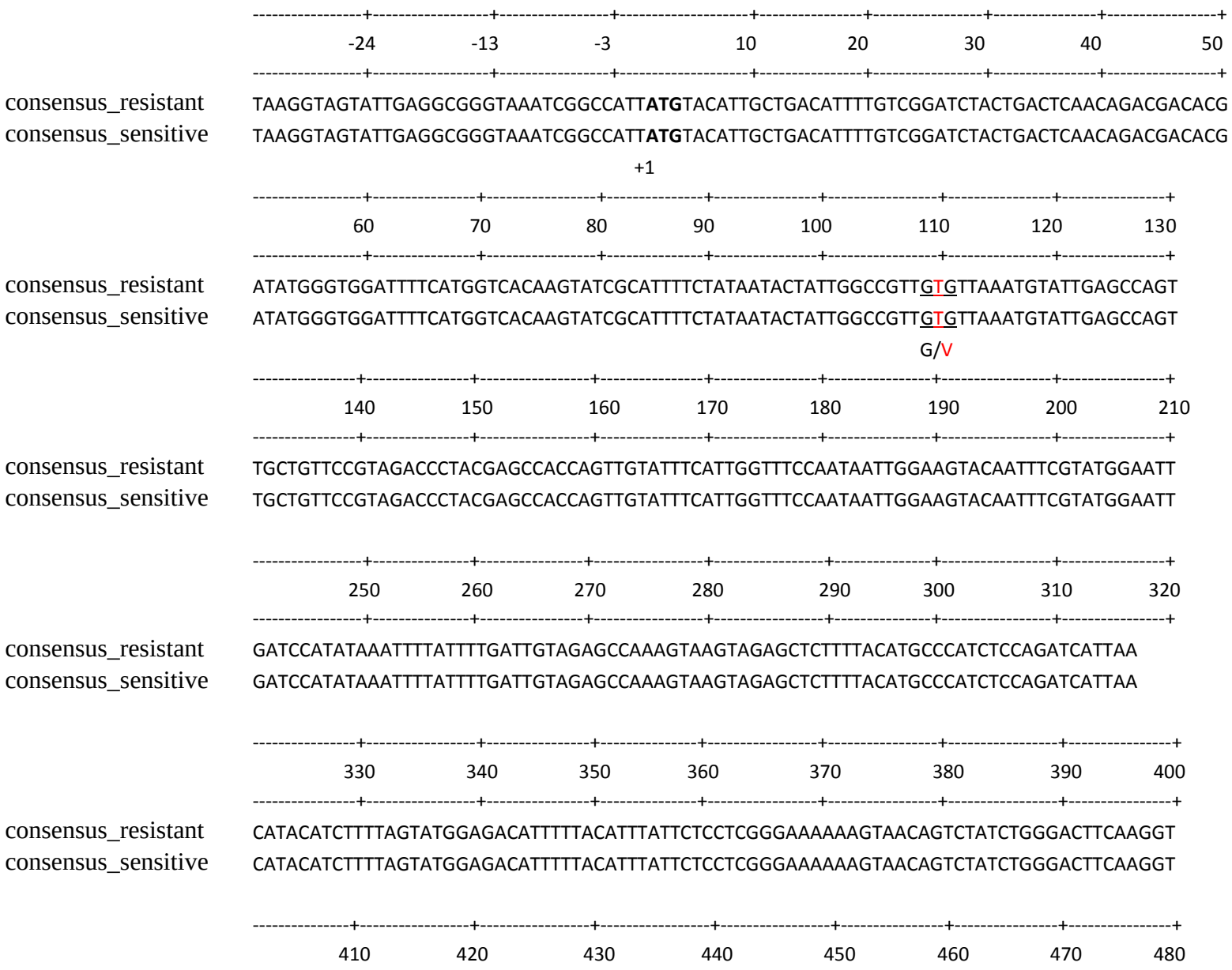
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Genotype	2	343.96569	171.983	8.3876	0.0021
Error	21	430.59170	20.504		
C. Total	23	774.55740			

Appendix Table 4.4. Pearson's correlation of RQ (gene expression level) and RF (resistance factor) for three fungicides.

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Significance Prob
RF-Teb	RQ Mean	0.7433	39	0.5589	0.8577	<0.0001
RF-Myc	RQ Mean	0.8049	41	0.6609	0.8917	<0.0001
RF-Fen	RQ Mean	0.6385	39	0.4044	0.7940	<0.0001

Appendix Table 4.5. Raw data for the correlation analysis of relative quantity of *cyp51* cDNA (RQ) and relative factor (RF) to different DMIs.

Isolate	Y136F Mutation	CYP51 genotype	RQ Mean	RF-Teb-2012	RF-Myc-2012	RF-Fen-2011
BLP4	absent	TAT	1	0.5	1.1	1.2
BLP4	absent	TAT	1	1.1	1.8	0.6
BLP4	absent	TAT	1	1.5	7.3	0.3
MVP9	absent	TAT	1.1	0.6	1.0	0.6
MVP9	absent	TAT	1.1	0.9	0.02	4.7
MVP9	absent	TAT	1.1	0.2	0.5	0.6
MVP9	absent	TAT	1.1	-	3.4	-
SCCP4	absent	TAT	1.2	2.6	-	1.7
SCCP4	absent	TAT	1.2	0.4	0.1	3.9
SCCP4	absent	TAT	1.2	2.4	0.1	0.2
PBP1	absent	TAT	1.3	0.1	0.9	2.4
PBP1	absent	TAT	1.3	2.0	1.1	0.4
PBP1	absent	TAT	1.3	0.9	0.2	2.3
BXP1A	present	TTT	6.0	8.5	7.9	5.9
BXPIA	present	TTT	6.0	4.6	6.9	4.1
BXP1A	present	TTT	6.0	5.5	14.7	9.5
BXP1A	present	TTT	6.0	-	3.9	-
MDMRP5	present	TTT	11.0	28.5	34.3	4.7
MDMRP5	present	TTT	11.0	13.6	33.3	8.9
MDMRP5	present	TTT	11.0	19.5	67.7	5.9
MDMRP7	present	TTT	12.9	14.2	65.7	16.6
MDMRP7	present	TTT	12.9	38.6	52.0	42.0
MDMRP7	present	TTT	12.9	18.6	149.1	12.4
VAHP1	present	TWT	1.9	12.5	3.9	4.7
VAHP1	present	TWT	1.9	7.5	9.8	3.6
VAHP1	present	TWT	1.9	7.5	7.9	5.3
AMP1	present	TWT	12.1	52.4	17.7	94.7
AMP1	present	TWT	12.1	88.6	34.3	42.0
AMP1	present	TWT	12.1	41.7	37.4	37.9
IVP4	present	TWT	10.7	20.2	49.0	26.6
IVP4	present	TWT	10.7	24.8	51.0	18.3
IVP4	present	TWT	10.7	18.7	77.5	40.0
IVP4	present	TWT	10.7	-	100.0	-
VAHP4	present	TWT	11.6	29.4	26.5	33.7
VAHP4	present	TWT	11.6	30.7	56.9	26.6
VAHP4	present	TWT	11.6	10.8	21.6	35.5
JRP4	present	TWT	18.9	31.2	65.7	41.4
JRP4	present	TWT	18.9	34.9	90.2	35.5
JRP4	present	TWT	18.9	40.4	95.1	2.4
IVP3	present	TTT	1.4	2.4	0.2	2.4
IVP3	present	TTT	1.4	1.7	0.2	1.8
IVP3	present	TTT	1.4	0.4	0.4	1.8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+1
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G/V
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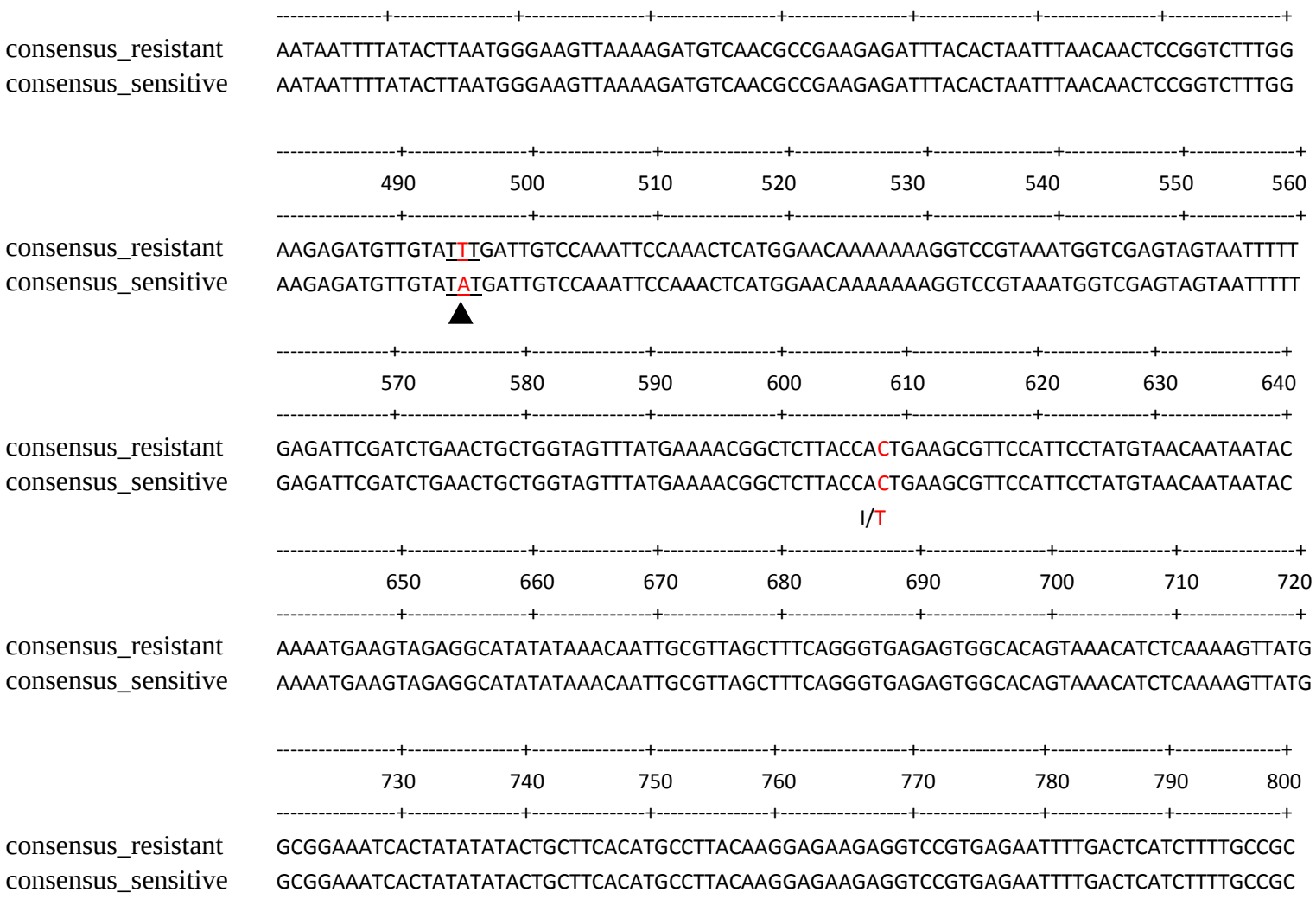
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consensus_sensitive   TCTTTATCATGATCTTGATATGGGATTTACACCGATCAACTTTACATTTTACTGGGCACCTCTACCTTGGAATCGTGCTC

                  ----------+----------+----------+----------+----------+----------+----------+----------+
                            890        900        910        920        930        940        950        960
                  ----------+----------+----------+----------+----------+----------+----------+----------+
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consensus_sensitive   RATAAGCATGATATAATGTGGGAGTTGATGCGTTCCACTTATAAAGACGGGACTCCGGTACCTGATCGAGAGATAGCGCA

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                  ----------+----------+----------+----------+----------+----------+----------+----------+
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consensus_sensitive   TATGATGATAGCCCTTCTGATGGCTGGACAACACTCTTCGTCATCCACGAGCTCATGGATTATGCTTTGGTTAGCCGCAC

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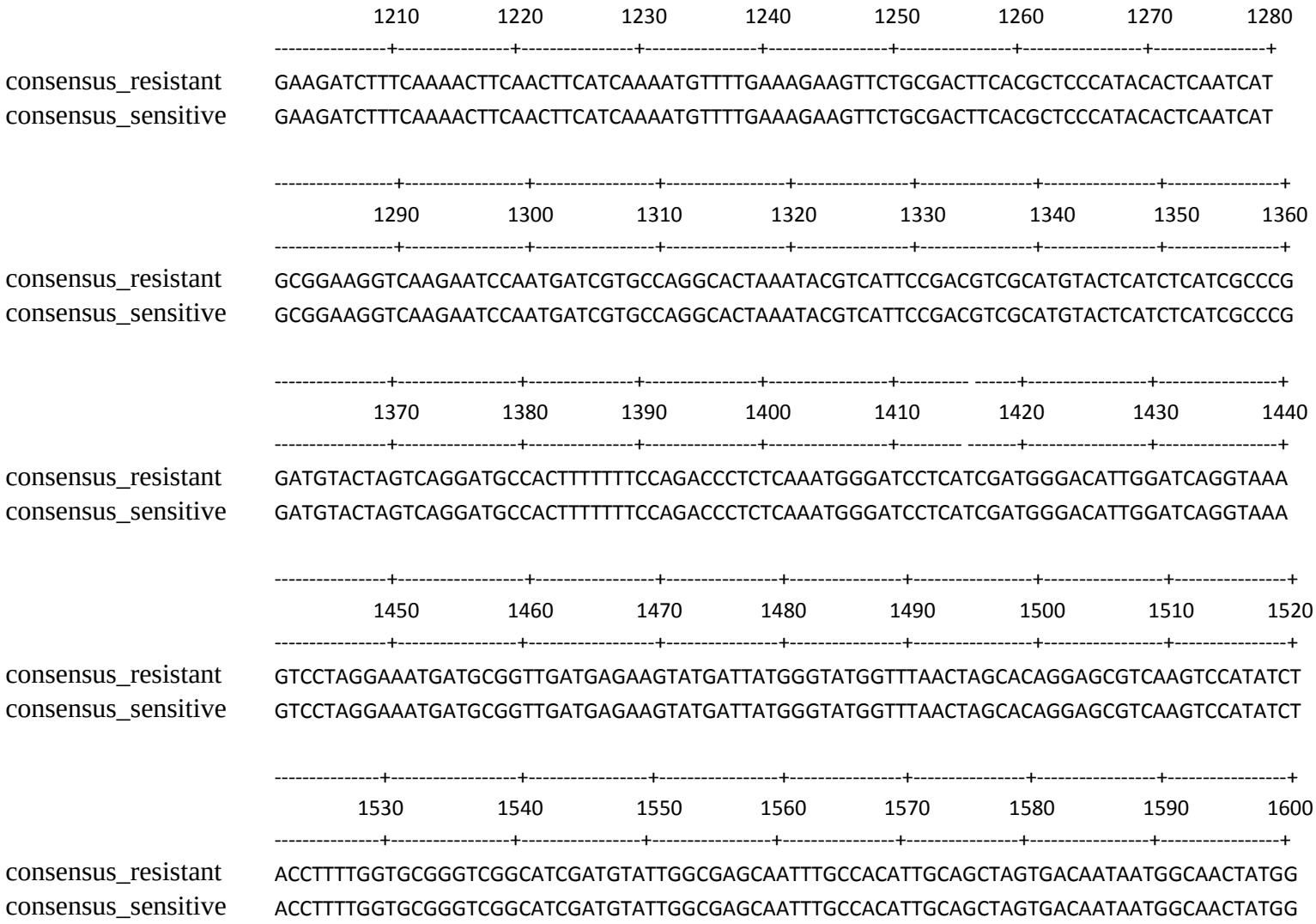
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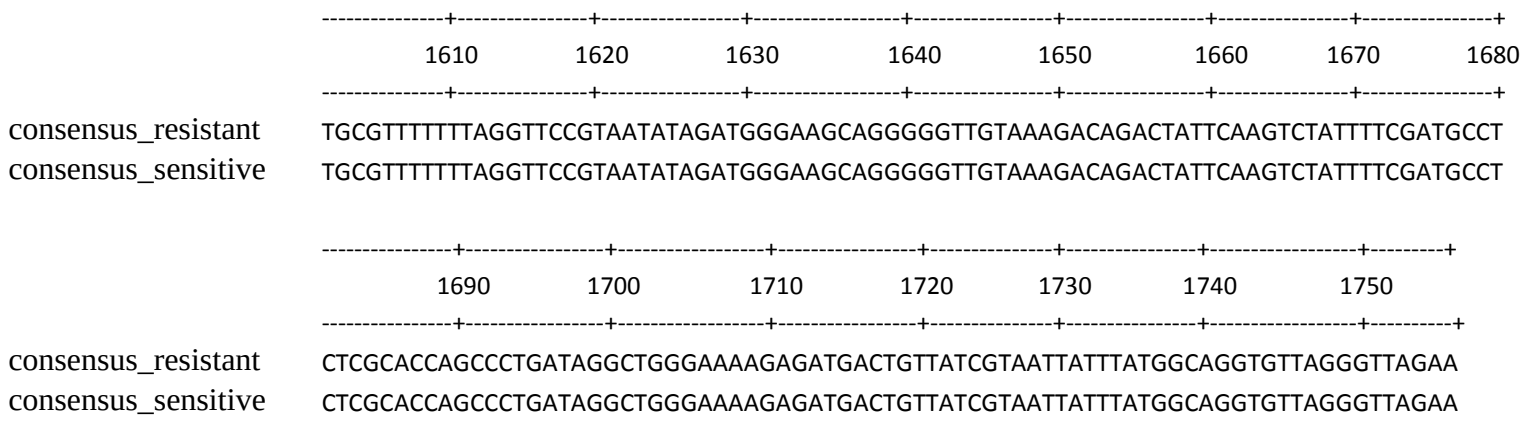
1530 1540 1550 1560 1570 1580 1590 1600
consensus_resistant ACCTTTTGGTGCGGGTCGGCATCGATGTATTGGCGAGCAATTTGCCACATTGCAGCTAGTGACAATAATGGCAACTATGG
consensus_sensitive ACCTTTTGGTGCGGGTCGGCATCGATGTATTGGCGAGCAATTTGCCACATTGCAGCTAGTGACAATAATGGCAACTATGG



Appendix Fig. 4.1. Alignment of consensus sequences of mutant and wildtype isolates of *Erysiphe* necator. Transcription start codon (ATG) is in bold. Nucleotides in red are different from a European sequence (Genbank accession no. U726567). Nucleotides in blue are polymorphic for the group but do not change the amino acid in that position. The black triangle indicates position of nt 495 which alters the 136th codon from TAT to TTT (underlined).

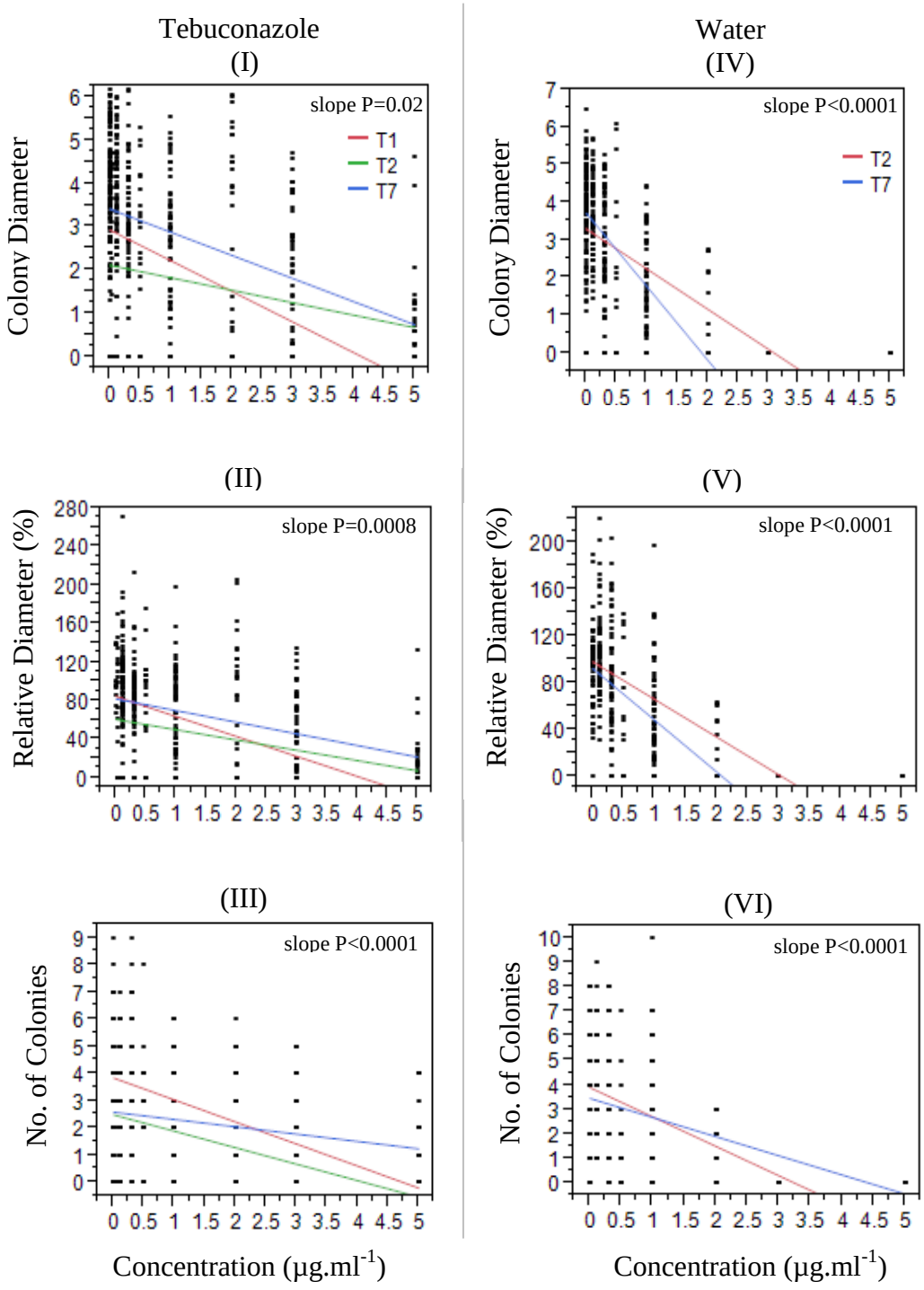
Appendices for Chapter 5

Appendix Table 5.1. Resistance factor (RF) of *Erysiphe necator* isolates to different DMI fungicides.

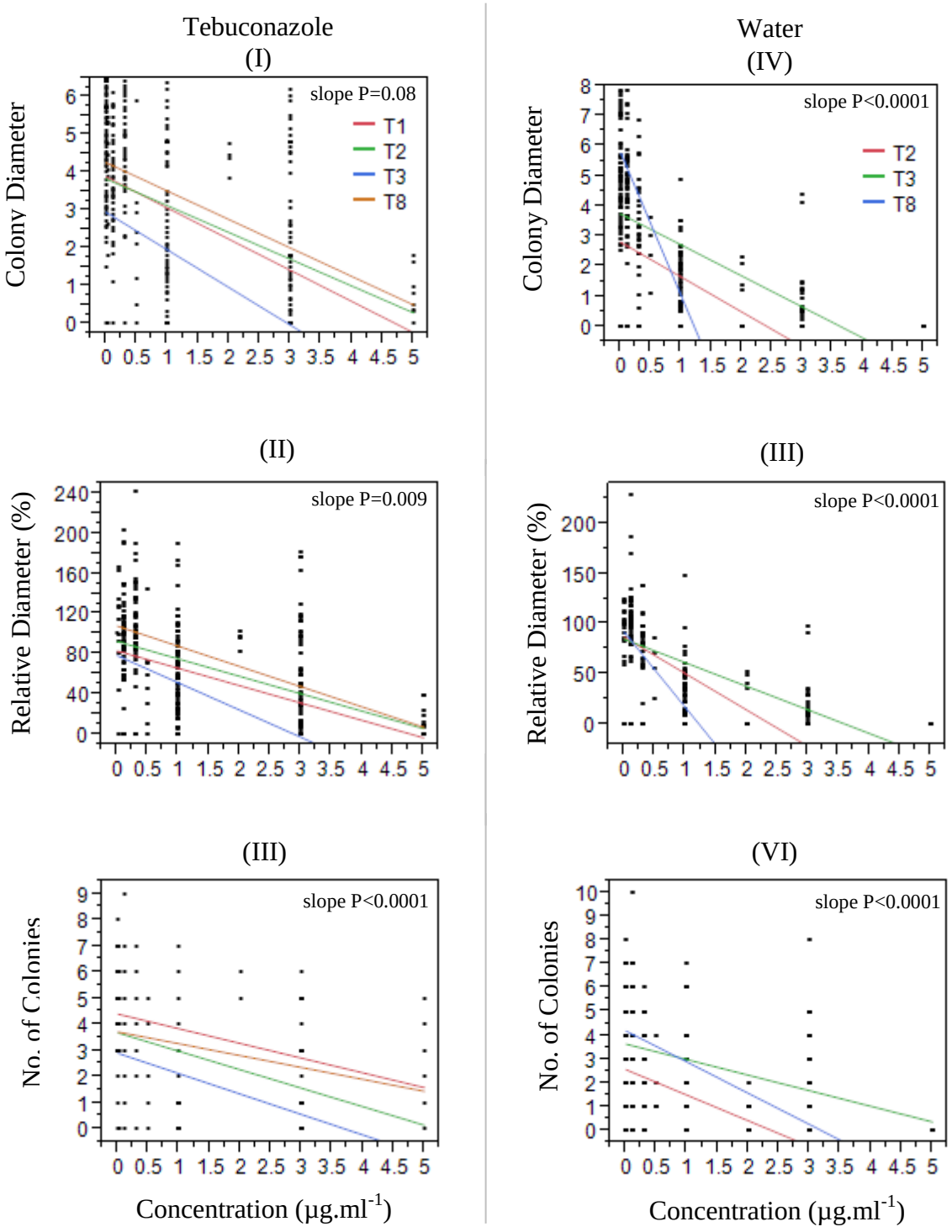
Isolates	RF-Tebuconazole				RF-Myclobutanil			RF- Fenarimol			RF-Triadimefon		RF- Triflumizole	
	2008	2009	2010	2011	2008	2009	2011	2008	2009	2011	2008	2009	2008	2009
BLP1	0.2	1.3	0.9	0.0	0.4	1.0	0.6	0.6	0.3		0.5	0.3	0.1	1.6
BLP4	0.3	1.2	0.9	0.9	1.0	0.6	3.1	1.7	2.2	0.8	0.8	2.7	0.2	2.3
BLP6	0.3	0.8	0.1					1.0	1.3	0.4	0.3	0.4	0.2	0.4
SCCP4	0.2	1.3		1.8	0.2		0.2	0.2		1.9			0.4	0.9
MVP9	0.0	0.6	0.6	0.7	0.2	1.0	0.6	0.3	0.7	2.6	1.1	0.2	0.1	0.5
PBP1	0.2			1.0	1.4		0.6	0.7		0.9				
BXPIA	514	204	7	6	52	73	7		9	6	20	27		
SUP13	246	428	9	11	1707	103	34	384	66	5	115	34	1453	62
JRP4	249	232	100	33	760	238	76	278	25	26	98	40		
JWP1	248	314	219			318	53	358	73	78	106	2	994	20
VAHP4	233	189	113	120	1640	91	32	391	44	32	343	60		
VAHP6	>112	285	60	23	204	83	39	132	33	20	84	34	1453	62
PRP7	>112	130	88	46	400	146	123	0	33	15	23	9	339	25
MDMRP7	99	476	55	25	212	81	9	25	24	36	38			
GRP15	68		56	12	96	255	0	112	57	17	97	17		
IVP3	82	164	2		272	29	0	76	3	2	32	30	76	0
IVP4		335	52	22	128	134	61	81	33	28	63	21	95	59
IVP11	48	201	45		276	131		150	30	15	157	12	170	42
GRP18	45	357	26	17	104	79	56	78	31	10	41	23		
MDMRP5	39	90	30	21	115	93	41	17	14	6	23	26		
AMP1	40	119	121	56	101	125	27	88	75	57	49	25	92	246
JRP3	35	350	86	29	160	291	80	164	77	44	58	73		
VAHP1	32	118	15	11	899	119	6	132	19	5	118	14		
SHP2	26	94	2		73	72		0.05		1	0.3	4	1	39
ROP14	13	118	120	27	39	75	58	0.1	44	11			18	

Appendix Table 5.2. T-test for relative quantity of *cyp51* cDNA in the presence or absence of tebuconazole, analysis by isolate, and trial (1 and 2) as block, confidence level= 0.95.

Isolate	Difference	Std. Err Difference	Upper CL Difference	Lower CL Difference	t Ratio	DF	Prob > |t|	Prob > t	Prob < t
AF-2	0.9767	1.3653	4.0200	-2.0667	0.7154	9.968	0.4908	0.2454	0.7546
AF-3	5.8500	0.6363	7.2718	4.4282	9.1933	9.801	<0.0001	<0.0001	1.0000
AF-4	1.7067	0.6854	3.6259	-0.2126	2.4901	3.916	0.0688	0.0344	0.9656
MR-B3	-0.4817	0.8237	1.4011	-2.3644	-0.5848	8.4270	0.5740	0.7130	0.2870



Appendix Fig. 5.1. Regression plots of growth (colony diameter, relative colony diameter, number of colonies) over over tebuconazole concentration (µg.ml^{-1}) at different transfers of AF11 isolates on tebuconazole-treated leaves (I to III) or water-treated leaves (IV to V1). T1, T2, T7 refer to number of transfers.



Appendix Fig. 5.2. Regression plots of growth (colony diameter, relative colony diameter, number of colonies) over tebuconazole concentration ((µg.ml^{-1}) at different transfers of MR11 isolates on tebuconazole-treated leaves (I to III) or water-treated leaves (IV to V1). T1 to T8 refer to number of transfers.