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Improving the understanding of fungicide resistance in Australian viticulture

FINAL REPORT TO WINE AUSTRALIA



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Project Leader: **Mark Sosnowski, Barbara Hall (SARDI)**

Project Supervisors**: Suzanne McKay (SARDI), Fran Lopez (CU), Markus Herderich (AWRI)**

Principal Investigators: **Ismail Ismail (SARDI), Lincoln Harper (CU), Anthony Borneman (AWRI)**

Research Organisations:
South Australian Research & Development Institute (SARDI)
Curtin University (CU)
Australian Wine Research Institute (AWRI)

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Project Title:	Improving the understanding of fungicide resistance in Australian viticulture
Project Leader:	Dr Mark Sosnowski, Barbara Hall South Australian Research and Development Institute GPO Box 397, Adelaide, South Australia 5001 Phone: (08) 8429 2281 Email: mark.sosnowski@sa.gov.au
Project Supervisors:	Dr Suzanne McKay (SARDI), Dr Fran Lopez (Curtin University), Dr Markus Herderich (AWRI)
Principal Investigators:	Dr Ismail Ismail (SARDI), Lincoln Harper (Curtin University), Dr Anthony Borneman (AWRI)

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1. Abstract

The grapevine diseases powdery mildew (*Erysiphe necator*), Botrytis bunch rot (*Botrytis cinerea*) and downy mildew (*Plasmopara viticola*) are managed with the use of fungicides. The development of fungicide resistance leads to reduced efficacy and field failure causing significant economic impacts. This project aimed to improve the understanding of fungicide resistance in Australian viticulture. In general, all three pathogens showed varying levels of reduced sensitivity to most of the fungicide groups tested, with resistance confirmed for fungicides from gp 13 for *E. necator*, gps 9, 12 and 17 for *B. cinerea* and gps 4 and 11 for *P. viticola*. It is recommended that fungicides be withdrawn from the spray program where resistance has been detected and that continued monitoring is carried out where reduced sensitivity has occurred. Techniques have been refined for improved monitoring of fungicide resistance with the development of a mini-greenhouse system to maintain and test isolates, rotorod spore traps to monitor for resistance, an in-field qPCR detection pipeline, Next Generation Sequencing to detect resistance and a heterologous yeast expression system to test resistance-associated mutations. For *E. necator*, there were strong relationships between phenotyping and genotyping for gp 11 (QoIs), but not for gp 3 (DMIs), and the resistance mutants G143A (QoI) and Y136F (DMI) were fit and stable for at least six months. The mutants H242R/Y (gp 7, SDHI, *E. necator*) and G1105S (gp 40, CAA, *P. viticola*) were not detected, but reduced sensitivity was recorded for both. Continued phenotypic and genetic monitoring will be crucial to manage and minimise fungicide resistance in Australian viticulture. Future research will develop in-field and high-throughput techniques in combination with rotorod spore trapping to achieve shorter turnaround and a high capacity for screening of known resistance-associated mutations.

2. Executive summary

Grapevine disease management can be compromised by resistance to fungicides used to control the most economically important foliar diseases in Australian viticulture; powdery mildew (*Erysiphe necator*), Botrytis bunch rot (*Botrytis cinerea*) and downy mildew (*Plasmopara viticola*). Fungicide resistant populations add to production costs by reducing fungicide efficacy, which can lead to the failure of spray programs for disease management. While laboratory testing has shown occurrences of fungicide resistance with these diseases in Australia, there is a need to evaluate the sensitivity of populations and hence the efficacy of the various fungicide products available to grape growers. The relationship between laboratory results obtained through bioassay (phenotyping), genetic analysis (genotyping) and field failure is still not well understood. In addition, the relative fitness of resistant populations compared with sensitive wild types (WT) in the field is still unclear. Several methods for monitoring fungicide resistance were investigated, such as the use of spore trapping, Next Generation Sequencing (NGS) and in-field real-time detection to increase the number of samples that can be processed. This project seeks to address these issues through the combined expertise of the research team in plant pathology and molecular diagnostics. The information generated will contribute to maintaining effective use of the available fungicide groups into the future, while reducing the economic and environmental impact of fungal diseases in Australian viticulture.

To determine the incidence and severity of fungicide resistance in Australian vineyards, isolates of *E. necator*, *B. cinerea* and *P. viticola* were collected between 2016 and 2021. A range of different collection methods were adopted and improved, together with a range of laboratory assays and genetic techniques. Analysis of sensitivity included the following fungicide groups: Quinone outside Inhibitors (QoIs, group 11), Demethylation Inhibitors (DMIs, gp 3), Amines (gp 5; spiroxamine), Aza-naphthalenes (gp 13; proquinazid and quinoxyfen), Succinate dehydrogenase inhibitors (SDHI, gp 7; boscalid and pydiflumetofen), Aryl-phenyl-ketones (gp 50; pyriofenone), Phenyl-acetamide (gp U6; cyflufenamid), Anilino-Pyrimidines (AP, gp 9), PhenylPyrroles (PP, gp 12), KetoReductase Inhibitors (KRI, gp 17), Carboxylic

Acid Amides (CAA, gp 40) and Quinone outside Inhibitor, stigmatellin binding type (QoSI, gp 45).

A new mini-greenhouse system was developed to maintain *E. necator* populations and effectively conduct experiments to obtain consistent results. In addition, field label rate concentrations were introduced to the fungal growth testing regime, leading to the new 'reduced sensitivity' category. In general, *E. necator* isolates showed varying levels of reduced sensitivity to most of the fungicides tested; QoIs (20-62% of samples collected), DMIs (9-50%), spiroxamine (17%), proquinazid (58%), boscalid (42%), pydiflumetofen (30%) and pyriofenone (41%), but resistance was only confirmed for quinoxyfen in 60% of samples collected from regions in Vic, SA and WA. However, all tested isolates were sensitive to cyflufenamid. Continued monitoring for *E. necator* resistance is required to detect any shifts in resistance, especially where reduced sensitivity was detected.

For *B. cinerea*, total resistance frequency for fungicides from gps 9, 12 and 17 was close to 16%. New genotypes associated with gp 9 and 17 resistance, not previously described in grape isolates in Australia, were characterised. These include mutations in *Pos5* (P293S), *Mdl1* (E407K, G408R) and *Erg27* (F412I/V). These new mutations are ideal targets for future development of qPCR assays for *in vitro* or in-field testing. Further resistance monitoring of these three key chemical groups is still required in all wine regions, and especially in regions where frequencies are elevated, such as Pemberton, WA and King Valley, Vic. Of particular interest is gp 12, where larger shifts in sensitivity are possible.

Resistance of *P. viticola* at field rate was detected for QoI fungicides (gp 11; 8% of isolates tested) and PhenylAmide (PA) fungicide (metalaxyl, gp 4; 19%), with the majority of resistant populations detected from regions in NSW and Victoria. In addition, reduced sensitivity was detected in 2-47% of isolates tested for fungicides from group 4, 11, 40 and 45. PA and QoI fungicides should be withdrawn from spray programs where resistance to metalaxyl and pyraclostrobin has been detected. It is recommended that monitoring for resistance be continued, especially where reduced sensitivity has been detected.

There were strong relationships between phenotyping and genotyping for QoIs. Bioassay results matched with presence of the mutant G143A (responsible for resistance to QoIs) with 92% and 90% agreement in powdery and downy mildew, respectively. This mutant will be an ideal candidate for high-throughput and in-field testing.

The relationship between phenotyping and genotyping for DMI fungicides in *E. necator* was weak and unclear as other factors may contribute to the resistance, such as gene copy number or other mutations. The Y136F mutant was detected in most of the isolates, but the phenotyping results showed only limited reduction in sensitivity. More research will be necessary before reliable genotyping can be performed for DMI resistance.

G143A and Y136F mutants were fit and stable for at least six months under laboratory and greenhouse conditions. This suggests that withdrawal of QoIs and DMIs from the spray program for six months may not be long enough to reduce mutant populations of *E. necator*.

The H242R/Y mutation linked to SDHI resistance was not detected in any *E. necator* populations, however reduced sensitivity was detected. Similarly, the G1105S mutant, linked to CAA resistance, was not detected in any isolates of *P. viticola*, however reduced sensitivity was again detected. Therefore, it will be important to continue monitoring for SDHI and CAA resistance in Australian vineyards.

The efficacy of DMI fungicides was evaluated when exposed to populations of *E. necator,* with varying sensitivity. The heterologous yeast expression system (HYES) revealed that there were significant shifts in sensitivity associated with the Y136F mutation to the DMI fungicides myclobutanil and triadimenol. These results support the findings that triadimenol is less effective at controlling Y136F isolates than WT isolates. These results have shown that the HYES is suitable for testing additional *Cyp51* mutations that may be identified in the future.

DMI fungicides differed in their efficacy regardless of the genetic profile (mutant or WT) of *E. necator.* Mefentrifluconazole and difenoconazole were more effective than myclobutanil and triadimenol in controlling powdery mildew in greenhouse conditions. Triadimenol was less effective when the Y136F mutant was present.

The relationship between copy number of the *Cyp51* gene and phenotypic resistance is not clear, however there was a trend indicating that high copy number may be associated with resistance to DMI fungicides. Further research is required to investigate this relationship, which may lead to the development of a marker for resistance to DMI fungicides.

Mixing sulfur with the DMI fungicides penconazole, myclobutanil and difenoconazole had no effect on the efficacy of these fungicides for controlling powdery mildew in grapevine under greenhouse conditions. This supports label recommendations for use of sulfur, and dispels any confusion raised by previous research of mixing sulfur with DMI fungicides.

Rotorod spore traps, mini-vacuum cyclone separators, washing infected leaves and cotton bud swabs were equally successful tools to detect genetic resistance of *E. necator*. However, the rotorod spore trap did not require the movement of plant material, and proved to be more sensitive and practical, especially in the early stages of infection when there were no visible symptoms to be collected using the other methods.

A rapid in-field qPCR detection pipeline was successful in estimating *B. cinerea* group 9 and 17 resistance frequencies in commercial vineyards. This pipeline can now be used as a rapid and cost effective 'first look' step to estimate frequencies at vineyards with known control issues. The pipeline can also be used for the monitoring of frequencies where there are no known control issues, or to estimate frequencies within a field trial setting. Additional assays for alternate group 9 and group 17 genotypes could now be developed. The in-field pipeline was also successfully developed to target the group 11 associated mutation G143A in *E. necator*.

Reduced sensitivity and resistance have been detected in Australian populations of *E. necator, B. cinerea* and *P. viticola* for some of the major groups of fungicides used to control grapevine diseases. Therefore, resistance management strategies are required to minimise and delay the onset of resistance. These strategies include regular monitoring (using bioassays and/or genetic analysis), rotating fungicide chemistries, applying chemicals only when necessary, applying multi-site fungicides and other alternatives such as inorganic fungicides (sulfur and copper) and biologicals (*Aureobasidium pullulans*). Future research will develop in-field and high-throughput techniques to achieve shorter turnaround and a high level of screening for known resistance associated mutations.

3. Background

3.1. Powdery mildew (*Erysiphe necator*)

Powdery mildew, caused by the fungal pathogen *Erysiphe necator* (Schwein.) Burrill, is one of the most economically important grapevine diseases worldwide. This obligate biotroph can infect all green tissues of a grapevine, affecting vine health and vigour as well as berry quality (Figure 3.1). As little as 1-5% infection can adversely affect wine sensory qualities (Stummer et al. 2003). As most commercial cultivars are susceptible to powdery mildew and the disease does not need highly specific environmental conditions for growth with a rapid disease cycle, the disease occurs in nearly all Australian grape-growing regions, in almost every season. Growers rely heavily on the use of fungicides for disease management. Although factors such as incorrect spray timing, insufficient spray coverage and adverse weather conditions can result in poor disease control, the development of fungicide resistance is also a major problem because fungicide-resistant populations add to production costs due to reduced fungicide efficacy and failure of spray programs to manage disease. In Australia, fungicides from eight different mode of action groups are used for control of powdery mildew (Table **3.1**).



Figure 3.1. Powdery mildew caused by *Erysiphe necator* on grapevine at different stages of infection.

3.1.1. Group 11, Quinone outside Inhibitors (QoIs)

Strobilurins, or Quinone outside Inhibitors (QoIs, FRAC 11) are derived from natural products, namely strobilurin A and oudemansin A produced by some basidiomycetes, which were further optimised to overcome the photo-liability and toxicity to mammals (Kunova et al., 2021). The QoIs are single site mode of action fungicides that work by inhibiting fungal respiration, specifically by blocking electron transport in the cytochrome bc complex located on the mitochondrial membrane, thereby inhibiting ATP or energy production. Currently, there are four active ingredients labelled within this group for powdery mildew control (i.e. azoxystrobin, trifloxystrobin, pyraclostrobin and trifloxystrobin), which are utilised in more than 14 commercial products (Essling & Lord, 2022). Although, several single point mutations linked to resistance have been identified (FRAC monograph 3) for most pathogens, including *E. necator*, a mutation at position 143 in the cytochrome b gene (*CytB*), results in a glycine for alanine substitution, referred to as G143A, is linked with complete resistance to the QoI fungicides (Gisi et al., 2002). However, Villani and Cox (2014) also found quantitative or partial

resistance responses to QoI fungicides in isolates of *Venturia inequalis* (apple scab pathogen) where isolates showed a range of phenotypic responses, with other mechanisms in addition to G143A, including heteroplasmy, likely responsible for resistance. To date, G143A is the only point mutation associated with resistance in *E. necator* (Rallos et al. 2014).

QoIs were first used commercially in agricultural systems in 1996 (Bartlett et al. 2002, Gisi et al., 2002, Ma & Michailides, 2005). In the USA, azoxystrobin was introduced to control powdery mildew in grapevines in 1997, trifloxystrobin in 2001 and pyraclostrobin in 2003 (Colcol and Baudoin, 2016) and five years after introduction, resistance was detected (Baudoin et al. 2008, Miles et al. 2012). Unfortunately, the frequent use of these fungicides eventually led to resistance development, with the first report of QoI fungicide resistant *E. necator* sub-populations occurring in New York (Baudoin et al. 2008; Wilcox et al. 2003). In the following years, QoI-resistant *E. necator* sub-populations were also observed in other grape-growing regions such as the eastern United States and France (Baudoin et al. 2008; Dufour et al. 2011). In Australia, resistance of *E. necator* to QoIs was first reported in South Australia and Victoria in 2010, approximately 10 years after the introduction of QoIs to Australia for powdery mildew control (Wicks et al. 2013). Field failure was observed and was strongly associated with high levels (>95%) of G143A. Cross-resistance has been shown between all fungicides within the QoI group with FRAC considering the QoIs at high risk of resistance development, given that G143A has already been identified and strongly linked to resistance and assays accurately detected each allele (100% agreement) and there was an absolute agreement between the presence/absence of the G143A mutation and resistance to QoIs in the *E. necator* sampled (Miles et al. 2021). Hence, molecular detection for QoI resistance is widely accepted.

3.1.2. Group 3, Demethylation Inhibitors (DMIs)

Demethylation inhibitor (DMI) fungicides are sterol inhibitors (SBI), class I fungicides, which target the cytochrome P450 lanosterol *C*-14α demethylase (CYP51) enzyme resulting in disruption of ergosterol synthesis (Ziogas and Malandrakis, 2015). Ergosterol is an essential component of fungal cell membranes, which when disrupted, reduces fungal growth. DMIs are single-site mode of action fungicides and are classified as medium risk of resistance development FRAC (2021). DMIs do not affect conidia germination, but very efficiently inhibit the fungal growth by affecting the membrane integrity and functionality. Their molecular mechanism of action has been studied in detail; DMIs bind to the heme iron of CYP51 with a nitrogen atom and inhibit the O2 binding and its transfer to lanosterol C14-methyl group, which is the main step in the lanosterol C14-demethylation process (Kunova et al., 2021). Four molecular mechanisms have been associated with DMI resistance in different pathosystems (Cools and Hammond-Kosack, 2013):

1. Single point mutations, e.g. at codon 136 in *Cyp51* causing the substitution of phenylalanine by tyrosine, Y136F (Delye, 1997);

2. Over-expression and/or copy-number variation of *Cyp51* (Ziogas & Malandrakis, 2015).

3. Increased efflux by over-expression of membrane-bound drug transporters, or reduced fungicide uptake, thereby reducing the accumulation of fungicide at the target site (de Waard and van Nistelrooy 1979, de Waard and van Nistelrooy 1988, del Sorbo et al 2000).

4. A mutation A1119C leading to synonymous substitution in the *Cyp51* sequence was associated with its over-expression and the azole resistance in *E. necator* (Frenkel, 2014).

Délye et al (1997) first associated the Y136F mutation with resistance in grapevine powdery mildew to triadimenol, and subsequently this mutation has been linked with azole resistance in *E. necator* from Virginia USA (Colcol et al. 2012), France (Dufour et al. 2011), Italy (Miazzi and Hajjeh 2011) and eastern USA and Chile (Frenkel et al. 2015). Recently, in addition to Y136F, Frenkel et al (2015) found another point mutation in *Cyp51*, A1119C, as well as an increased expression of *Cyp51*, both linked to myclobutanil resistance in isolates of *E. necator*

from eastern USA. However, they found no evidence for the effects of expression of efflux transporter genes on EC_{50} values. Rallos and Baudoin (2016) also found over-expression of *Cyp51* in Y136F mutants plus two synonymous mutations, one of which, A1119C, was present in isolates of *E. necator* with high *Cyp51* expression. However, it seems the slow step-wise development of resistance is a result of the accumulation of several mechanisms. Evidence for this is supported by findings of Délye et al. (1997), Miazzi and Hejjeh (2011) and Colcol et al. (2012) who found that not all resistant isolates carried Y136F and furthermore, Miazzi and Hajjeh (2011) found that not all isolates with Y136F were resistant. In contrast to the QoIs, where loss of efficacy is often complete, the DMIs often show partial efficacy. As there are large differences in the activity spectra of the different DMI fungicides (FRAC 2021), resistance to one DMI may not mean equal resistance to others. However, it is generally accepted that cross-resistance is present among the DMIs with the same pathogen (FRAC 2021).

DMIs were first used commercially in the late 1970s (Savocchia et al. 2004) and since, have been used widely in grapevines and other crops both in Australia and worldwide. Triadimefon (Bayleton) and fenarimol (Rubigan) were registered in Australia in the early 1980s for grapevine powdery mildew control (Savocchia et al. 2004), followed in 1990 by triadimenol (Bayfidan), which was widely used over the next 10+ years (Savocchia et al. 2004). Since then, other DMIs such as myclobutanil and penconazole have been more widely used. Resistance of *E. necator* to DMIs resulting in poor disease control was reported in France and Portugal (Steva, 1994), New York, USA (Erickson and Wilcox, 1997), California, USA (Gubler et al. 1994, Ypema et al. 1997), Europe and India (Delye, 1997), Ontario, Canada (Northover and Homeyer, 2001), mid-Atlantic USA states (Colcol et al. 2012), as well as Australia (Savocchia et al. 2004).

3.1.3. Group 5, Amines (Morpholines)

The Amines (Morpholines), SBI class II fungicides, affect the biosynthesis of sterols in cell membranes. Morpholines target Δ^{14} reductase, but they also inhibit squalene epoxidase, epoxysqualene cyclase, and sterol Δ^{8}– Δ^{7} isomerase (Kunova et al. 2021). Only spiroxamine fungicide is currently registered against grapevine powdery mildew. Spiroxamine was registered in 1999 and is used especially on cereals and grapes. It works as a protective, curative, and eradicative. Decreased sensitivity has been reported for powdery mildews and cross-resistance within the group generally found but not to other SBI classes, it is considered a low to medium risk (FRAC 2021). Similarly to DMI, the resistance to amines is presumed to be multigenic and of quantitative type. Miller and Gubler (2004) reported a shift in sensitivity to triadimefon (member of morpholines) in the Californian populations of *E. necator*, but it is in a stable situation in the European countries with low resistance factors towards amines and only with small regional fluctuations close to the baseline (FRAC, 2021). The molecular basis of the reduced sensitivity in *E. necator* is unknown. Fungicides e.g. Prosper®, containing the active constituent, spiroxamine, are registered in Australia for powdery mildew control in grapevines (Table **3.1**).

3.1.4. Group 13, Aza-napthalenes

Aza-naphthalenes comprise a novel group of fungicides with specific activity against powdery mildew. It has protectant activity with locally systemic and vapour phase redistribution and inhibits early stages of powdery mildew development such as spore germination and appressorium formation (Dietz and Winter, 2019). Aza-napthalenes affect or interfere with signal transduction but the mechanism is unknown (FRAC, 2021) and also, the exact site of the mode of action is unknown (Ouimette, 2011). Two fungicide groups, used for powdery mildew control in Australia, are quinoxyfen e.g. Legend ® and proquinazid e.g. Talendo®. FRAC state that resistance to quinoxyfen is known, and classifies it as a medium risk. FRAC also report that cross-resistance has been found in *E. necator* (FRAC, 2021).

Quinoxyfen was released for vines in Europe in 1998, Green and Gustafson (2006) carried out *in vitro* sporulation assays prior to quinoxyfen release to evaluate EC_{50} 'baselines'. EC_{50}

ranged from <0.03 to 2.6 µg/mL (from 56 isolates). Quinoxyfen laboratory resistant isolates were detected in 2013 at a single vineyard in western Virginia, USA (Feng et al. 2018). However, in a 3-year vineyard study from 2014-2017, quinoxyfen provided only slightly less control than that provided by myclobutanil and boscalid (Feng et al. 2018).

Proquinazid stimulates expression of host genes associated with resistance responses (ethylene mediated, phytoalexin, cell-wall strengthening and active O2), Crane et al. (2007) hypothesised that proquinazid modulates both pathogen and host. Genet and Jaworska (2009) tested 174 European isolates for baseline sensitivity to proquinazid, and EC_{50} values ranged from 0.001 to 0.3 µg/mL. EC_{50} values from proquinazid were significantly lower than for quinoxyfen indicating a higher intrinsic activity of proquinazid. Strong cross-resistance was observed to in terms of resistance management, both fungicides should be managed together (Genet and Jaworska, 2009).

Resistance has been reported for *Blumeria graminis* f.sp. tritici in wheat and *E. necator* in grapevine (Genet and Jaworska, 2009) and *E. graminis* f. sp. hordei in barley (Hollomon et al. 1996). Baudoin (2014) found field failure of quinoxyfen at one vineyard in Virginia. Isolates were tested for spore germination, and only sensitive isolates grew at <3.0 µg/mL. All others grew at 10, 30 and 100 µg/mL. No EC_{50} as no rate dependent effects. Also inoculated small potted vines with resistant isolates and sprays at 50 and 100 µg/mL did not control powdery mildew. Feng et al. (2018) found that despite the vineyard having high frequency ~ 65% of quinoxyfen resistant isolates (as reported by Baudoin (2014)), field efficacy was maintained following spray trials in 2015 to 2017.

3.1.5. Group 7, Succinate dehydrogenase Inhibitors (SDHIs)

Formally known as carboxamides, SDHIs inhibit succinate dehydrogenase (SDH) in complex II in the mitochondrial respiration. SDHI fungicides were discovered in the 1960s, Target site mutations can either directly affect binding of SDHI or affect binding by long range structural rearrangement of complex II (Stammler et al. 2015), which results in a range of sensitivity responses to SDHIs. In general, cross-resistance among SDHIs is thought to occur but again, a range of responses may occur (Stammler et al. 2015).

Various mutations in the three subunits involved in the formation of the ubiquinone-binding pocket have been described to be responsible for resistance to SDHI in different pathogens. Two mutations in two subunits were identified in *E. necator*, in *SdhB* there is a H242R/Y and I244V, and one mutation in *SdhC*, G169D/S (FRAC, 2022). Interestingly, these mutations have a different impact on resistance to different SDHI fungicides, for example H242R mutation confers resistance to boscalid (resistance factor RF > 100) while it has no or little impact on the effectiveness of fluopyram and fluxapyroxad (Cherrad et al. 2018). In 2014, single isolate from a single vineyard in Italy with decreased sensitivity to fluxapyroxad was described (FRAC), then spread in French populations of grapevine powdery mildew to boscalid was described (Cherrad et al. 2018). SDHI resistance was detected sporadically in different European countries, and in 2020, the monitoring detected only single sites in Hungary, France, and Germany (Cherrad et al. 2018, FRAC, 2022).

Different mutations can occur in a different codon but also different codons in different subunits can result in SDHI resistance, making genetic assays difficult to develop for sensitivity monitoring. Also, it is possible to get combinations of mutations under high selection pressure, Stammler et al. (2015) advises that classical laboratory sensitivity assays should be used for monitoring of resistance at the same time as genetic assays to ensure detection of the correct mutations linked with resistance. However, according to Stammler et al. (2015), genetic assays are sufficient for detecting resistance in *B. cinerea* as nearly all field isolates only carry a limited number of mutations. With new SHDIs, new mutations may become apparent or the frequency of existing known mutations may change.

Resistance is known for several fungal species with target site mutations in the *SDH* gene. They are classified as medium to high risk by FRAC (FRAC, 2022). Lower fitness has been observed for isolates with reduced sensitivity of *B. cinerea* (Stammler et al. 2015). Currently three group 7 products are registered in Australia for control of powdery mildew, namely Filan, Miravis and Luna Experience. Both Luna Experience and Miravis can be used for export wine, but Filan cannot (Table **3.1**) (Essling and Lord, 2022). Luna Experience is also registered from control of *Botrytis*.

3.1.6. Group 50 (previously U8), Aryl-phenyl ketones

Group 50, Aryl-phenyl ketones, are new-generation fungicides used almost exclusively for powdery mildew management. Currently, this group contains two fungicides; metrafenone (Vivando®) and pyriofenone (Kusabi®). The mechanism of actions are not completely understood, but the studies on cereal powdery mildew suggested the disruption of the actin cytoskeleton and the interference with hyphal morphogenesis, polarised hyphal growth, and cell polarity (Schmitt et al. 2006). Resistance was observed, first in *B. graminis f. sp. tritici* (Felsenstein et al. 2010), then by *E. necator* (Kunova et al. 2016), moderately- and highly-resistant isolates were observed in grapevine powdery mildew. Highly resistant strains to metrafenone were able to grow and sporulate at the concentration 1250 mg/L (10-times higher than the recommended field application) (Kunova et al. 2016). Resistant strains are nowadays distributed in different European countries at fluctuating frequencies and are not homogeneously distributed (Kunova et al. 2021).

3.1.7. Group U6, Phenyl-acetamide

Group U6, phenyl-acetamide (cyflufenamid), has preventive and curative activity against powdery mildews in cereals, specialty crops and brown rot on stone fruits (Dietz and Winter, 2019, Sano et al. 2007). The mode of action is still unknown, and therefore it remains classified as a class U06 (FRAC, 2021). Cyflufenamid does not inhibit the spore germination and appressorium formation, of but strongly inhibits haustorium formation of *B. graminis f. sp. tritici* (Haramoto et al. 2006). Resistance was described in cucurbit powdery mildew in 2006 in Japan(FRAC, 2021), followed by report from Italy (Pirondi et al. 2014) and the US (McGrath and Sexton, 2018). However, until now, resistance has not been observed in grapevine powdery mildew. Although resistance risk is unclassified, resistance management is required (FRAC code list 2021).

3.1.8. Other fungicides

Other fungicides used for powdery mildew control in Australia are from the M or multisite groups, e.g. M1 (copper-based compounds), M2 (Sulfur-based compounds, potassium bicarbonate and calcium polysulfide compounds) and M3 (the Dithiocarbamates). These are all classed as low risk of resistance development (FRAC 2021) as many have been used for hundreds of years without any signs of resistance developing. Due to the low risk of resistance development and the lack of reports about loss of sensitivity, none are included in resistance testing in this project. However, they are key components of fungicide programs to control fungal diseases, including powdery mildew. Not only providing excellent control when used correctly, they are an important part of resistance management. However there are also drawbacks to their use, for example, they have no systemic activity and need to be applied regularly, and there are potential adverse health and environmental impacts with some of these products, particularly some copper and sulfur based products. Other products have negligible health and environmental effects e.g. Ecocarb, but they are also relatively expensive compared to other fungicides.

Table 3.1. The activity group table for fungicides used to control powdery mildew in Australia (Essling and Lord, 2022).

FRAC code	Group Name	Active constituent	Some registered products
3	Demethylation Inhibitors (DMI) (SBI class I)	difenoconazole	*Digger, Digger EW
		tebuconazole	Buzz Ultra 750WG, Laguna Xtreme 800WG, Orius 430 SC,Tebucon 430 SC, Ultrateb 750WG, Zolo 430SC
		mefentrifluconazole	Belanty
		penconazole	Azotic, Delos, Pearl, Ruby 100EC, Topas 100 EC
		tetraconazole	*Domark 40ME, Mettle 40ME
		triadimefon	Triadimefon 125
		triadimenol	Allitron, Bayfidan 250 EC, Triadimenol 250 EC, Tridim 250 EC
		myclobutanil	Myclonil WG, *Mycloss Xtra, Stamina
5	Amines (“morpholines”) (SBI class II)	spiroxamine	*Prosper 500 EC, Spire 500 EC
7	Succincate dehydrogenase inhibitors (SDHI)	pydiflumetofen	*Miravis
		boscalid	*Filan,
11	Quinone outside Inhibitors (QoI)	azoxystrobin	Affix 250SC, *Amistar 250SC, A-star 250 SC, Avior (250SC, 800 WG), Azoxy 250, AzoxyGuard 250 SC, Azoxys 250 SC, Azoxystrobin (250, 250 SC, 500 WG), Connect 800 WG, Galoxy 250SC, Mirador 250 SC, Spartacus (250 SC, 500WG), Stellar, Supernova 250SC
		trifloxystrobin	*Flint 500 WG, Invictus 500 WG
		pyraclostrobin	*Cabrio, Pavo 250 EC, Roadster 500 EC, Symbio 250 EC, Vipyr 250 EC
13	aza-naphthalenes	quinoxyfen	*Legend, Quinfen 250 SC, Vitae
		proquinazid	*Talendo
50	aryl-phenyl-ketones	pyriofenone	*Kusabi 300 SC
		metrafenone	*Vivando
3+11	DMI+QoI	tebuconazole + azoxystrobin	Aztec, Custodia
7 + 3	SDHI+DMI	fluopyram + tebuconazole	Luna Experience
1M2	inorganic (electrophiles)	sulfur	Brimflo 800, Cosamil, Cosavet WG, Flosul 800, InnoSulph 800 WG, Sulphur, Kumulus DF, Microsul WG Elite, *Microthiol Disperss, Nimbus WG, Sulfur, Sulfur 800 WG, Sulgran (80WG, WG), Sulphur (Spray, 800 WG, WG), *Thiovit Jet, Top Wettable Sulphur, Uni- Shield, Wettable Sulphur, Zulfa 800WG
		potassium bicarbonate	EcoCarb and Plus
M2 + 3	inorganic (electrophiles) +DMI	sulfur + tebuconazole	Unicorn 745WG
U6	phenyl-acetamide	cyflufenamid	*Flute 50 EW

*fungicides tested in this project

3.2. Botrytis bunch rot (*Botrytis cinerea*)

3.2.1. Introduction

Botrytis cinerea, the causative agent of botrytis bunch rot (BBR), is a necrotrophic fungal pathogen responsible for one of the most economically important diseases of grapevines worldwide (Figure 3.2). In combination with other bunch rots, BBR can have an economic impact of AU$50M per annum to the Australian grape and wine industry (Emmett and Nair, 1991, Scholefield and Morison, 2010). In Australia, BBR and other bunch rots can cause yield losses of up to 30% depending on the climatic zone (Scholefield and Morison, 2010). *Botrytis cinerea* can cause primary (latent) infections by entering scar tissue in grape flowers after capfall (Keller et al. 2003). Once sugar levels in the berry rise and/or the host immune system wanes, latent infections can express (Meyer et al. 2000; Sanzani et al. 2012). *Botrytis cinerea* can also produce secondary infections, whereby conidia from external sources (e.g. bunch detritus, expressing primary infections) can infect berry wound sites (Elmer and Michailides, 2007). Globally, BBR is managed by cultural practices and the application of single and multi-site fungicides. However, resistance to single-site fungicides has been described across several wine grape growing regions worldwide (Avenot et al. 2018, Beresford et al. 2017, Campia et al. 2017, Latorre and Torres, 2012, Sang et al. 2018). *Botrytis cinerea* is prone to resistance due to a high sporulation rate and short life cycle (Brent and Hollomon, 1998, Grimmer et al. 2015). Currently in Australia the key single-site groups registered for *B. cinerea* control in wine grapes are FRAC groups 9, 12 and 17. Fungicides from FRAC group 7 are rarely used because of maximum residue level (MRL) restrictions for this group in export wine. Fungicides from FRAC group 3 and 11 are not widely used for *B. cinerea* control in grapes due to low efficacy. In Australia, single-site fungicides for *B. cinerea* control are applied before flowering through to when berries are peppercorn sized (EL-29).

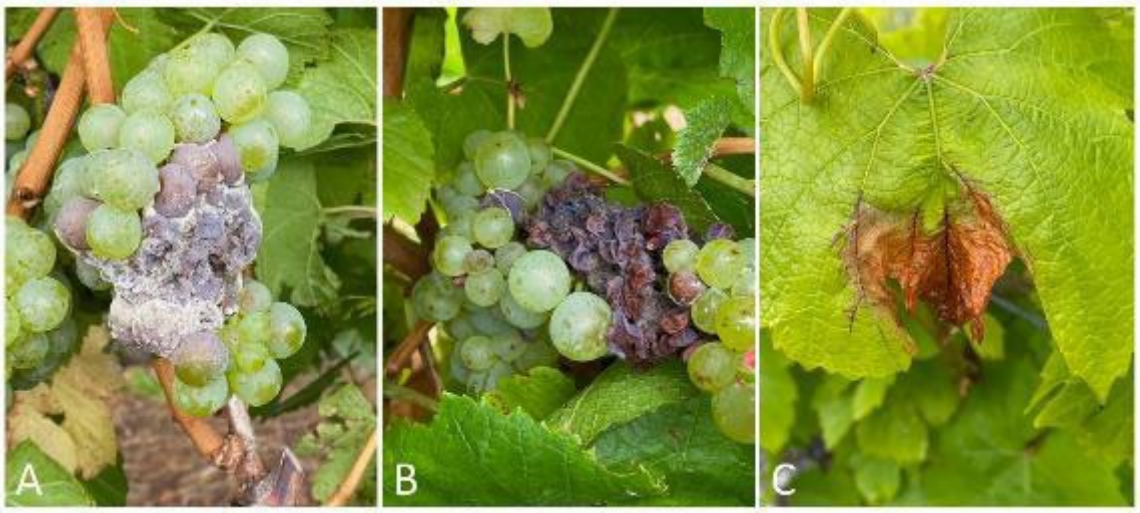


Figure 3.2. (A-B) Botrytis bunch rot and (C) leaf infection (Photographs courtesy of Scott Paton).

3.2.2. Anilino-pyrimidine (AP) fungicides - FRAC Group 9

Group 9 fungicides (cyprodinil and pyrimethanil) are commonly used for the control of *B. cinerea* on fruits and vegetables (Mosbach et al., 2017). It is proposed that this class of fungicides disrupts the methionine biosynthesis pathway (Fungicide Resistance Action Committee). The fungicide class was originally registered overseas between 1992 – 1995 and in Australia in 1996 (Mosbach et al. 2017; Australian Pesticides and Veterinary Medicines

Authority). Resistance to these fungicides was first reported in strawberries soon after registration (Forster and Staub, 1996, Hilber and Schuepp, 1996). Resistance has since been described in several wine grape reports worldwide (Beresford et al. 2017, Campia et al. 2017, Leroch et al. 2011, Toffolatti et al. 2020, Walker et al. 2013). Resistance in Australian grapevine was first reported in 2002 (Sergeeva et al. 2002). To identify the molecular mechanism for resistance, initial studies sequenced the cystathionine β-lyase and cystathionine g-synthase-encoding genes in the methionine biosynthesis pathway but could not associate any mutations with resistance (Fritz et al. 2003, Sierotzki et al. 2002). Recently, mutations associated with a high level of resistance in grape and strawberry field isolates have been described in the genes *Pos5* and *Mdl1*. *Pos5* and *Mdl1* encode the two mitochondrial based gene products; an NADH kinase and ABC transporter, respectively (Mosbach et al., 2017). These mutations include V273I, P293S, P319A, G408R/V and L412F/S/V in *Pos5* and E406K in *Mdl1* (Mosbach et al. 2017). An Australian grapevine report associated the *Pos5* changes V273I/L with medium resistance, and P319A or L412F/V with high resistance (Harper et al. 2021). The presence of mutations in *Pos5* and *Mdl1*, suggests that group 9 fungicides may interfere with mitochondrial respiration (Mosbach et al. 2017).

3.2.3. Phenylpyrrole (PP) fungicides - FRAC group 12

The target for phenylpyrrole fungicides is the MAP/histidine kinase within the osmotic signal transduction pathway (FRAC). The phenylpyrrole fungicide fludioxonil was first registered in Australia in 2000 as a product in combination with the group 9 fungicide cyprodinil (Australian Pesticides and Veterinary Medicines Authority). Resistance to fludioxonil has been described in several wine grape reports worldwide (Chapeland et al. 1999, Leroch et al. 2011, Toffolatti et al. 2020, Walker et al. 2013). The molecular mechanisms of resistance to fludioxonil were first described in *B. cinerea* in a grape report from Germany (Kretschmer et al. 2009). It was shown that over-expression of the ABC transporter ATRB (MDR1), the MFS transporter MFSM2 (MDR2), or a combination of both (MDR3), can result in resistance to fludioxonil (Kretchmer et al. 2009). Over-expression of *AtrB* and *MfsM2* is caused by mutations in the transcription factor gene *Mrr1* and a transposon insertion in the promoter of *MfsM2*, respectively (Fernández-Ortuño et al. 2015, Kretschmer et al. 2009, Leroch et al. 2013, Li et al. 2014, Rupp et al. 2017). Recently, MDR1 isolates have been reported in an Australian wine grape study (Harper et al. 2021). In general, the MDR phenotype is associated with low to medium RF values (2.1 – 15) to fludioxonil (Harper et al. 2021, Kretschmer et al. 2009, Latorre and Torres, 2012, Leroch et al. 2013, Li et al. 2014). However, recent reports have identified field isolates with very high resistance factors (>57) to fludioxonil (Fernández-Ortuño et al. 2015, Sang et al. 2018). Sang et al. 2018 reported changes in *Os1*, not *Mrr1*, that were associated with very high shifts in sensitivity to fludioxonil.

3.2.4. Ketoreductase inhibitor fungicides (FRAC group 17)

Group 17 fungicides interfere with the 3-keto reductase enzyme, which is a component of the sterol biosynthesis pathway (Fungicide Resistance Action Committee). The group 17 fungicide fenhexamid was first registered in Australia in 1999 (Australian Pesticides and Veterinary Medicines Authority). Resistance to fenhexamid has been described in several wine grape reports (Beresford et al. 2017, Leroch et al. 2011, Toffolatti et al. 2020, Walker et al. 2013). Group 17 resistance has been recently reported in an Australian wine grape study (Harper et al. 2021). The group 17 resistance mechanism involves changes to target gene *Erg27* which encodes the 3-keto sterol reductase enzyme. Several non-synonymous changes in *Erg27* have been reported to confer moderate resistance (Grabke et al. 2013, Toffolatti et al. 2020) (Albertini and Leroux, 2004). The T63I and F412C/I/S/V changes in *Erg27* have been associated with high levels of resistance (Fillinger et al. 2008, Grabke et al. 2013, Harper et al. 2021).

3.3. Downy mildew (*Plasmopara viticola*)

Plasmopara viticola, the causal agent of grapevine downy mildew disease (Figure 3.3), is a native species from North America that was introduced several times into Europe in the late 1870s. Originally classified in the Kingdom Fungi, *P. viticola* is now classified as an oomycete in the Kingdom Heterokonta. Oomycetes are closely related to photosynthetic organisms such as brown algae and diatoms than to true fungi. Oomycete cell walls comprise cellulose rather than chitin as with fungi and in their vegetative state, and have diploid (two) nuclei, whereas the nuclei of analogous ascomycete fungi are haploid (one nuclei) (Gessler et al. 2011). Many oomycetes produce self-motile zoospores with two flagella (Gessler et al. 2011). There are major differences in the epidemiology, disease cycles and management of the oomycetes compared to true fungi because of their structural and morphological features. In addition, *P. viticola* is an obligate host-specific biotroph, which affects the handling and logistics of working with this pathogen in a research capacity.

Plasmopara viticola spread rapidly through most of continental Europe in the 1900s resulting in one of the most important grapevine diseases (Taylor et al. 2019). The first Australian report of *P. viticola* causing downy mildew was in several commercial vineyards in Rutherglen, Victoria in 1917 (de Castella and Brittlebank, 1917). The pathogen spread rapidly within grape-growing regions of eastern Australia in 1918, South Australia and Queensland in 1920 and 1921, respectively, Tasmania in 1959 and Western Australia in 1997 (Emmett, 1992, Taylor et al. 2019). Genetic analysis shows that the Australian and South American populations of *P. viticola* are more closely related to the European population than the North American population, the reported source of origin of the pathogen (Taylor et al. 2019). The Western Australian population had similarities to the South Australian population, and the tight clustering of samples suggests a single introduction into Western Australia (Taylor et al. 2019). Annual costs of $7.3 million per year have been reported, equivalent to a 1% drop in the gross value of Western Australia grape growing industries (Taylor and Cook, 2018). It has been estimated that the costs of disease management and yield losses to the Australian wine industry are A$63 million per year (Scholefield and Morison, 2010). Downy mildew can cause 50–100% foliage and inflorescence loss in grape vines (Emmett et al. 1992). Chemical control is the most effective measure currently used to protect grapes from downy mildew disease. However, *P. viticola* is a pathogen characterised by high mutation rate and high asexual sporulation efficiency; factors that probably contribute to the rapid adaptation to single-site fungicides (Maddalena et al.). Resistance to single-site fungicides is one of the main issues in downy mildew management (FRAC pathogen risk list, www.frac.info) and resistance against several fungicide classes has been reported (Maddalena et al.). Several reports indicate resistance to different fungicide mode of action groups such as Quinone outside Inhibitor (QoI) fungicides, Carboxylic Acid Amide (CCA), fungicides (Quinone outside Inhibitor, stigmatellin binding type (QoSI), Phenylamide (PA) and carboxylic acidamide (CAA) fungicides. Fungicide resistance has been detected in WA, VIC and NSW in several vineyards (Hall et al., 2017). In Australia, fungicides from nine different mode of action groups are used for control of powdery mildew and four fungicides from four groups were used to test the sensitivity (Table 3.2).



Figure 3.3. Downy mildew caused by *Plasmopara viticola* on grapevine at different stages of infection.

3.3.1. Group 11, Quinone outside Inhibitors (QoIs)

Downy mildew is widely controlled by QoI fungicides, however resistance to this chemical group can develop relatively quickly. For example, QoI fungicides were introduced into France for pest management in viticulture in 1998, and two years later QoI fungicide resistance was discovered and the G143A mutation was detected in *P. viticola* populations using quantitative PCR (Chen et al. 2007). Furthermore, a comparative study of the cytochrome b gene structure in various plant pathogen species suggested that the F129L substitution of the cytochrome b gene could also be involved in resistance to QoI fungicides in *P. viticola* (Chen et al. 2007). Pyraclostrobin, from the strobilurin chemical family in the QoI group, is registered for use against grapevine downy mildew as Cabrio (pyraclostrobin; Nufarm Australia Ltd) and has had widespread use in Australian vineyards since its introduction in 2003. QoI fungicides are used for preventative control and they actively stop energy production within mitochondria, therefore stopping any cellular activity within the fungal cells and spores from germinating. As these fungicides have been used for the past 17 years, it was expected that some isolates would display reduced sensitivity to this fungicide, particularly where downy mildew is an issue from season to season as in regions with high humidity. Several studies found that *P. viticola* isolates were considered resistant when growing on pyraclostrobin at 3 µg/mL (Giraud et al. 2013) and 390 µg/mL (Gauthier and Amsden, 2014). Baseline sensitivities of *P. viticola* of up to 0.94 µg/mL pyraclostrobin were reported by Wong and Wilcox (2000). In the previous project (SAR 1204; Hall et al. 2017), 58% of isolates collected from the Hunter Valley, NSW and 50% of the isolates from the Tamar Valley, Tasmania were still viable at the highest test concentration (0.1 µg/mL), as well as the isolates from Margaret River, Western Australia and one from Wagga Wagga, NSW (Hall et al. 2017).

3.3.2. Group 4, Phenylamide (PA)

The most common fungicide from this group is metalaxyl-M (mefenoxam) which has been available for use in grapevines in Europe since 1977, in the US since 1993 and more recently in Australia since 2005 (Wicks et al. 2005). Metalaxyl-resistant *P. viticola* isolates were detected as early as 1983 in France (Colcol and Baudoin, 2016). Metalaxyl-M is effective against plant pathogenic oomycetes such as *P. viticola* but not against true fungi such as the powdery mildews. Metalaxyl-M inhibits the ribosomal RNA polymerase of the target pathogen, and is categorised as high risk of developing resistance by the Fungicide Resistance Action

Committee (FRAC 2020). In the previous project (SAR 1204; Hall et al. 2017), six of the 10 samples tested from Hunter Valley, NSW were resistant to metalaxyl, with EC_{50} values of 10 µg/mL or greater. The mechanism of resistance to metalaxyl is unknown.

3.3.3. Group 45, Quinone outside inhibitor stigmatellin binding type (QoSI)

Ametoctradin is the only fungicide belonging to the QoSI group (Nanni et al. 2016). Target-site resistance to ametoctradin was first detected in 2015 in France and is likely caused by a single point mutation in the cytochrome b gene, leading to the S34L substitution (Fontaine et al. 2019). The authors described an amino acid substitution on the Qi site of cytochrome b, namely S34L, which only affects sensitivity to ametoctradin. They developed a molecular biological tool to enable detection of the mutant allele in both sporangia and downy mildew lesions. A recent study using controlled inoculation in a field assay showed that a high proportion of alternative oxidase (AOX)-related resistance strains can substantially affect the efficacy of ametoctradin (Fontaine et al. 2019).

3.3.4. Group 40, Carboxylic acidamides (CAAs)

The CAA fungicide group includes dimethomorph, iprovalicarb, benthiavalicarb, valifenalate and mandipropamid. Dimethomorph (Acrobat) first registered for use against grapevine downy mildew in Australia in 2001 and mandipropamid (Revus) is a new mandelic acid amide fungicide, introduced in 2005, expressing high activity against foliar infecting oomycetes, including *P. viticola*. All the members of the CAA class are considered cross-resistant and are classified by FRAC as medium-risk fungicides for the development of resistance (Toffolatti et al. 2018, FRAC info 2020). Dimethomorph was the first CAA fungicide used to control the downy mildews (Gisi et al. 2007). Currently, *P. viticola* strains resistant to CAAs have been detected in Japan, India and European countries including Italy and France (Toffolatti et al. 2018). Single amino acid exchanges in CesA3 protein conferring mandipropamid resistance in *P. viticola* were identified. The G1105S mutation leading to an amino acid change from glycine to serine at codon 1105 was detected in 2010 (Blum et al. 2010) and the G1105V mutation leading to an amino acid change from glycine to valine at the same codon 1105 was detected in *P. viticola* (Sierotzki et al. 2011).

Table 3.2. The activity group table for fungicides used to control downy mildew in Australia (Essling and Lord, 2022).

FRAC code	Group Name	Active constituent	Some registered products
M1	Multi-site activity inorganic (electrophiles)	ammonium acetate	Cop-IT
		ammonium complex	Copperguard, Liquico
		cuprous oxide	Nordox 750 WG, Red Copper WG
		hydroxide	Blue Shield DF, Champ (500WG, Dry Prill WG), Copper Hydroxide 400 WG, Flo-Bordo, Flowcop 500WG, Hydrocop WG, Kocide (Blue Xtra, Opti), Vitra 400 WG
		octanoate	Tricop
		oxychloride	Cobox 500 WP, Copper (Oxychloride, Oxychloride WP), Coppox (WG, WP), Cupro 375WG, EcoCopper 375WG, Isacop 500WP, Neoram 375 WG, Oxydul DF
		oxychloride + hydroxide	Airone WG
		sulfate tribasic	Bordeaux WG, Cuprofix Disperss, Tri-Base Blue, Tribasic Flowable, Tribasic Liquid
M3	Multi-site activity dithiocarbamate and relatives (electrophiles)	metiram	Fruitcote, Polyram DF
		zineb	Zineb
		mancozeb	Dithane Rainshield Neo Tec, Fortuna Globe 750WG, Greenshield 750WG, Kencozeb 750DF, Manco 750 WG, Mancozeb (750 DF, 750 WG, 800 WP, DF), Manic WG, Mantra 750WG, Manzate (750 WG, DF), Manzeb, Penncozeb 750DF, Sinozeb 750 WG, Unizeb (420 SC, Disperss 750 DF
M5	Multi-site activity chloronitriles (phthalonitriles) (unspecified mechanism)	chlorothalonil	Applonil 720, Barrack (720, Betterstick), Barrow (900WG, Stick 720SC), Bravo Weather Stik, Caster (720SC,900 WG), Cavalry (Dry, Weatherguard), Cheers 720 Weathershield, Chlornil 720 SC, Chloro (720, 900WG), Chloronil Pro, Chlorostar 900 WG, Chlorothalonil (720, 720 SC, 900 WG), Chlortan 720, Clash (Dry 900 WG, Storm Guard 720 SC), Conan (720, Sticks 720SC), Echo (500SC, 720, 900 WDG), Mueso (720, 900 WG, Stick 720), Whack (720, 900 WG
M1+M3	Multi-site activity inorganic (electrophiles) + Multi-site activity dithiocarbamate and relatives (electrophiles)	Copper sulfate tribasic + mancozeb	Copman DF
4	PA – fungicides (phenylamides)	Metalaxyl	*Ridomil Gold 480 SL
11	Quinone outside Inhibitors (QoI)	azoxystrobin	Affix 250SC, Agristar 250SC, Amistar 250SC, A-star 250SC, Avior (250 SC, 800 WG), Azoxy 250, AzoxyGuard 250 SC, Azoxys 250 SC, Azoxystrobin (250, 250 SC, 500WG), Connect 800 WG, Galoxy 250SC, Mirador (250 SC,625), Spartacus (250, 250 SC, 500WG), Stellar
		trifloxystrobin	(suppression only) Flint 500 WG, Invictus 500 WG

FRAC code	Group Name	Active constituent	Some registered products
		pyraclostrobin	Cabretta 250EC, *Cabrio, Pavo 250 EC, Roadster 500 EC, Symbio 250 EC, Vipyr 250 EC
3+11	DMI+QoI	tebuconazole + azoxystrobin	Aztec, Custodia (Forte)
40	CAA - fungicides (carboxylic acid amides)	dimethomorph	*Acrobat SC, Dimethomorph 500 SC, Downright, Meta Morph 500SC, Sphinx
		mandipropamid	*Revus
40+45	CAA + QoSI - fungicides (quinone outside inhibitor, stigmatellin binding type)	dimethomorph +*ametoctradin	Zampro
4+M3	PA – fungicides (phenylamides) + Multi-site activity dithiocarbamate and relatives (electrophiles)	Metalaxyl + mancozeb	Axiom MZ WG, Ridomil Gold MZ WG, Axiom MZ 720, Maxyl, Metal-Man MZ 720, Metman 720 WG, Zeemil (720WG, MZB 720 WP
		oxadixyl + propineb	Rebound W
21+M1	QiI - fungicides (quinone inside inhibitors) + Multi-site activity inorganic (electrophiles)	misulbrom + tribasic copper sulfate	Amicus Blue WG

*fungicides tested in this project

4. Project aims and performance targets

Determine the sensitivity of *E. necator, B. cinerea* and *P. viticola* to selected fungicides

Evaluate isolates of *Erysiphe necator*, *Botrytis cinerea* and *Plasmopara viticola* for sensitivity to a range of fungicides, to provide information about the incidence and severity of fungicide resistance in Australian vineyards.

Increase knowledge about the relationships between phenotype, genotype and field efficacy

Improve understanding of how phenotypes relate to genotype results and how field efficacy can be determined. Understand the relationship between laboratory results and field efficacy of fungicides. Investigate the fitness of a mutant genotype compared to a wild type.

Evaluate spore trapping technologies

Determine which, if any, spore trapping technologies are useful for the practical detection and quantification of fungicide resistance in *E. necator* and *B. cinerea*.

Examine the efficacy of Demethylation Inhibitor (DMI) fungicides when exposed to populations of *E. necator* with decreased sensitivity

Determine the relative efficacy of DMIs when applied to populations of *E. necator.* The efficacy of the DMIs will be evaluated on sensitive strains of *E. necator* and strains containing the Y136F mutation, associated with decreases sensitivity to the DMIs.

The effect of sulfur on the efficacy of demethylation inhibitor (DMI) fungicides on powdery mildew (an extra aim for the project added as requested by the Industry Reference Group)

Investigate the effect of mixing DMIs with sulfur, using two types of *E. necator* populations (wild type and mutant), on the control of powdery mildew.

Evaluate in-field methods to detect resistance in *B. cinerea*

Assess a range of methods to detect and/or quantify resistance in *B. cinerea* in the field to work towards a quick and reliable in-field test for resistance.

5. Sensitivity of *E. necator, B. cinerea* and *P. viticola* to selected fungicides

5.1. *Erysiphe necator*

Erysiphe necator is an obligate host-specific biotroph and that causes grapevine powdery mildew disease. Chemical control is the most effective measure to protect grapes from the disease. However, resistance to single-site fungicides is one of the main issues in powdery mildew management (FRAC pathogen risk list, www.frac.info; (Maddalena et al. 2020). Several international reports indicate resistance to different fungicide mode of action groups as outlined in Section 3.1. Improved management of powdery mildew in vineyards, including understanding and the management of fungicide resistance is critical to the sustainability of the Australian wine industry. The aims of this study were to:

1. Collect isolates of *E. necator* from Australian wine regions.

2. Determine the sensitivity distributions to a range of fungicides.

3. Investigate the relationship between phenotype and genotype.

5.1.1. Material and methods

5.1.1.1. Sample collection

Infected plant material was either collected by collaborators and sent to the laboratory or collected directly by project team members. Sampling was not targeted to particular regions or vineyards, but obtained from viticulturists and growers who responded to calls for samples and were interested in contributing to the project. The samples came from a variety of vineyards, ranging from those with poor field performance of fungicides from unknown causes, suspected resistance, hot spots of disease, organically managed vineyards and home gardens. Six isolates, all from the Adelaide Plains, were obtained from vines in home gardens, not previously exposed to any fungicides and will be referred to in this report as isolates from the 'unexposed reference group' (Table 5.4). Sample collections were generally targeted to problematic vineyards and therefore results obtained from this study may be skewed and should not be interpreted as being representative of a region.

5.1.1.2. Maintenance of potted vines

Grapevines, *Vitis vinifera,* cv. Cabernet Sauvignon, were grown in pots in a controlled environment room (Figure 5.1) to provide a year-round source of young, disease-free leaves. Plants were generated from rooted cuttings obtained from dormant vines and were grown in potting media comprising 50% Bio Grow® (Van Scheiks, Mount Gambier SA) and 25% UC mix and 25% cocopeat. To maintain vigorous vine growth, plants were pruned and fertilised regularly using slow release Osmocote® (Evergreen Garden Care, Australia), applied four times per year, and a liquid formulation of Nitrosol® (Amgrow Pty Ltd) or Manutec foliage feeder (Manutec, Adelaide, Australia), applied once a week, and grown at 25ºC with a 16 h photoperiod. To prevent the growth of powdery mildew and to control insect pests, plants were sprayed weekly with a mixture of Eco-Fungicide at 4 g/L (potassium bicarbonate 940 g/kg ai, Organic Crop Protectants, Australia) and Eco-oil at 2.5 ml/L (emulsifiable botanical oils 850 g/L ai, Organic Crop Protectants, Australia) or with a monthly spray of Microthiol Disperss (800 g/kg sulfur, Nurfarm). To control black fly and fungus gnat, the biological control predators, *Stratiolaeleps scimitus* and *Neoseiulus cucumeris* mites obtained from Biological Services, Loxton, SA, were applied to the potting media when required as well as yellow sticky traps were placed to capture insects.



Figure 5.1. Controlled Environment Room (CER) at the SARDI Plant Research Centre, Urrbrae, used to maintain grapevine and supply fresh leaves for experiments.

5.1.1.3. Establishment and maintenance of *E. necator* isolates

To establish *E. necator* cultures, young glossy leaves (from nodes 3-4 from the growing tip) (Figure 5.2 A) were collected and surface disinfested in 0.125% sodium hypochlorite (White King® Bleach) for 3 mins, then washed 3-4 times in sterile distilled water (SDW). Excess moisture was removed by blotting between two layers of sterile paper towel then leaves were air-dried in a laminar flow cabinet. Leaves were placed on top of two sterile wooden toothpicks which were placed on the surface of tap water agar (TWA; Figure 5.2 B) amended with 0.4 µL/mL of pimaricin (2.5% aqueous suspension, Sigma) and benzimadazole (50 µg/L) contained in 100 x 20 mm petri dishes described by (Evans et al., 1996).

Isolates were initially established by transferring sporulating *E. necator* from diseased material to detached, surface disinfested leaves on TWA and grown for 10-14 days at 22ºC, 12 h day/night cycle. Single-spore isolates were then established by transferring a single conidium or a conidial chain (~ up to 10 spores/chain) using a single bristle of a sable hair paintbrush, disinfested in 99% ethanol for 10 mins, or an acupuncture needle (Tianmen 0.22 x 30 mm, San Acupuncture Supplies, Queensland) (Figure 5.2 C) under a dissecting microscope, to fresh detached leaves (Figure 5.2 D and E). One or two subsequent single spore or chain transfers were carried out, then the single spore-derived isolates were maintained by transferring spores on to fresh detached leaves by gently touching the sporulating colony on to the upper surface of a fresh leaf. Sub-culturing was carried out approximately every 2-3 weeks as required. To avoid cross-contamination, individual isolates were kept separately in plastic trays sealed with a clear plastic bag.

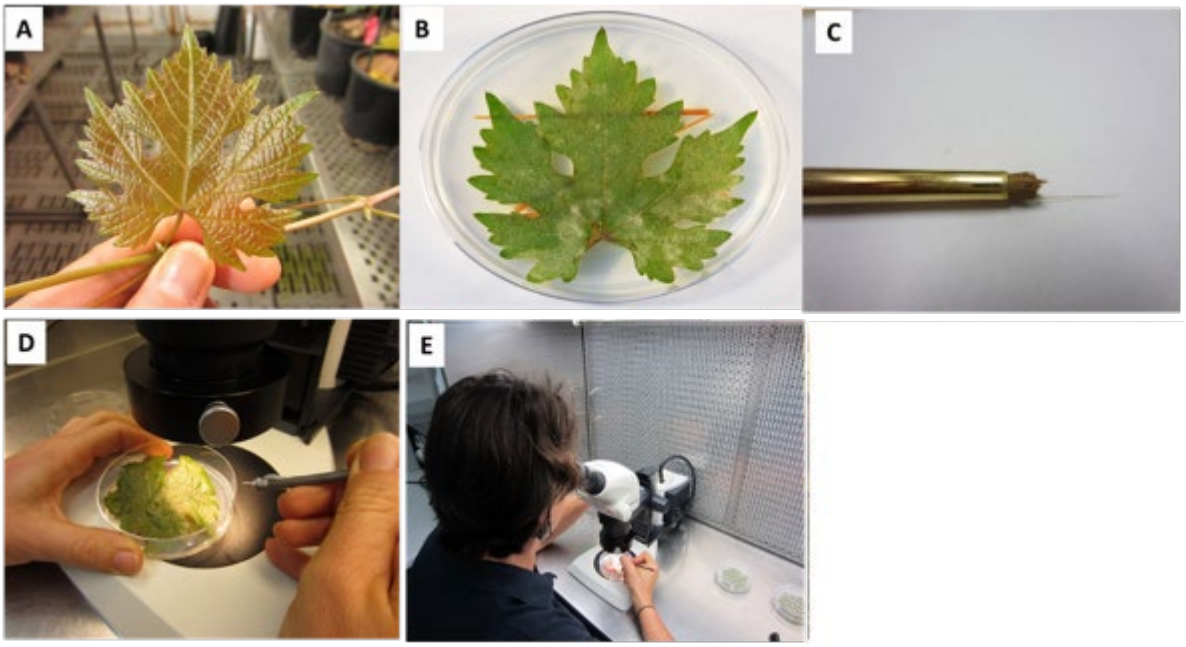


Figure 5.2. (A) Representative fresh leaf used for sub-culturing and testing. (B) Detached grapevine leaf used to maintain *E. necator* in the laboratory. (C) A single bristle of a sable hair paintbrush used to isolate single spore. (D and E) Sub-culturing single-spores under a dissecting microscope in the laminar flow hood.

5.1.1.3.1. Double-petri dish method

Detached leaves growing on TWA senesce after two weeks and contamination by saprophytic fungi e.g. *Penicillium* and *Paecilimyces* spp., is common. To reduce this problem, a trial of a double petri dish method (DPD), a technique developed by W. Mahafee and T. Nield, USDA, Corvallis was tested. The bases of two deep 100 x 20 mm petri dishes were glued together using a hot glue gun. A 5-mm-diameter hole was made through the two glued bases using an electric soldering iron (Figure 5.3 A). Twenty millilitres of SDW was pipetted into the base of the bottom petri dish (Figure 5.3 B). Leaves were prepared as described in section 5.1.1.3. The petiole of a dried, surface disinfested leaf was cut to reveal a fresh surface, then inserted through the hole made in the two petri dishes, to rest in the SDW in the bottom petri dish (Figure 5.3 B). This system prevents the leaf contact with the water and minimises the humidity suitable for saprophyte contamination. This system was used regularly used to clean up contaminated cultures.

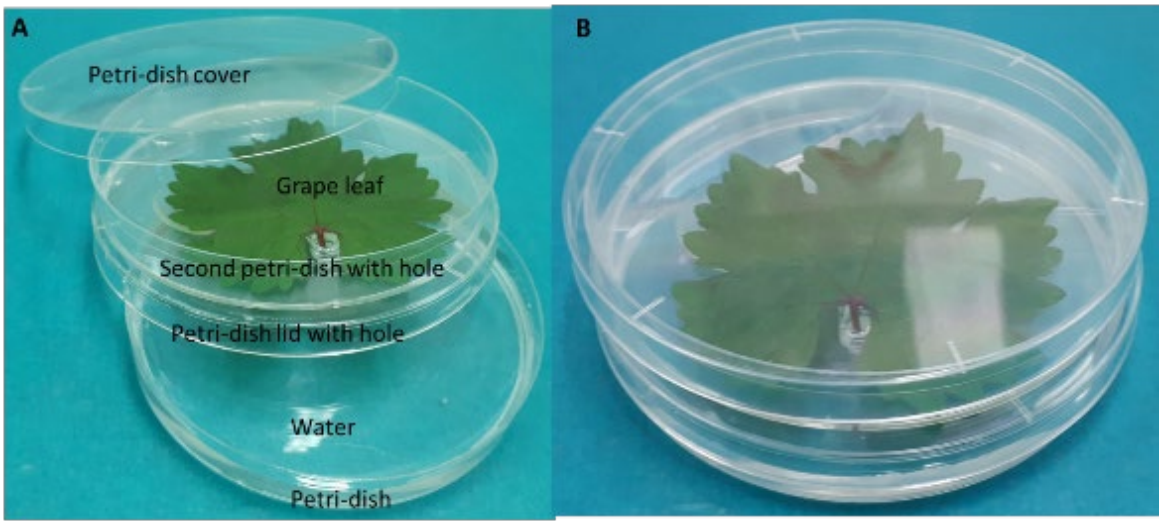


Figure 5.3. Detached leaves growing on water in a double petri dish method (DPD). (A) the parts of DPD system where two petri dishes glued together on top of each other with hole for the grape leaf. (B) assembly of the DPD system ready to be placed in the incubator.

5.1.1.3.2. Self-maintained system for grapevine powdery mildew (SMSGPM)

To reduce the time required for culture maintenance, a prototype self-sub-culturing system was developed; the self-maintained system for grapevine powdery mildew (SMSGPM) (Figure 5.4). The aims of the system were to;

- provide a continuous supply of fresh grape leaves to enable continuous growth of *E. necator*,
- prevent cross contamination among isolates,
- require minimal maintenance
- be cost effective.

The SMSGPM consists of three main components; 1. A foliage growth chamber (3 L cylinder plastic jar with lid, The Plastic Man, Australia), 2. A hydroponic root chamber (500 mL plastic bottle, The Plastic Man, Australia) and 3. An irrigation system to circulate fresh water and nutrients to the root system and for disposal of waste-water (Figure 5.4 B).

The growth camber was modified by cutting two holes in the side of the jar and two holes in the lid using a hot 20 mm cork borer. These were subsequently covered by a 25 µm pore size mesh fabric to allow ventlation and to prevent cross contamination (Figure 5.4 A). The root chamber consisted of a 500 mL plastic bottle that was modified by connecting 9 mm plastic tube at 8 cm from the base of the bottle) through a 9 mm hole to allow water disposal. Rooted grapevine cuttings, cv. Cabernet Sauvignon, ~150 mm length, and irrigation tubes (4-mm diameter) were inserted into their respective holes of a silicone lid (Boon Snug Straw lid, Baby Bunting, Australia) which cover the root chamber. The silicon lid prevents water leaking and provides flexibility for the cane to move and grow without damage. The root chamber was inserted inside the growth chamber and fixed to the bottom of the growth chamber using Velcro tape, and the whole unit was fixed onto plastic tray using Velcro tape (Figure 5.4 A). Growth chambers were connected to the water pump to supply fresh water three times a week and the waste-water was connected to a draining system (Figure 5.4 A and B). Each tray can hold 10 growth chambers, which contain one plant each, trays were connected with each other to supply fresh water and drain the waste-water. The whole system was set up inside and incubator at 22^{o}C, with 12h day/night cycle.

1. 3L plastic jar
2. Lid
3. Ventilation hole
4. 500mL plastic bottle
5. Silicon lid
6. Fresh water tube
7. Grape canes
8. Velcro tape
9. Waste water tube
10. Waste water irrigation pipe
11. Plastic tray



Figure 5.4. (A) Diagram for Self-Maintained System for Grapevine Powdery Mildew (SMSGPM). (B) System inside the laboratory incubator and connected to the irrigation and waste-water system.

5.1.1.3.3. Mini-greenhouse system

The aim of this method was to maintain *E. necator* isolates naturally on whole plants in the greenhouse. Rooted cuttings, cv. Cabernet Sauvignon, were grown in 20 cm diameter pots filled with potting mix as described in section 5.1.1.2. Two tier mini-greenhouse (Naturallife green house, Australia) was modified to improve air flow and maintain the temperature around 20- 25°C (Figure 5.5). Three square holes (20 x 20 cm) were cut in the plastic cover of the middle of both sides and the top of the mini-greenhouse, and subsequently covered with two layers of muslin fabric (pore size?) to prevent cross contamination between isolates and improve air circulation. A layer of black polyethylene sheet underneath a polystyrene board lined the base of each mini-greenhouse to provide stability for the plants in pots. Plants in pots were obtained from a disease-free controlled environment room (Section 5.1.1.2) and placed inside the mini-greenhouses. Plants were inoculated by rubbing infected leaves of an individual culture (from the detached leaf culture collection (section 5.1.1.3) onto the new leaves of potted plants. Visible infection occurred after 4-5 days and infections readily established on new leaves and shoots as they grew over time. There were up to three potted plants contained within each mini-greenhouse for each isolate. The mini-greenhouses were all located inside a greenhouse, maintained between 20 - 26ºC. Watering was carried out by inserting a stainless-steel watering wand under the front opening of the mini-greenhouse to fill the pot saucer with water. Fertilising and pest control were conducted as per (5.1.1.2) except for controlling powdery mildew. Thirty individual isolates were established and maintained using this system.

To test for cross contamination among isolates, PM disease free sentinel plants were placed in a mini greenhouse and assessed regularly for the presence of PM. In addition, spores from selected isolates, with known G143A and Y136F allele frequencies, were collected and sent for NGS (section 5.1.1.7) to determine any change of allele frequencies, indicating possible cross-contamination.



Figure 5.5. Mini greenhouse system for culturing powdery mildew on grapevine. Three ventilation holes on both sides and on top of the tent.

5.1.1.4. Fungicide sensitivity assays

5.1.1.4.1. Leaf disc bioassay

Leaf-disc bioassays, as described by (Erickson and Wilcox, 1997, Evans et al. 1996, Gubler et al. 1994, Wong and Wilcox, 2002) with modifications, were used to test fungicide sensitivity. Young, glossy, disease-free leaves from the 3rd or 4th node were collected from grapevines grown in the CER. The leaves were surface disinfested as described previously and up to five 10 mm diameter discs were cut from each leaf using a sterile cork borer (Figure 5.6 A). In sensitivity tests carried out from 2013 to 2019 (Hall et al. 2017), the discs were randomised and placed adaxial surface downwards in a 15 x 150 mm diameter glass petri dish containing 9 mL of given concentration of fungicide solution. Discs were soaked, with periodic gentle agitation for 30 min, with all fungicides tested except for 60 min soak time for Mycloss™ Xtra (Frenkel, 2014, Wong and Wilcox, 2002). Discs were blotted dry between two pieces of sterile paper towel, then placed adaxial surface up on the surface of TWA in 50 mm diameter petri plates (Figure 5.6 B).

From 2019 to present, the fungicide application method was changed as follows: fungicide suspensions were sprayed onto the adaxial and abaxial surface of each leaf, using a small 100 mL hand sprayer (Figure 5.6 C), until just before run-off. This modification avoided phytotoxicity commonly seen with concentrations of ≥ 20 µg/mL using the soak method. This change allowed for sensitivity testing to be carried out at higher concentrations (Colcol and Baudoin, 2016) that were approximately equivalent to label rates. This procedure was carried out inside a fume containment cabinet. Leaves were allowed to dry in the cabinet, then transferred to a laminar flow cabinet where the leaf discs were cut using a cork borer as previously described. There were up to five discs/plate and two or three replicate plates for each fungicide concentration/isolate combination. Each disc was inoculated in the centre with powdery mildew spores (~300 spores), by touching the end of a sterile cotton tip on to the surface of a 14-day-old sporulating colony of *E. necator* to collect spores on the cotton tip then the spores were deposited on the centre of a disc by touching once with the tip (Figure 5.6 D & E).

In 2020, the inoculation procedure was further modified to tapping an infected the leaf over the leaf discs which improved time efficiency. Inoculated plates from each concentration were kept in a separate plastic tray sealed in a clear plastic bag to prevent any vapour effects (Figure 5.6 F). Fourteen days after inoculation, the percentage of each disc covered in sporulating powdery mildew was assessed under a dissecting microscope at x100 magnification. The mean percentage of growth for each concentration/isolate combination was calculated and the EC_{50} µg/mL was determined for each isolate by Probit analysis using Genstat (18th edn, VSN International Ltd). Fungicide sensitivity was measured as per section (5.1.1.5), where a full description of all statistical analysis methods are described.

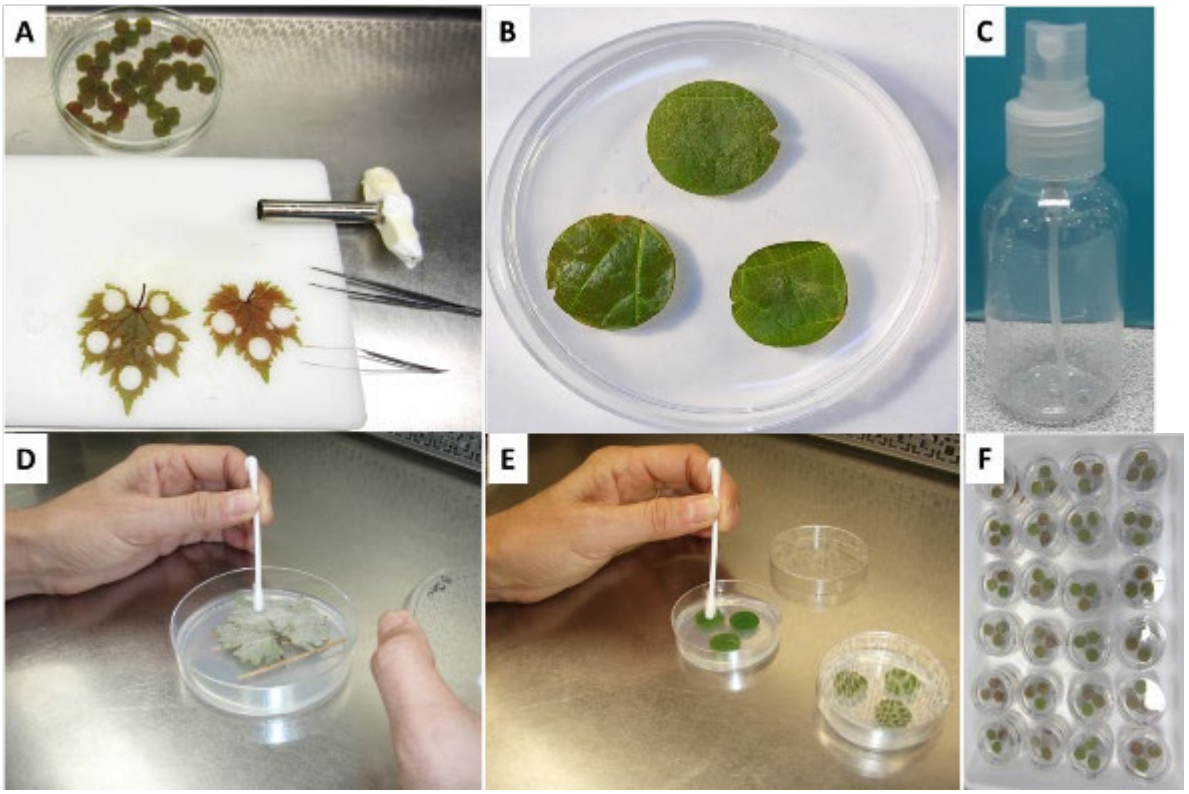


Figure 5.6. Leaf-disc bioassays material; (A) leaf discs cut using cork borer, (B) leaf discs on top of TWA, (C) small 100 mL hand sprayer used to spray fungicide solutions, (D) infected leaf of grape used for inoculation, cotton buds used to transfer the powdery inoculum, (E) leaf discs on top of TWA inoculated with powdery spores, (F) TWA plates contain treated discs inside tray.

5.1.1.4.2. Discriminatory dose bioassay

Discriminatory dose (DD) bioassays were carried out in the same manner as the previously described leaf disc bioassay except a limited number of concentrations were used, commonly 1.0 and 10.0 µg/mL, as well as a no fungicide control. The advantages of carrying out a DD bioassay are that it is faster than a full bioassay and a greater number of isolates can be tested at once. Although, an indication of resistance can be obtained using a DD bioassay, the limitations are that other measures of resistance i.e. EC_{50}, Minimum Inhibitory Concentration (MIC) and resistance factors (RFs) cannot be determined due to the limited number of concentrations used. DD bioassays are useful for quick results but the results are less useful for understanding whole populations or collections of isolates and for monitoring changes to resistance status in populations.

5.1.1.4.3. Whole leaf bioassay

The whole leaf bioassay is a modification of the leaf disc bioassay, whereby whole leaves instead of leaf discs are used to test sensitivity. Either a full range of concentrations or DD concentrations can be used. In a similar manner to the method to prepare the leaf discs, whole leaves were surface disinfested, blotted dry and either soaked in or sprayed with fungicide suspension then placed on to TWA in a 15 x 90 mm petri dishes (Figure 5.7). Inoculation was carried out using cotton tips as per the leaf disc assay, however, five individual isolates were inoculated on to one leaf at five different areas on each leaf (Figure 5.7). Three replicate leaves per fungicide concentration were used in each bioassay. Colony diameter was measured after 12 to 14 days of growth (mm) to a maximum of 15 mm diameter in controls without fungicide. Colony diameter on fungicide treated leaves was then measured and converted to a percentage of maximal growth on controls to obtain % relative growth. In addition to % relative growth, EC_{50} values, MIC and RFs could be calculated for assays using ≥ 4 concentrations

including the control, whereas reliable EC_{50} values, MIC and RFs cannot be calculated with DD bioassays as previously described.

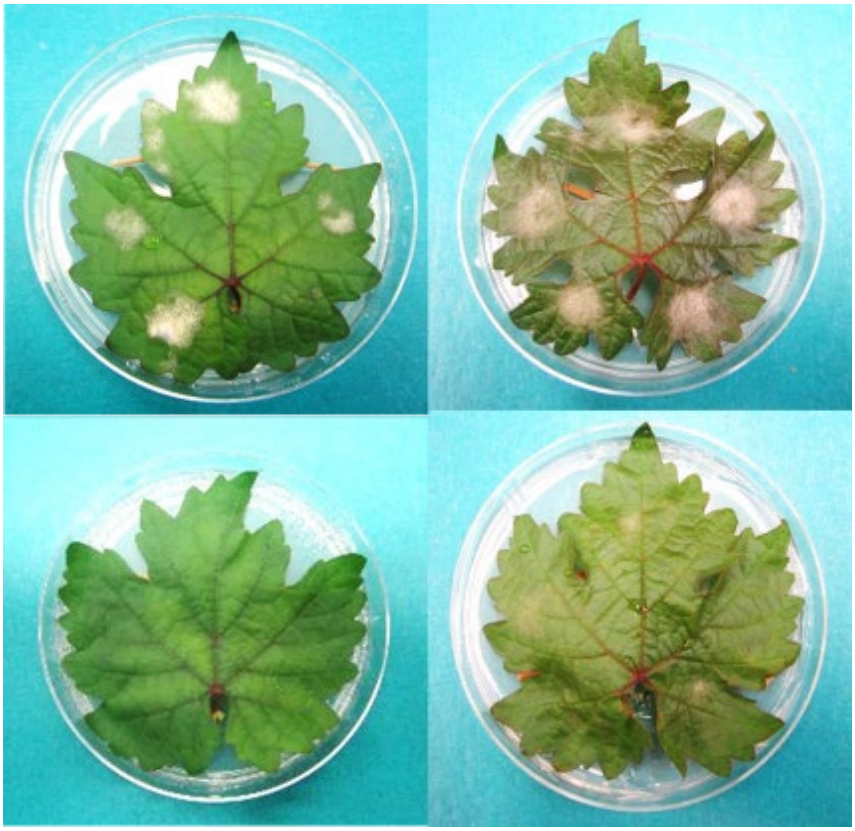


Figure 5.7. whole leaf bioassay, up to five isolates can be tested using same leaf. Each leaf represent one concentration of the fungicide.

5.1.1.4.4. *In-vitro* spore germination assay

An *in-vitro* assay was conducted on 20 isolates of *E. necator* with Cabrio® to determine fungicide sensitivity by measuring spore germination. The assay method was adapted from Hoffman and Wilcox (2003) and Miles et al. (2012) and the laboratory protocols of Walt Mahaffee (USDA, Corvallis, USA pers comm.) and Wayne Wilcox (Cornell University, NY State, USA, pers comm). The isolates chosen contained either 0 or 100% G143A. Each isolate was tested once with eight of these also tested two or three times. Five mg of salicylhydroxamic acid (SHAM) is dissolved in 5 mL of 1:2 volumes of methanol: acetone and stored at -20˚C for future use. SHAM is necessary to block the alternative oxidation pathway (AOX) that the QoI fungicides can use *in-vitro* (Miles et al, 2012, Wood and Hollomon, 2003). A stock suspension of Cabrio at 10,000 µg/mL was prepared in 100% acetone then serially diluted in sterile Milli-Q (SMQ) water to 100, 20, 10, 1.0, 0.1 and 0.01 µg/mL. Cabrio stock suspension was stored at -20ºC for future use. The test agar comprised 5 mL of fungicide suspension added to a bottle of 45 mL of warm 1.5% TWA, to which 50 µL of SHAM stock solution was added. The amended agar was gently mixed and kept at ~60º C in a water bath until required as well as the non-fungicide control agar containing SHAM only. Warm amended TWA (1 mL) was pipetted onto a microscope slide, the surface smoothed with a sterile glass rod and allowed to cool. Once cool, the outer two thirds of the agar was removed by cutting with a sterile scalpel, leaving only the central third of the slide containing amended agar. Three replicate slides were prepared for each fungicide concentration, as well as the control. The microscope slides were placed under a spore settling tower and inoculated with isolates of *E. necator* (Hall et al. 2017; SAR 1204). Slides from each concentration were placed in 90 mm glass petri dishes and incubated at room temperature for 24 h. Using a compound microscope

at x 100 magnification, 50 conidia were observed for germination (Figure 5.8). A conidium was considered germinated if the germ tube was equal in length to at least half the width of the conidium (Miles et al., 2012). The percentage of germination was calculated and EC_{50} µg/mL was then determined by Probit analysis using Genstat.

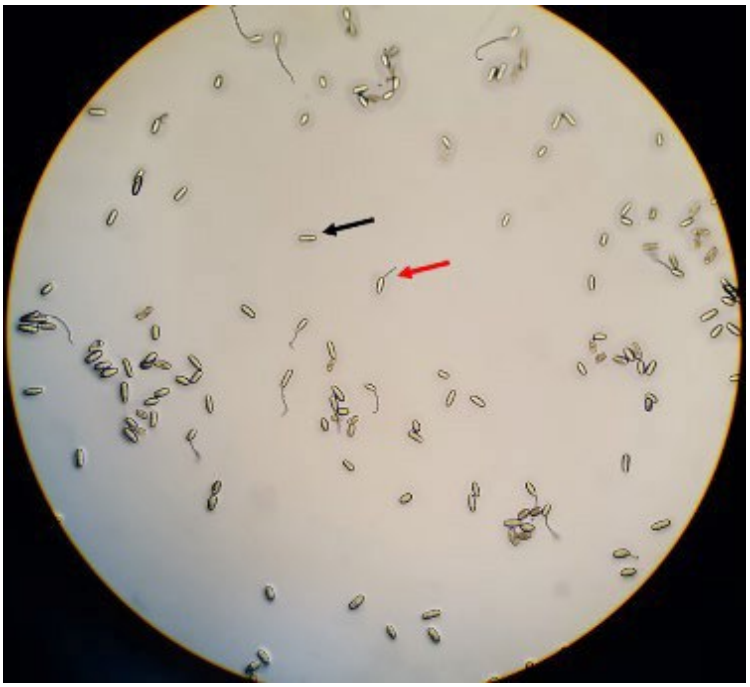


Figure 5.8. *In-vitro* spore germination assay for *E. necator* spores, which were considered germinated if the germ tube was present (red arrow) and not germinated if there was no germ tube (black arrow).

5.1.1.4.5. Comparison of commercial product to technical grade active ingredient

Following discussion with the IRG in 2018, it was decided to determine whether there were differences in fungicide sensitivity results between fungicide commercial products and the corresponding technical grade active ingredient. Due to time constraints, only three products from group 3 were tested. Three isolates of *E. necator,* #215, 216 and 222, with different Y136F genetic profiles (Table 5.1) were tested with both the commercial product and technical grade of penconazole, myclobutanil, and triadimenol (Sigma Aldrich, Australia). The commercial products were prepared as previously described in 5.1.1.4.1. For each active ingredient, 1000 µg/mL stock solutions were prepared in 100% acetone. Serial concentrations, 0.01, 0.1, 1.0, 10.0 µg/mL and label rate of active ingredients, were prepared in SMQ. Commercial grade fungicides were used for all bioassays for fungicide sensitivity as follows; Topas® (penconazole 100 g/L ai, Syngenta), Mycloss® TM Xtra (myclobutanil 200 g/L ai, Dow AgroSciences) and Bayfidan® (triadimenol 250 g/L ai, Bayer). Fungicide suspensions were freshly prepared immediately prior to each assay. Leaf discs were sprayed with each chemical and inoculated with *E. necator* spores as per section 5.1.1.4.1. The mean percentage of growth for each concentration/isolate combination was calculated and the EC_{50} µg/mL was determined for each isolate by Probit analysis using Genstat (18th edn, VSN International Ltd). A full description of all statistical analyses methods are described in Section 5.1.1.5.

Table 5.1. Isolates of *Erysiphe necator* used for testing the sensitivity to both commercial products and technical grade active ingredients for three DMI fungicides

Isolate	Y136F %	Exposure
215	100	likely
216	38	yes
222	4	no

5.1.1.5. Measuring the fungicide sensitivity

There is inconsistency in the literature about how to measure the fungicide sensitivity in *E. necator* populations and how to categorise these populations from sensitive to resistant (Table 5.2). The EC_{50} values in this study were somewhat variable and did not give a sufficiently clear definition of sensitivity as did using growth measures, a similar finding to Colcol et al (2016). Therefore, sensitivity was mainly measured based on the growth, when an isolate grew more than 5% on 1 µg/mL of active ingredient it was considered to have reduced sensitivity, and resistant if it grew at field rate. In addition, EC_{50}, MIC and RF were also considered to evaluate the isolates.

5.1.1.6. Statistical analyses

EC_{50} is defined as the concentration that reduces growth to a value of 50% of that growth in the absence of the fungicide (Russell, 2010). EC_{50} values were calculated using probit analysis as recommended in the FRAC guidelines (KÜNG, 1991) using the statistical software, Genstat 20th edn (www.vsni.co.uk).

The minimum inhibitory concentration (MIC) is the lowest concentration of a fungicide that gives total control (Russell, 2010). MIC values were determined directly from the data.

Growth was assessed by rating the % of sporulating *E. necator* covering each leaf disc at each concentration, then the average growth was calculated.

Resistance factors (RFs) are a measure of the extent of resistance of an isolate compared to a known unexposed population, in that, it is a comparative measure or number useful to detect changes in sensitivity of populations over time. The calculation is as follows:

RF=EC_{50} selected isolate / mean EC_{50} of selected reference isolates (Russell, 2010).

The isolates used as the denominator in the calculation can be a single standard reference isolate (Russell, 2010), or a variety of isolates and terms to describe them e.g. a collection of unexposed isolates (Rallos and Baudoin, 2016), a collection of sensitive isolates (Vielba-Fernández et al. 2020) or a collection of unexposed sensitive isolates (Rallos and Baudoin, 2016). In this research, RFs were calculated using a) the mean EC_{50} of unexposed isolates and b) the mean EC_{50} of the unexposed and sensitive isolates combined as follows:

A. RF=EC_{50} selected isolates/mean EC_{50} unexposed isolates and

B. RF=EC_{50} selected isolates/mean EC_{50} unexposed and sensitive isolates combined

To determine whether there was a significant difference or a shift in EC_{50} values between two populations e.g. sensitive and resistant, unexposed and sensitive, the distributions of the $\log_{10}$ EC_{50} values from both populations were tested for normality using the Shapiro-Wilk test. If the distributions were normal, the significance of any shift was tested using a two-sample t-test. If the two data sets were not normally distributed, data were analysed using the Mann Whitney non-parametric two-sample test (Genstat 18th edn VSN International Ltd).

Cross-resistance between fungicides was analysed using either Pearson's correlation coefficient of $\log_{10}$ EC_{50} values if data were normally distributed or Spearman's rank correlation coefficient if distributions were not normal.

Table 5.2.Summary of fungicide sensitivity assays and the parameters used to determine the sensitivity/resistance of *E. necator* populations.

FRAC Group	Group name	Active/product	Test type	EC_{50} µg/mL sensitive	EC_{50} µg/mL resistant	Parameters	Location	Reference
11	QoI	trifloxystrobin/Flint	spore germination	Unexposed 0.3 Exposed 0.1	Unexposed >1000 Exposed >1000	Spore germination % 50 spores used Appressorium was visible and germ tube longer than the width of the conidia	Michigan/USA	(Miles et al., 2012)
		trifloxystrobin/Flint	spore germination	0.003-0.01	>100	Spore germination % 50 spores used Appressorium was visible and germ tube longer than the width of the conidia	Oregon & California /USA	(Miles et al., 2021)
		kresoxim-methyl	spore germination	0.02-0.05	>100	Spore germination % Appressorium was visible and germ tube longer than the width of the conidia	Oregon & California /USA	(Miles et al., 2021)
		pyraclostrobin/ Cabrio	Leaf discs	≤1.0	≥1.0	EC_{50} % relative growth	Virginia and North Carolina/ USA	(Colcol, 2008, Rallos et al., 2014)
50	aryl-phenyl-ketones	metrafenone	plantlets of *Vitis vinifera*	<12.5	125	Mycelium growth (mm) and sporulation (conidia cm^{-2})	Italy	(Kunova et al., 2016)
3	DMI	myclobutanil	Leaf discs	<1.9	1.9-14.67	EC_{50}		
50	aryl-phenyl-ketones	pyriofenone/ Kusabi	plantlets of *Vitis vinifera*	<12.5	125	Mycelium growth (mm) and sporulation (conidia cm^{-2})	Italy	(Kunova et al., 2016)
5	amines ("morpholines") (SBI: Class II)	spiroxamine	Leaf discs	<0.365	>0.365	EC_{50} % growth disease incidence for incidence analyses	California /USA	(Miller & Gubler, 2004)

The range, mean, standard deviation of the mean, median of the EC_{50} values, MIC, RFs growth % at different concentrations, and G143A and Y136F % were calculated using MS Excel. Ranges for these values were also determined directly from the data.

5.1.1.7. Genotype analyses

A. Sanger sequencing

The Sanger sequencing method was used in SAR 1204 and early SAR 1701 to determine the presence or absence of G143A and Y136F mutants. The full method is described in the final report (Hall et al 2017; SAR 1204). Results from this sequencing, specifically those for G143A, were later found to be unreliable based on problems with the initial primer design carried out in SAR 1204. Further work was carried out to address this and all subsequent results are based on the redesigned primers (see section D below). The outcome is that now the %G143A is a good indicator of sensitivity and matches well to phenotypic data when using % fungal growth as the measure of sensitivity.

B. Pyrosequencing

Prior to the development of methods to detect and quantify mutations associated with resistance in this project, in 2014, a selection of isolates was sent to the laboratories of Dr Gerd Stammler, BASF, Limbergerhof, Germany, where pyrosequencing to determine the % G143A was carried out (Hall et al. 2017). Technical details of the method were based on (Miessner, 2015).

C. qPCR

Dr Walt Mahafees' team at the USDA, Corvalis, Oregon, USA analysed 22 isolates from Australia by qPCR, and of these, 16 were also analysed by NGS (SAR 1204; Hall et al. 2017).

D. Next Generation sequencing (NGS)

DNA was extracted from samples using the Mag-Bind Environmental DNA 96 Kit (Omega Biotek). To detect *E. necator*, an NGS-based fungal profiling methodology was applied (Sternes et al. 2017). This uses a DNA-barcoding strategy based on the fungal internal transcribed spacer (ITS) region to determine the proportions of species present in mixed environmental samples.

For resistance allele testing regions of the *CytB, Cyp51 and SdhB* genes that potentially contained resistance mutations were amplified by polymerase chain reaction (PCR). Proportions of resistant and sensitive alleles were determined from analysis of NGS data (Ramaciotti Centre for Genomics, UNSW Sydney, Australia) produced from the pooled the PCR products.

New primer sequences were established for *E. necator CytB* based on the published genome sequence (Table 5.3).

Table 5.3. PCR primers used in this study.

Primer	Sequence
En_cytB_193_F	TTGTTCTAGAGGCAGCAAAGC
En_cytB_405_R	CCCCGCACCTACTTAAAGC
En_cytB_83F	CGCTACAGACTGGGTCACTG
En_cytB_517R	AGTCTCTTAGGGCCCCCATT
En_sdhB_H242X_3417F	TGCAAAGCTATAGATGGTTAGCAG
En_sdhB_H242X_3615R	CCTACTATTTGACTTCAAGAAAATGC
En_sdhB_H242X_3353F	TCAACGTACATCTCCCTCACC
En_sdhB_H242X_3743R	AATTTCCTACCCCTCCATGC

E. necator sdh primer design

Primers were designed to target the H242R and H242Y mutations in *E. necator SdhB* that provide resistance to SDHI-based fungicides (Boscalid). Gene sequences were obtained from the *E. necator* genome and as well as published sequences from both resistant and sensitive isolates (Cherrad et al. 2018; Jones et al. 2014).

E. Whole genome analysis of *E. necator* isolates

A total of thirteen *E. necator* isolates were subjected to whole-genome sequencing (Illumina Nextera Flex library, NextSeq 2x150bp chemistry; Ramaciotti Centre for Functional Genomics), with sufficient sequencing data ultimately obtained from 12 of these. Raw sequence data were aligned to the existing draft *E. necator* genome (Jones et al. 2014) using bowtie2 (Langmead and Salzberg, 2012). Copy number (CN) of the *Cyp51* locus (contig JNVN01001694.1) was determined from these aligned sequences by calculating the relative coverage of JNVN01001694.1 compared to the median average sequencing depth (10 kb windows, 5kb step) across the entire genome.

5.1.3. Results

5.1.3.1. Isolate collection

One hundred and thirty four samples of diseased leaves and/or berries were obtained from 115 sites, comprising vineyards from many of the major winegrape growing regions of South Australia, New South Wales, Tasmania, Victoria and Western Australia as well as from home gardens in Adelaide during the years 2014 to 2020 (Table 5.4). Identification of sample locations is not provided due to confidentiality. Since the previous project SAR 1204, the successful isolation of *E. necator* from samples has improved, with better methods of sample packaging and collection, and transport implemented (data not shown).

Table 5.4. Powdery mildew samples received during the years 2014 to 2020 from different states and regions of Australia,

State	Region	Number of samples received	Number of sites
South Australia	Adelaide Hills	11	7
	Adelaide Plains	20	9
	Barossa Valley	13	13
	Coonawarra	1	1
	Langhorne Creek	12	10
	McLaren Vale	1	1
	Mount Benson	1	1
	Padthaway	2	2
	Riverland	11	11
New South Wales	Hilltops	4	4
	Hunter Valley	8	8
	Orange	7	7
	Riverina	4	2
Tasmania	East Coast	1	1
	Tamar valley	3	3
Victoria	Bendigo	2	2
	Geelong	1	1
	Goulburn Valley	1	1
	King Valley	5	5
	Mornington Peninsula	4	4
	Strathbogie Ranges	3	3
	Yarra Valley	6	6
Western Australia	Great Southern	3	3
	Margaret River	9	9
	Swan District	1	1
TOTAL		**134**	**115**

5.1.3.2. Culture maintenance

The standard method of culture maintenance of *E. necator* on detached leaves (on TWA +/- toothpicks) was successful and provided a constant supply of fungal material for bioassays and genetic analysis. However, it was time consuming and labour intensive and it was isolate type dependent where some isolates were more difficult to maintain than others and some do not survive regular sub-culturing. Also, as detached leaves senesce, saprophytic fungi such as *Penicillium* and *Paecilomyces* spp. grow and contaminate the cultures, which results in the time-consuming activity of regularly re-creating single-spore derived cultures. Some modifications have been implemented following attendance at the 19th Reinhardsbrunn symposium and the field laboratories of BASF, hosted by Dr Gerd Stammler, in 2018 (WAT 1803; (McKay, 2019)). These modifications included not blotting dry the leaves and elimination of toothpicks used on the TWA, which saved time and reduced the contamination risk by some saprophytic fungi, and wet leaves were simply placed directly on the TWA and air dried in the laminar flow cabinet.

The double petri dish method (DPD) method resulted in less contamination and the time between sub-culturing was extended as most leaves survived longer than on TWA. However, offsetting these benefits, there were several issues; the logistics of moving DPD cultures resulting in water spillage, many cultures did not grow as well on the leaves in the DPDs compared to those on TWA, resulting in a more cultures dying than when using TWA and greater care was needed when handling the leaves to put in to the DPD hole, as it was easy to damage the petiole resulting in rapid leaf death. Therefore, the DPD method was carried out only when cultures were contaminated by saprophytes for the purpose of clean up cultures.

5.1.3.2.1. Self-maintained system for grapevine powdery mildew (SMSGPM)

Results showed that SMSGPM was successful system for self-maintaining the PM on grapevine leaves for up to six months for some isolates with minimal maintenance (Figure 5.9 A). system proved to be easy to handle as every unit (isolate) was removable and securely transferable in the laboratory to collect samples. Although, the system provided optimal condition for PM infection, severe nutrient deficiencies (Figure 5.9 B) were observed after six months resulting in plant death. Several attempts to correct these deficiencies were tested including; watering with 1:500 mL of Seaweed solution (Seaweed Health Tonic Concentrate, Seasol, Australia): water and watering with hydroponic fertiliser (Uni-one 4-3-6 Uber, Australia) 200 µL/mL SDW water, however none of these treatments ameliorated the deficiencies. Although promising, the use of this system ceased, as more time and research is needed to optimise the system.

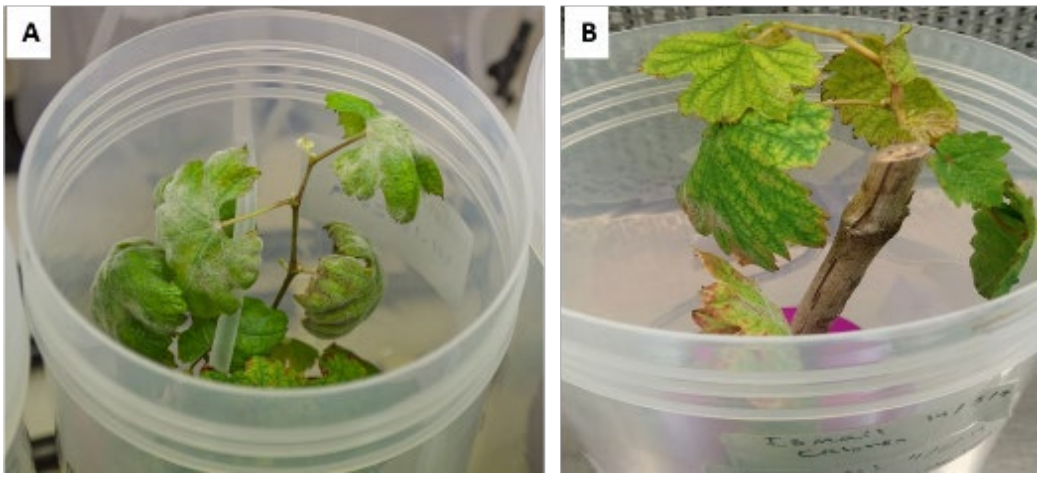


Figure 5.9. (A) Powdery mildew symptoms on a grapevine cutting using the self-maintained system for grapevine powdery mildew and (B) symptoms of nutrient deficiencies after six months growth.

5.1.3.2.2. The mini-greenhouse system

Potted vines have been successfully grown using the mini-greenhouse system for over two years. Powdery mildew was well established (Figure 5.10), with a large supply of fresh inoculum constantly available. The system has been providing optimal conditions for the powdery mildew to grow all year around with minimal maintenance resulting consistent supply for fungal material for NGS analysis and bulk inoculum for other experiment. This system has been used successfully in experiments (sections 6.1.2.2 and 9.2). The NGS analysis revealed no cross-contamination among isolates in short-term sub-culturing (e.g. up to six months) (Figure 6.12), however long term sub-culturing (up to 12 months) using this system resulted in some changes in the frequencies of Y136F and G143A mutants (Table 5.5). Improvements have been made for the system to minimise the cross contamination. Currently, 22 isolates are being maintained using this system and regular check for mutations frequency of Y136F and G143A is ongoing (Table 5.5).

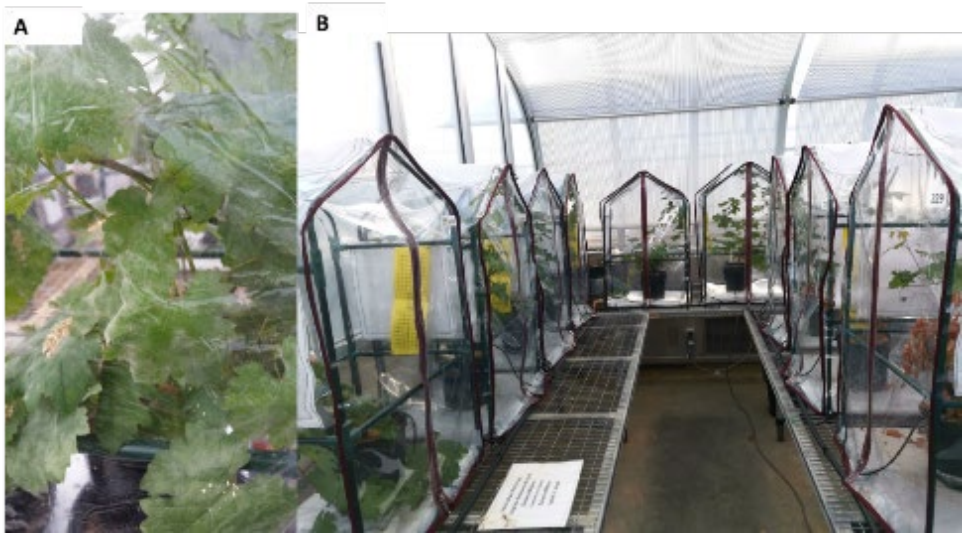


Figure 5.10. Mini-greenhouse system, (A) symptoms of powdery mildew inside the mini-greenhouse, (B) multiple mini-greenhouses inside the large greenhouse.

5.1.3.3. Whole leaf assay

Sixteen isolates were tested with both the whole leaf assay and leaf disc assay. Although, the EC_{50} values were vastly different, the resistance classification was the same for 13 of the isolates for both leaf disc and whole leaf assays.

5.1.3.4. Spore germination assay

The relationship between EC_{50} values calculated from the spore germination assays and % G143A was not clear, but there was 65 % agreement between % growth on leaf disc and spore germination percentage. Hence there was no complete agreement between spore germination percentage and growth rate, spore germination assay was discontinued.

5.1.3.5. Comparison of commercial product to technical grade active ingredients

The EC_{50} values for both the commercial product and active ingredient for isolates #215, 215 and 222 were similar, mostly within the same decimal point/10-fold concentration (Table 5.6). The percentage of Y136F had no effect on the EC_{50} values for the each isolate and there was no obvious relationship between EC_{50} values and Y136F% with neither the commercial product nor the technical grade active ingredient.

Table 5.5. The percentage of frequency of Y136F and G143A mutants before and after sub-culturing *E. necator* isolates in mini-greenhouses.

Isolate	% of Y136F		% of G143A	
	Before mini-greenhouse	After 12 months of subculturing in mini-greenhouse (2022)	Before mini-greenhouse	After 12 months of subculturing in mini-greenhouse (2022)
137	100	100	0	0
143	100	100	0	0
158	100	100	0	0
164	100	100	0	0
170	39.0	66.2	100	100
172	100	100	100*	0*
194	NT	100	**NT	0
195	100	100	0	0
197	35*	90.9*	100*	8.3*
200	100	100	0	0
203	100	100	0	0
206	100	100	0	0
209	100	100	100*	0*
213	72	97.2	100*	1*
215	100	97.2	0	0
216	38*	100*	100*	0*
217	100	100	0*	1.1*
219	100	100	0	0
222	4*	73.1*	0*	16*
224.1	100	100	0	0
228	100	100	0	0
230	100	100	100*	0*

* considerable changes in the % of mutation during the sub-culturing in mini-greenhouses.** not tested

Table 5.6. The EC_{50} µg/mL of three isolates of *Erysiphe necator* when tested with the commercial product and the technical grade active ingredient (ai) for three Demethylation Inhibitor fungicides in a leaf disc bioassay.

Isolate	%Y136F	EC_{50} µg/mL					
		Bayfidan	triadimenol ai	Mycloss	myclobutanil ai	Topas	penconazole ai
215	100	0.2	0.1	0.4	0.3	0.3	0.2
216	64	0.01	0.02	0.05	0.2	0.2	NT
222	0	0.1	0.09	0.3	0.03	0.4	0.2

5.1.3.6. Quinone outside Inhibitors (QoIs)

Reliable phenotype data was obtained for a total of 125 isolates out of 153 tested. Genotype results were available for %G143A, using NGS (24 isolates; Section 5.1.1.7) and pyrosequencing (22 isolates; SAR1204)

Forty-five isolates were classified as phenotypically sensitive and 80 with reduced sensitivity to pyraclostrobin. For the sensitive isolates (excluding and including the group previously unexposed to fungicides), no growth was observed at ≥1.0 ug/mL and MIC values were all 0.1 µg/mL (Table 5.7). The populations of both the 'unexposed reference group' and sensitive isolates were normally distributed about the mean, as determined by a Shapiro-Wilk test for normality. A two-sample t-test showed that the mean EC_{50} values for each group were not significantly different from one-another (p=0.1). Therefore, the data from these two groups can be combined to make an overall sensitive group (Table 5.7; Table 5.8).

Table 5.7 Analysis of phenotyping and genotyping of sensitive isolates (excluding and including the group previously unexposed to fungicides) of *Erysiphe necator*, phenotyping testing conducted using pyraclostrobin (Cabrio).

Unexposed group	Statistical parameters	EC_{50} µg/mL	Mean % growth on 1.0 µg/mL	MIC µg/mL	G143A% NGS n=6	G143A% pyrosequencing n=7
Excluding n=39	Mean (sd)	0.02 (0.2)	0	0.1 (0)	5 (11)	7 (16)
	Median	0.009	0	0.1	0	0
	Range	0.001–0.01	0	0.1	0–30	0–47
Including n=45	Mean (sd)	0.02 (0.02)	0	0.1 (0)	3 (9)	5 (15)
	Median	0.007	0	0.1	0	0
	Range	0.001–0.01	0	0.1	0–30	0–47

Table 5.8. Classification of fungicide sensitivity based on growth of *Erysiphe necator* in a leaf-disc bioassay.

Classification	level	Culture growth
Sensitive		no growth on ≥ 1.0 µg/mL
Reduced sensitivity	low	growth on ≥ 1.0 µg/mL with no growth at ≥ 10.0 µg/mL
	high	growth on ≥ 10.0 µg/mL
Resistance		Growth on field rate

Based on growth at 1.0 and 10.0 µg/mL, the 80 isolates with reduced sensitivity were further classified as low or high. Thirty-six isolates that grew on 1.0 µg/mL and not at the higher concentrations were classified as low reduced sensitivity (Table 5.9; Table 5.8). The G143A frequency for this group ranged from 0 to 100% with a median of 90%. Forty-four isolates classified as high reduced sensitivity grew at 10 and/or 20 µg/mL and all contained 100% G143A using NGS. The MIC values were ≥ 10 µg/mL for all resistant isolates.

Table 5.9. Analysis of phenotyping and genotyping of isolates with reduced sensitivity of *Erysiphe necator*, phenotyping testing conducted using pyraclostrobin (Cabrio). Standard deviation shown in parentheses.

Reduced sensitivity level	Statistical parameters	EC_{50} µg/mL	Mean % growth at µg/mL			MIC µg/mL	G143A% NGS n=6	G143A% pyrosequencing n=7
			1	10	20			
All (n=80)	Mean (sd)	1.8 (2.7)	47 (25)	12 (14)	7 (10)	15 (5)	70 (43)	91 (23)
	Median	0.8	47	10	3	10	100	99
	Range	0.03–14.4	4–85	0–73	0–40	10–≥20	0–100	0–100
Low (n=36)	Mean (sd)	1.6 (4.1)	31 (23)	0	0	10 (0)	56 (45)	49 (49)
	Median	0.05	30	0	0	10	90	49
	Range	0.005–14.4	1–85	0	0	10	0–100	0–99
High (n=44)	Mean (sd)	1.8 (2.2)	56 (23)	15 (14)	9 (9)	18 (4)	100 (0)	96 (12)
	Median	0.9	59	12	5	20	100	99
	Range	0.06–10	9–85	1–73	2–40	10–≥20	100	47–100

RFs were calculated using two different denominator values; 1. the mean EC_{50} value for the 'unexposed reference group' i.e. 0.004 µg/mL and 2. the mean of the overall sensitive population ('unexposed reference group' and sensitive isolates combined) i.e. 0.02 µg/mL. When using the first value of 0.004 µg/mL, the RF values were considerably higher i.e. mean (sd) 448 (687), median 200 and range 0-3600 than when using the second value of 0.02 µg/mL i.e. mean (sd) 91 (137), median 40 and range 2-720 (Table 5.10).

Table 5.10. The resistance factors (RF) of isolates of *E. necator* resistant to Cabrio (pyraclostrobin), calculated using EC_{50} values. Standard deviation shown in parentheses.

Source of isolates	EC_{50} µg/mL	Mean RF (sd)	Median RF	Range RF
unexposed reference isolates	0.004	458 (687)	200	0 to 3600
Combination unexposed & sensitive isolates	0.02	91 (137)	40	0 to 720

The frequency distribution of the log_{10} median EC_{50} values of isolates tested with Cabrio was continuous but bimodal (Figure 5.11), ranging from log_{10} values of -3.5 to 1.0. The Shapiro-Wilk test revealed that the frequency distribution of the isolates with reduced sensitivity was not normal (p=0.04) so the nonparametric Mann Whitney test was used to determine whether the median EC_{50} values of the sensitive and reduced sensitivity group differed. There was a highly significant difference (P<0.001) between the median EC_{50} values for the overall sensitive subgroup (0.007 µg/mL) and the reduced sensitivity group (0.8 µg/mL).

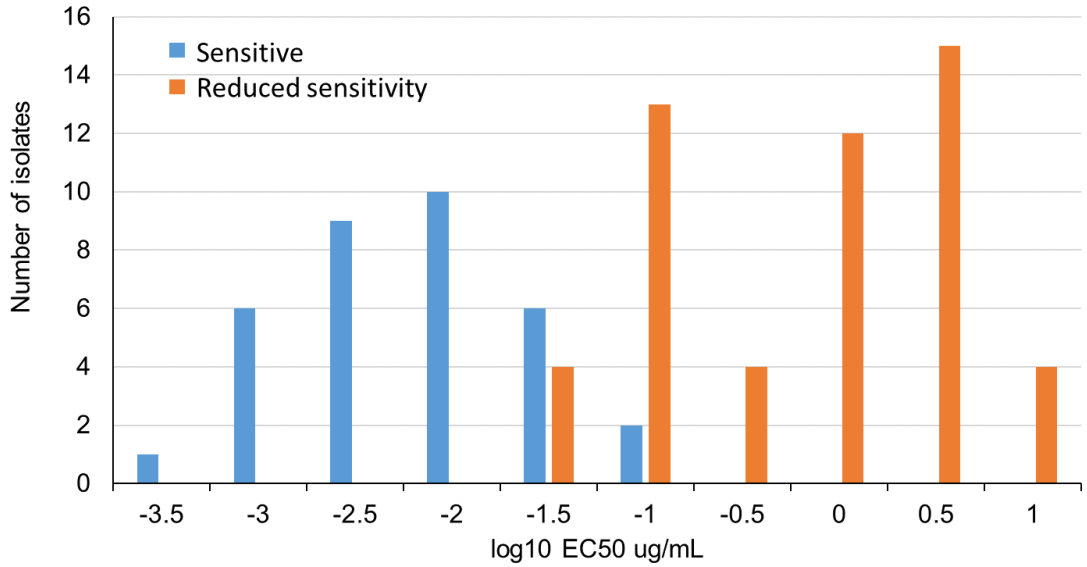


Figure 5.11. The frequency distributions of the log10 EC_{50} µg/mL values for the sensitive and reduced sensitivity populations of *E. necator* tested for sensitivity to pyraclostrobin.

One hundred and fifty-three single spore derived isolates were prepared from powdery mildew infected samples obtained from the majority of viticultural regions from each state growing wine grapes, except for Queensland. Of these, 125 isolates were tested and 62% were classified as resistant (Table 5.11) and (Figure 5.12).

Table 5.11. Powdery mildew samples obtained from the majority of viticultural regions from each state growing wine grapes, *E. necator* tested for sensitivity to pyraclostrobin (Cabrio)

State	Viticultural region	Resistance category (number of isolates)		Total tested	Total obtained
		Sensitive	reduced sensitivity		
South Australia	Adelaide Hills	6	8	15	21
	Adelaide Plains	11	5	16	24
	Barossa	4	9	14	15
	Coonawarra	1	0	1	1
	Langhorne Creek	0	7	9**	12
	Mount Benson/Limestone Coast	1	0	1	1
	Padthaway	0	2	2	2
	Riverland	7	7	14	12
Victoria	Bendigo	0	0	1*	4
	Geelong	0	1	1	1
	Goulburn Valley	0	1	1	1
	King Valley	1	4	5	5
	Mornington Peninsula	2	2	4	4
	Strathbogie Ranges	0	3	3	3
	Yarra Valley	0	5	5	5
Tasmania	East Coast/Bream Creek	0	1	1	1
	Tamar Valley	0	3	3	3
New South Wales	Hilltops	1	2	3	4
	Hunter	1	2	3	8
	Orange	1	6	7	7
	Riverina	3	1	4	4
Western Australia	Great Southern	1	3	4	3
	Margaret River	4	5	9	11
	Swan District	1	0	1	1
Total		**45**	**77**	**125**	**153**

*Excluded from analyses ** two isolates were excluded from analyses

5.1.3.6.1. Spore germination assays – Cabrio (pyraclostrobin)

The percentage of germination of each replicate within each concentration was similar with little inter-replicate variation. Although, not statistically compared, there appears to be considerably less variability among replicates with this assay than for replicates in leaf-disc assays (data not shown). The EC_{50} values for the spore germination assays were; mean (sd) 0.2 (0.5), median 0.01 and range 0.005 to 1.8 (Table 5.12), which were similar to those EC_{50} values for the same 19 isolates tested in leaf-disc assays, *viz*. mean (sd) 0.3 (0.6), median 0.03 and range 0.001 to 2.3. Although results of these statistical tests were similar for both assay methods, regression analysis revealed a poor correlation (R^2=0.06) between EC_{50} values for individual isolates tested with both assays (Figure 5.13).

Table 5.12. The mean (sd), median and range of EC_{50} values for 19 isolates of *Erysiphe necator* tested for sensitivity to pyraclostrobin (Cabrio) using in-vitro spore germination and leaf-disc bioassays.

Statistical parameters	Spore germination	Leaf-disc bioassay
mean (sd)	0.2 (0.5)	0.3 (0.6)
median	0.01	0.03
range	0.005 to 1.8	0.001 to 2.3

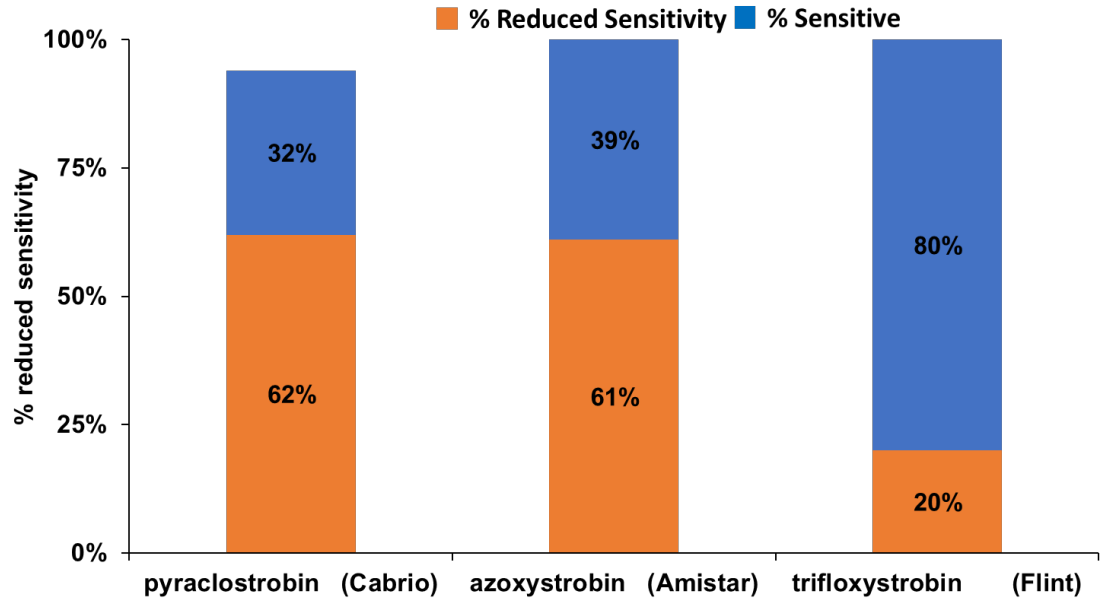


Figure 5.12. Summary of sensitivity of *Erysiphe necator* isolates to QoIs, isolate growing more than 5% on 1 µg/mL of active ingredient considered with reduced sensitivity.

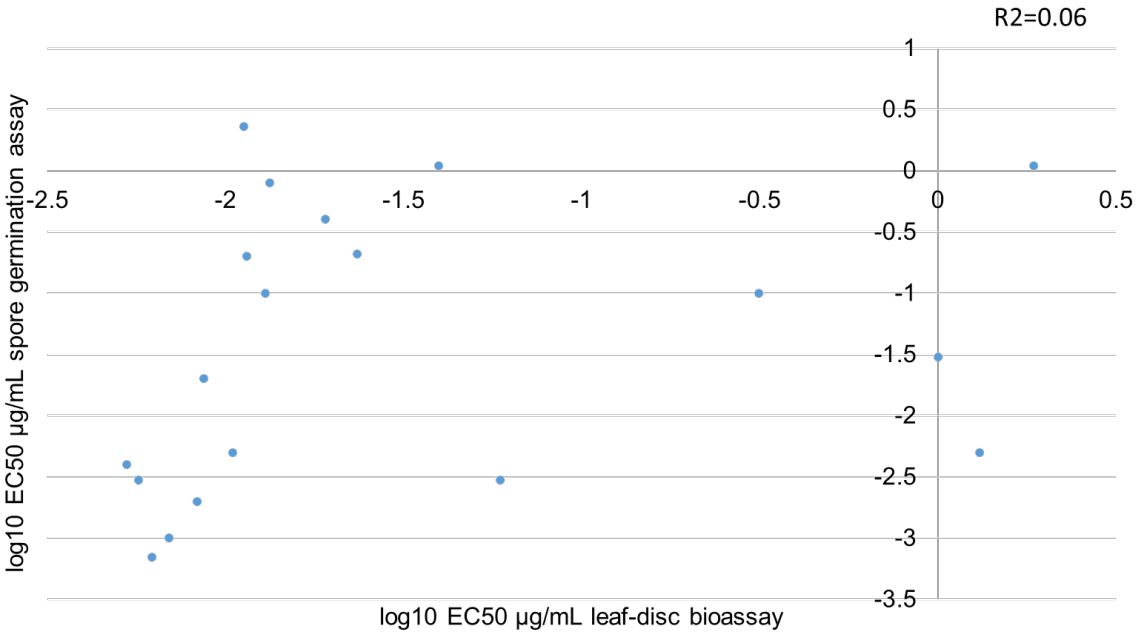


Figure 5.13. The log10 EC_{50} µg/mL values for 19 isolates of *Erysiphe necator* tested for sensitivity with pyraclostrobin (Cabrio) using *in vitro* spore germination and leaf-disc bioassays.

5.1.3.6.2. Genotype analyses

Following the development of accurate and reliable primers to detect and quantify G143A, 39 isolates that were previously phenotyped in SAR 1204 for sensitivity to pyraclostrobin were analysed. The results show that the G143A frequency is positively associated with the mean EC_{50} value, and the mean growth at both 1.0, 10.0 and 20.0 µg/mL (Table 5.13). There was a good relationship between the G143A frequency and the sensitive and reduced sensitivity isolates. For the isolates classified in the low reduced sensitivity category, the relationship is less clear, with some isolates containing high frequency and some low frequency of G143A. However, with a larger set of data, it is envisaged that the relationships between the variables would be clearer.

Table 5.13. Comparison of the mean EC_{50} (sd) growth at 1.0, 10.0 and 20.0 µg/mL, MIC and G143A frequency obtained by next generation sequencing (NGS) and pyrosequencing, for 39 isolates of *Erysiphe necator* classified as sensitive and reduced sensitivity to Cabrio (pyraclostrobin).

Classification	Mean EC_{50} µg/mL (sd)	Mean % growth* at µg/mL			MIC	G143A NGS (%)	G143A pyro (%)
		1.0	10.0	20.0			
Sensitive	0.02 (0.02)	0	0	0	0.1 (0)	3 (9)	5 (15)
Reduced sensitivity	1.8 (2.7)	47 (25)	12 (14)	7 (10)	15 (5)	70 (73)	91 (23)

* percentage of leaf discs covered with sporulating *E. necator*

Dr Walt Mahafees' team at the USDA, Corvalis, Oregon, USA analysed 22 isolates by qPCR, and of these, 16 were also analysed by NGS. Two isolates were 'mixed' (contained both G143 and G143A with unspecified percentages) by qPCR, which equated to 10% G143A frequency by NGS. Five isolates had no G143A from both NGS and qPCR. The remaining nine isolates were 100% G143A from both NGS and qPCR. These qPCR results show that NGS data were robust and reliable.

5.1.3.6.3. Amistar (azoxystrobin)

Thirteen isolates were phenotyped for sensitivity using Amistar (azoxystrobin) at 0, 0.001, 0.01, 0.1, 1.0, 10.0 and 20.0 µg/mL. Fungicide was applied using the soaking method (5.1.1.4.1). Phytotoxicity was sometimes observed at 10.0 µg/mL and often at 20 µg/mL, therefore data for 20 µg/mL were not included in analyses. Five isolates were classified as sensitive (39%), with EC_{50} values ≤0.07 µg/mL, MIC values ≤0.01 µg/mL and no growth at 1.0 and 10.0 µg/mL, while eight isolates had reduced sensitivity (61%) (Table 5.14) and (Figure 5.12). G143A frequency (using NGS) for the three sensitive isolates, *viz.* from the Riverina, Riverland and Adelaide Hills, was 0, 0 and 47 % respectively. Eight isolates were classified as reduced sensitivity, with EC_{50} values 0.9 to >10.0 µg/mL and growth at both 1.0 and 10.0 µg/mL (Table 5.14). The MIC for all eight resistant isolates was ≥10.0 µg/mL. The eight phenotypic resistant isolates were from the Barossa, Langhorne Creek, and the Adelaide Hills in SA, Margaret River in WA, Tamar Valley in Tas and Orange in NSW). Genotype data (qPCR performed at the USDA laboratory in Corvallis, Oregon) for two out of the eight phenotypic resistant isolates revealed 0 and 100% G143A respectively.

Table 5.14. The mean, median and range for EC_{50} µg/mL, and growth at 1.0 and 10.0 µg/mL for isolates classified as either sensitive or reduced sensitivity to Amistar (azoxystrobin).

Classification	Statistical parameters	EC_{50} µg/mL	Mean % growth* at µg/mL	
			1.0	10.0
Sensitive n=5	Mean (sd)	0.03 (0.02)	0	0
	median	0.03	0	0
	range	0.01 to 0.07	0	0
Reduced sensitivity n=8	Mean (sd)	6.7 (3.8)	48 (14)	38 (17)
	median	>10	54	45
	range	0.9 to >10.0	23 to 74	18 to 65

percentage of leaf discs covered with sporulating *E. necator*

5.1.3.6.4. Flint (trifloxystrobin)

Five isolates were tested for sensitivity with Flint (trifloxystrobin), one isolate with reported loss of field efficacy to Flint from the Adelaide Hills (#230), two unexposed sensitive isolates from unsprayed vines in the Adelaide Plains (#222) and McLaren Vale (#229), plus two isolates

from the Adelaide Hills (#216) and the Adelaide Plains (#215) that were previously found to be having reduced sensitivity to Cabrio (pyraclostrobin).

The two unexposed isolates were sensitive to trifloxystrobin (20%) with EC_{50} values of 0.01 and 0.03 µg/mL and no or minimal growth at 1.0 or 10.0 µg/mL (Table 5.15) and (Figure 5.12). However, the EC_{5O} values of the suspected resistant isolate from a commercial vineyard in the Adelaide Hills was 2.7 µg/mL with a RF of 130. Although the EC_{50} value of the other isolate from the Adelaide Hills (resistant to Cabrio) was only 0.2 µg/mL, the RF was 10, and growth was observed at 10 and 20.0 µg/mL, therefore this isolate was also considered to be resistant to Flint.

Table 5.15. The mean EC_{50} and growth at 1.0 and 10.0 µg/mL for five isolates classified as either sensitive or resistant to Flint (trifloxystrobin). R = resistant, S = sensitive.

Isolate code	Region	EC_{50} µg/mL	Mean % growth at µg/mL			G143A % using NGS	pyraclostrobin resistance classification (EC_{50} µg/mL)
			1.0	10.0	20.0		
215	Adelaide Plains Research Centre	0.04	7.5	0	n/a	100	R (2.4)
216	Adelaide Hills	0.2	17	14	19	100	R (1.1)
222	Adelaide Plains home garden	0.01	0	0	0	0	S (0.001)
229	McLaren Vale	0.03	7	0	0	n/a	n/a
230	Adelaide Hills	2.7	28	23	14	n/a	n/a

5.1.3.6.5. Cross-resistance in the QoIs.

Five isolates were tested both with Cabrio and Amistar. Two isolates were considered phenotypically resistant and two were sensitive to both products indicating cross-resistance. However, one isolate from the King Valley was resistant to Cabrio yet not to Amistar (Table 5.16). This isolate was genotypically resistant with 99% G143A. A greater number of isolates need to be tested to confirm cross-resistance among our isolates.

Table 5.16. Cross-resistance test for *Erysiphe necator* isolates collected from different regions in Australia.

Isolate code	Viticultural region	EC_{50} µg/mL		MIC		G143A % *
		Cabrio	Amistar	Cabrio	Amistar	
33a	Barossa, SA	14.4 (R)	0.9 (R)	>10	>10	97
62a	Riverland, SA	0.03 (S)	0.01 (S)	0.1	0.1	0
79a	King Valley, Vic	0.9 (R)	0.01 (S)	>20	0.1	99
108b	Adelaide Hills, SA	0.03 (S)	0.07 (S)	0.1	0.1	47
128	Langhorne Creek, SA	0.7 (R)	1.1 (R)	≥10	>10	S

* G143A % was determined by pyrosequencing carried out by BASF, Germany, except for isolate #128, where genotype status of S=sensitive was determined by qPCR carried out by USDA, Coravalis, USA.

5.1.3.6.6. Whole leaf assays – Cabrio (pyraclostrobin)

Twenty isolates were tested for sensitivity to Cabrio (pyraclostrobin) in a whole leaf assay, using the discriminatory concentrations of 0, 0.1, 1.0 and 10.0 µg/mL. Resistance status was classified using mean growth at 1.0 and 10.0 µg/mL, as previously described. Ten isolates were classified as phenotypically sensitive and the other 10 as resistant (Table 5.17). Although the mean and median G143A frequency were low for the sensitive group, and high for the resistant group, the ranges overlapped.

Table 5.17. Results from 20 *Erysiphe necator* isolates tested for sensitivity to Cabrio (pyraclostrobin) in a whole leaf assay.

Classification	Statistical parameters	EC_{50} µg/mL	Mean % growth at µg/mL		G143A %
			1.0	10.0	
Sensitive	Mean	0.5	2.0	0	25
	median	0.5	1.5	0	0
	range	0.3–0.9	0–5	0	0–100
Resistant	Mean	4.5	11.9	2.5	87
	median	2.3	12.5	0–11	100
	range	0.6–13.8	8–15	0	0–100

5.1.3.7. Demethylation Inhibitors (DMIs)

5.1.3.7.1. Topas (penconazole)

Forty-nine isolates were tested for sensitivity to Topas (penconazole) using the leaf disc bioassay. EC_{50} values, MICs and RFs were determined, as was % growth at 1.0 and 10.0 µg/mL. Detection and quantification of the Y136F allele associated with decreased sensitivity to the DMIs was carried out using both Sanger sequencing (for isolates obtained in SAR 1204) and NGS.

Eight isolates were not previously exposed to fungicides and are labelled the 'unexposed sensitive group'. Results of leaf disc assays and Y136F sequencing are shown in Table 5.18. The mean (sd) EC_{50} value for seven unexposed isolates was 0.12 µg/mL (0.12), median 0.1 and range from 0.002 to 0.2 µg/mL (Table 5.19). Low level of reduced sensitivity was recorded in *E. necator* populations (Figure 5.14).

Table 5.18. Mean EC_{50} µg/ml, growth at 1.0 µg/mL, results of both Sanger and Next Generation Sequencing (NGS) for eight isolates of *Erysiphe necator* not exposed to fungicides, i.e. the 'unexposed sensitive group'

Isolate code	EC_{50} µg/mL	Mean % growth at 1.0 µg/mL	MIC	Sanger sequence	Y136F % using NGS
TW1	0.1	0	1	*	*
TW2	0.4	0	1	Y136	0
ID1	0.1	0	1	Y136	*
27a	0.01	0	1	Y136/Y136F	59
195	*	*	*	*	0
217	0.04	11	10	*	100
218	0.002	0	1	*	4
222	0.2	35	10	*	4

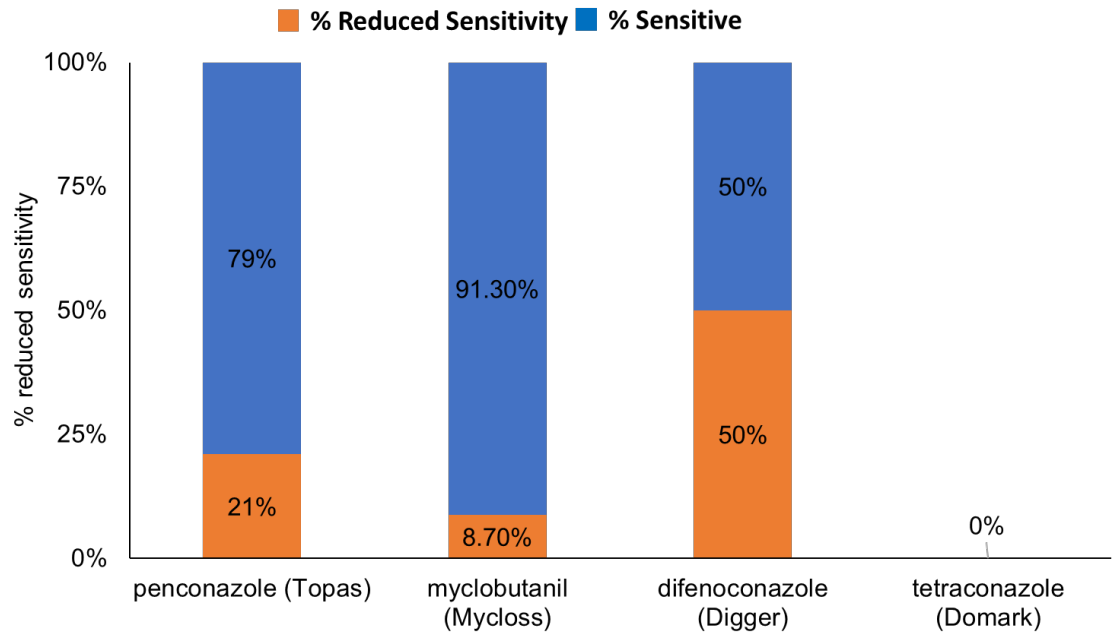


Figure 5.14. Summary of sensitivity of *Erysiphe necator* isolates to DMI fungicides. Isolates growing more than 5% on 1 µg/mL of active ingredient are considered to have reduced sensitivity.

For the 41 isolates exposed to fungicides, the mean (sd) and median EC_{50} values were 0.15 (0.13) and 0.1 respectively with a range of 0.01 to 0.5 µg/mL. The MIC values for all isolates were either 1.0 or 10.0 µg/mL, including those from the unexposed sensitive group (Table 5.19). The RF values ranged from 0.08 to 4.1. These RFs are low and generally not indicative of resistance. However, several isolates grew up to 35% on 1.0 µg/mL and the MIC values were 10.0 µg/mL, which may indicate a low level loss of sensitivity.

Table 5.19. Results for the unexposed and selected isolates of *E. necator* following testing on leaf disc bioassay with Topas (penconazole).

Classification	Statistical parameters	EC_{50} µg/mL	Mean % growth (%) at µg/mL		MIC	RF	Y136F % using NGS
			1	10			
Unexposed	mean (sd)	0.12 (0.12)	7 (12)	0	3.5(4)	1(1)	33 (40)
	median	0.1	0	0	1	0.8	4
	range	0.002 to 0.2	0 to 35	0	-	0.3-3.3	0 to 100
Selected	mean (sd)	0.15 (0.13)	14 (15)	0	6.6(4)	1.2(1.1)	75 (31)
	median	0.1	9	0	10	0.8	90
	range	0.01 to 0.5	7 to 52	0	-	0.08-4.1	5 to 100

The population distribution was normal for both the unexposed and selected group according to a Shapiro Wilk test. An independent two sample t-test was run on the log_{10} data with a 95% confidence interval for the mean differences. The mean log EC_{50} of the selected group was the same as that of the unexposed group, therefore the two population means are the same, indicating that the selected population is not significantly different to the unexposed sensitive group. The frequency histogram of the log_{10} EC_{50} values displays the unexposed sensitive and the selected populations values, showing the overlap of ranges for both populations (Figure 5.15).

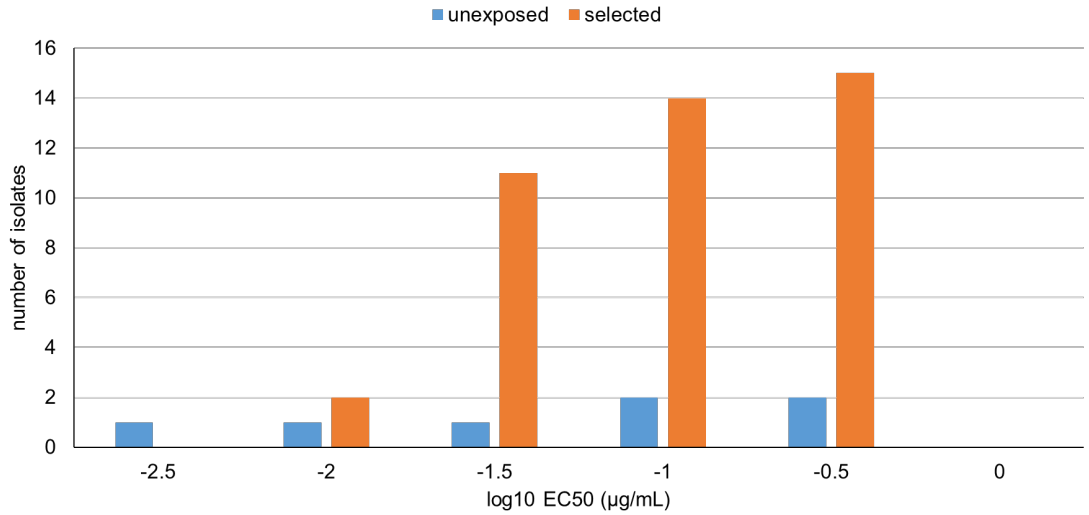


Figure 5.15. The distribution of sensitivities to Topas (penconazole) among populations of *E. necator* (i) with no history of DMI fungicide exposure 'unexposed' (blue) and likely exposed to DMIs 'selected' (orange).

Whole leaf assay

Ten isolates were tested for sensitivity to Topas using the whole leaf bioassay, with four being from the unexposed group (viz, #195, 217, 218 and 222) (Table 5.15). There was no difference between mean, median nor range of EC_{50} values for the unexposed and selected groups (Table 5.20) and (Figure 5.16). Although the RFs range from 0.02 to 1.5 indicating no loss of sensitivity when comparing selected isolates to unexposed based on EC_{50} values, two isolates grew well (>25%) on 1.0 µg/mL indicating a likely low-level loss of sensitivity.

Table 5.20. Results for the unexposed and selected isolates of *E. necator* following testing on a whole leaf bioassay with Topas (penconazole).

Classification	Statistical measure	EC_{50} µg/mL	Mean % growth at µg/mL		Y136F % using NGS
			1.0	10.0	
unexposed	mean (sd)	0.4(0.3)	16 (12)	0	27 (42)
	median	0.4	13	0	4
	range	0.04 to 0.7	2 to 35	0	0 to 100
selected	mean (sd)	0.4 (0.2)	20 (20)	0	70 (25)
	median	0.3	11	0	71
	range	0.01 to 0.8	1 to 60	0	41 to 99

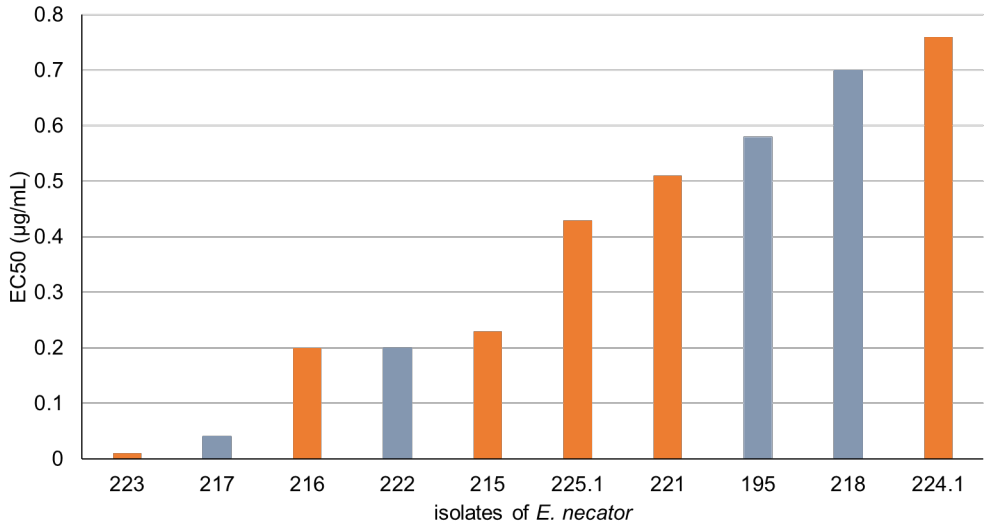


Figure 5.16. The EC_{50} µg/mL of unexposed (blue bars) and selected (grey bars) isolates of *E. necator* tested for sensitivity to Topas (penconazole) in a whole leaf assay.

When comparing the leaf disc and whole leaf bioassay results, the EC_{50} values are approx. 3-4-fold higher with the whole leaf assay (Table 5.20) than with those of the leaf disc assay (Table 5.19). This demonstrates the effect of using different methods, and that EC_{50} values are best used when referring to populations of isolates and in the detection of shifts in sensitivity for the DMIs. However, the whole leaf assay is useful for high throughput screening of isolates to give an indication of sensitivity loss.

A total of 54 isolates were tested in either a leaf disc or whole leaf bioassay from a range of viticultural regions in Australia, however, many areas are not represented or have low representation (Table 5.21). Of the 54 isolates phenotypically tested, Sanger sequencing or NGS, or both, were carried out on 39. Seventy three percent of these isolates contained the Y136F allele at ≥41% (as determined by NGS) and were a mix of Y136/Y136F or only Y136F as determined by Sanger (Table 5.21), which is considered as genotypically resistant.

Table 5.21. The isolates tested for sensitivity in either leaf disc or whole leaf bioassays to Topas (penconazole).

State	Viticultural region	Number phenotyped	Number genotyped	Genotypic resistance *
South Australia	Adelaide Hills	3	3	3
	Adelaide Plains	12	7	4
	Barossa	7	4	3
	Langhorne Creek	4	4	4
	Padthaway	2	2	2
	Riverland	8	5	5
Victoria	Bendigo	2	2	1
	King Valley	2	2	0
	Goulburn Valley	1	1	0
Tasmania	Tamar Valley	3	3	1
New South Wales	Hilltops	1	1	1
Western Australia	Great Southern	2	2	2
	Margaret River	6	3	3
	Swan Valley	1	1	0
Total		54	39	29

* Y136F ≥41% using NGS or Y13F/Y136F, based on median value for %Y136F in wild type/Y136

Genotype

Sanger sequencing was carried out on 35 of the 54 phenotyped isolates, and of these, 10 were Y136, nine were mixed and 16 were Y136F. There was no obvious relationship between Sanger results and EC_{50}, MIC nor RF values (data not shown).

NGS was carried out on 27 of the 54 phenotyped isolates to determine the Y136F frequency. To determine whether there was any relationship between %Y136F and any of the following parameters, E_{50} values, $\log_{10}$ EC_{50} values, MIC, RFs and growth at 1.0 µg/mL, simple linear regression was undertaken. There was no linear relationship between %Y136F and any of the variables (all R_2 values <0.02) (data not shown).

To determine whether the two methods of sequencing support one another, all isolates (58 in total) with both Sanger and NGS data were analysed (Table 5.22). Apart from one outlier (isolate 16b, Y136F = 59%), results for the Y136F Sanger category (mutant) and those from NGS were consistent. In this case, if only Sanger sequencing were available, then this would be a reliable method to determine whether the mutant is present in a sample. The mixed Sanger (Y136/Y136F) results show the frequency of Y136F is >50%. The results for the wild type/ Y136 category are less clear. In theory, Sanger wild type indicates that no mutation is present, however, the NGS results indicate otherwise. In the Sanger Y136/wild type group, the Y136F % using NGS ranged from 0 to 67 %.

Table 5.22. The Y136F mutation frequencies determined by next generation sequencing for isolates classified by Sanger sequencing as Y136 (wild type), mixed (Y136/Y136F) or mutant (Y136F).

Statistical parameters	Sanger Y136 (n=13)	Sanger Y136/Y136F (n=19)	Sanger Y136F (n=26)
Mean (sd)	29 (23)	69 (15)	97 (9)
Median	36	75	100
Range	0 to 67	50 to 100	59 to 100

Relationship to genotype

Phenotype, irrespective of the method used and the statistical measure used to classify 'resistance', does not show any clear relationship to genotype, using either Sanger or NGS to detect and quantify the Y136F mutation. Therefore, there is a poor relationship between phenotype and genotype.

5.1.3.7.2. Mycloss (myclobutanil)

Eighty isolates were tested for sensitivity to Mycloss (myclobutanil) using the leaf disc assay, of which two, #222 and TW2, were from the unexposed sensitive group described previously (Table 5.18). Isolate 222 contained 4% Y136F (using NGS) and TW2 was Sanger wild type (Table 5.23), these two isolates were used in the calculation of RFs.

Table 5.23. Two unexposed isolates tested for sensitivity to Mycloss (myclobutanil) in a leaf-disc assay

Unexposed isolate code	EC_{50} ug/mL	Mean of % growth at ug/mL				MIC	Genotype status
		1.0	2.0	4.0	16.0		
222	0.5	26	20	17	19	20	4 % Y136F*
TW2	0.01	0	0	*	0	1	Y136**

*based on next generation sequencing, ** based on Sanger sequencing

The results for 78 exposed isolates are shown in Table 5.24. The RFs for seven isolates (out of 78 isolates) ranged from 10 to 53, with EC_{50} values from 1.9 to 10.0 µg/mL and all MICs ≥ 10.0 µg/mL. These isolates showed decreased sensitivity to myclobutanil. Of these, decreased efficacy of the DMIs has been reported for four of these vineyards, with one, isolate from the Yarra Valley (RF 34, MIC 20) specifically reporting a loss of field efficacy for myclobutanil. NGS analysis of Y136F% for six isolates revealed a mean (sd) of 71 (15), median 72 and the range was from 50 to 85%.

Table 5.24. Sensitivity of 78 isolates of *Erysiphe necator* tested with Mycloss (myclobutanil) in a leaf-disc assay

Statistical parameters	EC_{50}	Mean of % growth at µg/mL						MIC	RFs	Y136F %
		1.0	2.0	4	10	16	20			
Mean (sd)	0.8 (1.6)	28 (23)	24(24)	12(15)	14 (20)	2 (4)	3 (7)	8.5 (6)	4.2 (5.7)	76 (22)
Median	0.3	22	15	7	0	0	0	10	1.6	80
Range	0.002 to 10	0 to 87	0 to 98	0 to 69		0 to 17	0 to 18	0.2 to >20	0.01 to 52	3 to 100

Results from the other isolates are less clear to interpret, with no obvious groupings. Those (25 isolates) with RFs of < 1 have low EC_{50} values (0.002 to 0.1 µg/mL), MICs range from 0.2 to 16 µg/mL and most did not grow at 1.0 or 4.0 µg/mL. The mean (sd), median and range of Y136F% (21 isolates out of 25) was 20 (25), 76 and 3 to 100, respectively. Here, these isolates are mostly phenotypically sensitive yet many contain a high % of Y136F.

The remaining 46 isolates have RFs of between 1.1 and 8.4, EC_{50} values between 0.2 and 1.6 µg/mL, MICs from 4 to 20 µg/mL and the majority growing at 1.0 and 4.0 µg/mL. For this group, the mean (sd), median and range of Y136F% (38 out of 46 isolates) was 15 (20), 82 and 16 to 100, respectively. In this group, some of the isolates show decreased sensitivity but the cut-off point between phenotypic sensitive and decreased sensitivity is not clear. The Y136F% is similar to the 25 putative phenotypic sensitive isolates.

In summary, 80 isolates of *E. necator* were mostly sensitive (91%) to myclobutanil (Figure 5.14) and there was no relationship between phenotype and genotype results from the 78 isolates tested.

There was an obvious lack of sensitivity especially for those with RFs ≥10 (Figure 5.17). The EC_{50} µg/mL data were log_{10} transformed and show a continuous distribution, consistent with qualitative resistance as found in the DMIs. Looking at the population tested, there is a gradual shift from sensitive to decreased sensitivity (Figure 5.17). The log_{10} data can also be grouped into categories and shown as a frequency distribution graph (Figure 5.18).

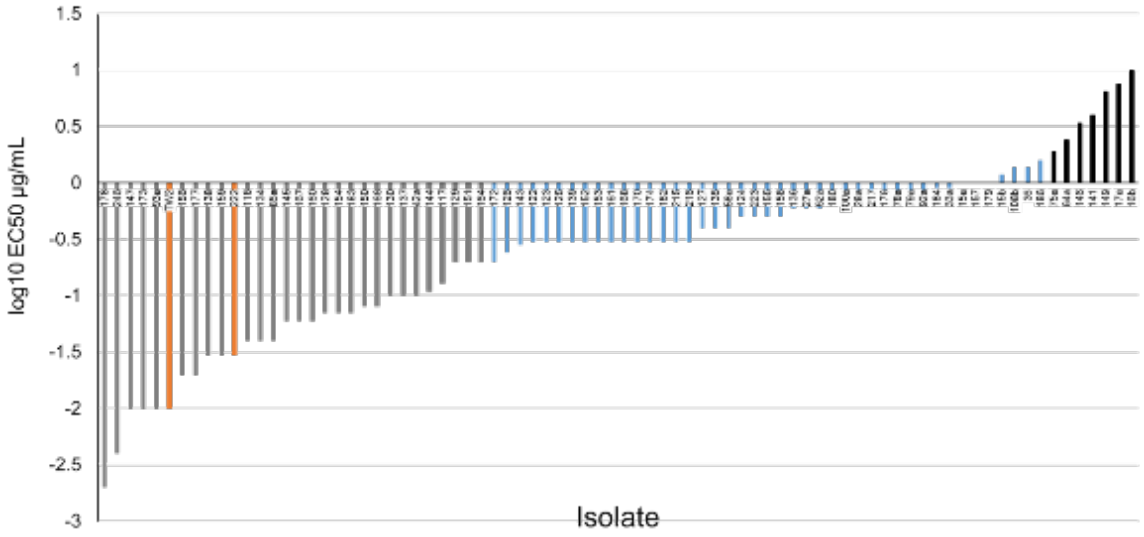


Figure 5.17. Log_{10} EC_{50} µg/mL of 80 isolates tested with Mycloss (myclobutanil) in a leaf-disc assay. Isolates with an RF ≥10.0 (black bars); RF between 1.1 and 9 (blue bars); RF ≤1.0 (grey bars) and the unexposed wild type isolates (orange bars).

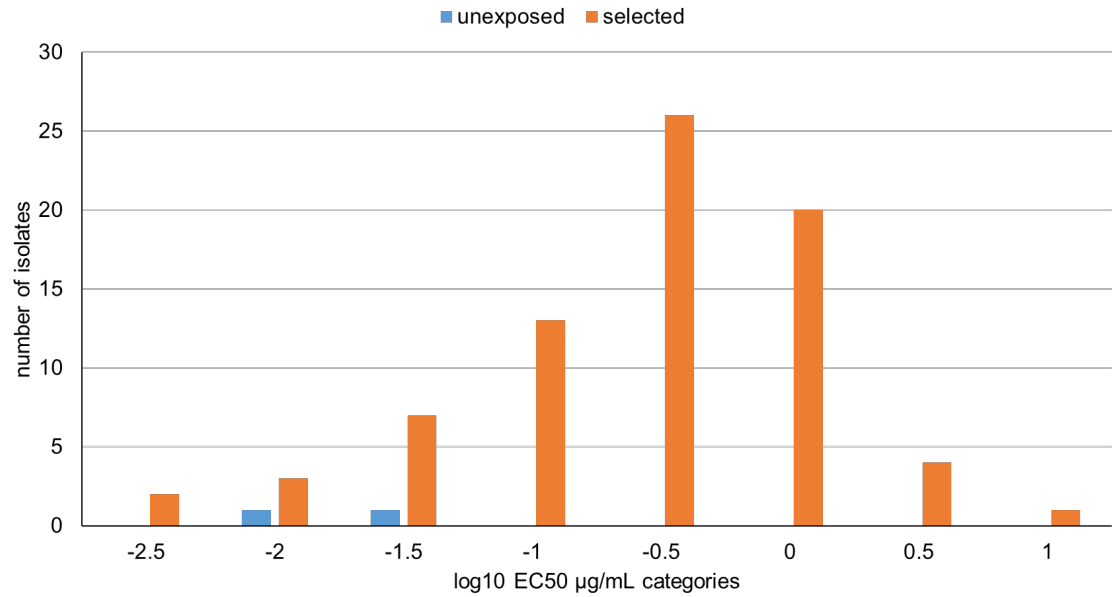


Figure 5.18. The frequency distribution of the log_{10} EC_{50} µg/mL values for two unexposed wild type and 78 selected isolates tested with Mycloss (myclobutanil) in a leaf-disc assay.

There were five isolates from the total of 80 tested that were wild type i.e. 0% Y136F. These were from both the unexposed and selected group. The frequency distribution of log_{10} EC_{50} µg/mL of both wild-type and isolates containing Y136F were analysed and showed there is no clear relationship between lack of Y136F and the log_{10} EC_{50} µg/mL value (Figure 5.19).

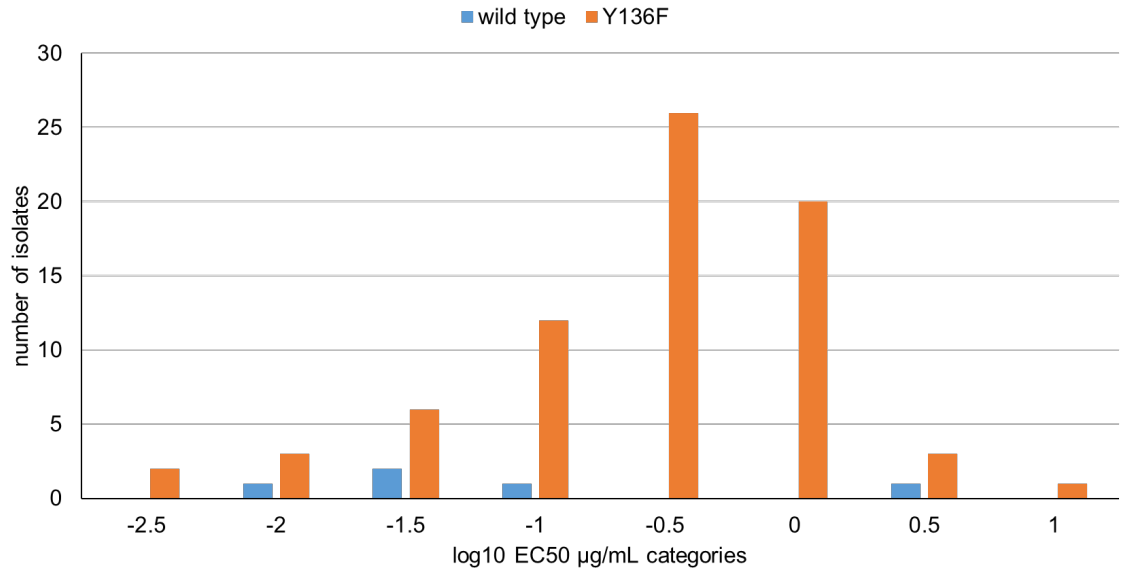


Figure 5.19. The frequency distribution of the log_{10} EC_{50} µg/mL values for the wild type and *Erysiphe necator* isolates containing Y136F tested with Mycloss (myclobutanil).

Whole leaf assay

Seventeen isolates, including seven from the shadehouse experiments (6.1.2.1) were tested for sensitivity to myclobutanil using the whole leaf assay using three concentrations *viz.* 0.1, 1.0 and 10.0 µg/mL plus a no fungicide control. Two unexposed isolates were used in the RF calculations (Table 5.25), however later NGS analysis showed that one of them (isolate #201) has 100% Y136F.

The results for 15 isolates showed that RFs ranged from 0.001 to 11.5, with EC_{50} values from 0.0001 to 1.1 µg/mL, minimal growth at 10 µg/mL and all MICs ≤ 10.0 µg/mL (Table 5.26). These isolates show sensitivity to myclobutanil. Although genotyping analysis showed a medium to high range (38-100%) of Y136F mutant, these isolates were sensitive to myclobutanil. Therefore, no link was found between phenotyping and genotyping.

Table 5.25. Two unexposed isolates used in whole leaf assay with Mycloss (myclobutanil)

Unexposed isolate code	EC_{50} ug/mL	Mean of % growth at ug/mL		MIC	Genotype status (%Y136F)
		1.0	10.0		
201	0.06	13	0	10	*100
222	0.13	0	0	0	4

*initial sample information suggest that this isolate was not exposed to DMI and later NGS analysis showed it has 100% Y136F

Table 5.26. Results for 15 isolates tested with Mycloss (myclobutanil) in a whole leaf assay

Statistical parameters	EC_{50} ug/mL	Mean of % growth at ug/mL		MIC	RF	Genotype status
		1.0	10.0			
Mean (sd)	0.2 (0.3)	21 (18)	0.06 (0.2)	7.8 (4)	2.6 (2.9)	79 (29)
median	0.2	13	0	10	2	100
range	0.0001 to 1.1	1 to 60	0 to 1	0.1 to 10	0.001 to 11.5	38 o 100

5.1.3.7.3. Digger (difenoconazole)

Ten isolates were tested for sensitivity to Digger (difenoconazole) using a leaf-disc assay; two were unexposed and contained low frequencies of Y136F (0 and 4 %), three were from

commercial vineyards and the remaining five were obtained from experiments carried out in the shadehouse at the Waite campus (6.1.2.1).

EC_{50} values were low, ranging from 0.006 to 0.3 µg/mL and there was no growth on 10 µg/mL, but growth range was 0 to 80 at 1 µg/mL (Table 5.27). RF varied and low to high frequency (4-100%) of the Y136F mutant was detected. Based on the % of growth at 1 µg/mL, 50% of the tested isolates were considered as having reduced sensitivity to difenoconazole (Figure 5.14).

Table 5.27. Results for 10 *Erysiphe necator* isolates tested with Digger (difenoconazole) in leaf-disc assay

Statistical parameters	EC_{50} ug/mL	% growth at ug/mL		RF	Y136F for final analysis
		1.0	10		
mean	0.06 (0.09)	27 (31.5)	0	8.62 (15)	47.67 (37)
median	0.03	8.00	0	3.33	46.50
range	0.006 to 0.3	0-80	0	0-45	4-100

5.1.3.7.4. Domark (tetraconazole)

Twenty-four isolates were tested for sensitivity to tetraconazole (Domark) in a leaf-disc bioassay, which includes only one unexposed wild type, #222. No fungal growth was observed at 1.0 or 10.0 µg/mL. The mean (sd) for EC_{50} was 0.06 (0.15), median 0.008 and the range of EC_{50} values were from 0.0008 to 0.7, MICs ranged between 0.01 to 0.1 and RFs ranged between 0.001 to 1 (Table 5.28). Total analysis showed that all tested isolates were sensitive to tetraconazole (Figure 5.14). NGS analysis identified the Y136F mutant and it ranged between 4 to 100%. There was no relationship between EC_{50}, mean growth at 1.0 or 10.0 µg/mL and Y136F frequency. Overall, based on phenotype, no decreased sensitivity was found, but based on >80% Y136F frequency, 13 isolates are considered genotypically resistant.

Table 5.28. Results for 24 *Erysiphe necator* isolates tested with Domark (tetraconazole) in leaf-disc assay

Statistical parameters	EC_{50} µg/mL	MIC	Mean % growth at ug/mL		RF	Y136F% (n=21)
			1.0	10.0		
Mean (sd)	0.06 (0.15)	0.1 (0.2)	0	0	0.08 (0.2)	76 (23)
Median	0.008	0.1	0	0	0.01	80
Range	0.0008 to 0.7	0.01 to 0.1	0	0	0.001 to 1.0	4 to 100

5.1.3.8. Other fungicide groups

5.1.3.8.1. Group 5, Prosper (spiroxamine)

Twenty-nine isolates, including five sensitive unexposed, were tested in a leaf disc assay for sensitivity to spiroxamine. The results reveal that the EC_{50} values, growth parameters and MIC values overlap between the sensitive unexposed group and the exposed group (Table 5.29), indicating that most isolates are sensitive, with only 17% of the isolates showing reduced sensitivity (Figure 5.20). Two individual isolates i.e. # 215 and 216 with EC_{50} values of 0.7 and 0.9 µg/mL respectively were those heavily exposed to fungicides and one, #230, with an EC_{50} of 0.7 µg/mL, was from vines with powdery that had been sprayed with nine consecutive sprays over two years with Prosper (6.1.2.1). These data are a useful beginning as a reference data set for future testing and assessment of decreased sensitivity with spiroxamine. The mechanism associated with resistance is not known, therefore genotype analyses is not yet possible.

Table 5.29. Results for 25 *Erysiphe necator* isolates tested with Prosper (spiroxamine) in a leaf-disc assay

Statistical parameters	EC_{50} µg/mL	Mean % growth at µg/mL		MIC	RF
		1.0	10.0		
Mean (sd)	0.2 (0.3)	20.6 (20.5)	2.8 (7.0)	6.2 (4.4)	1.5 (2.1)
median	0.05	16	0	10	0.3
range	0.005 to 0.9	0 to 62	0 to 30	0.1 to >10	0.06 to 5.4

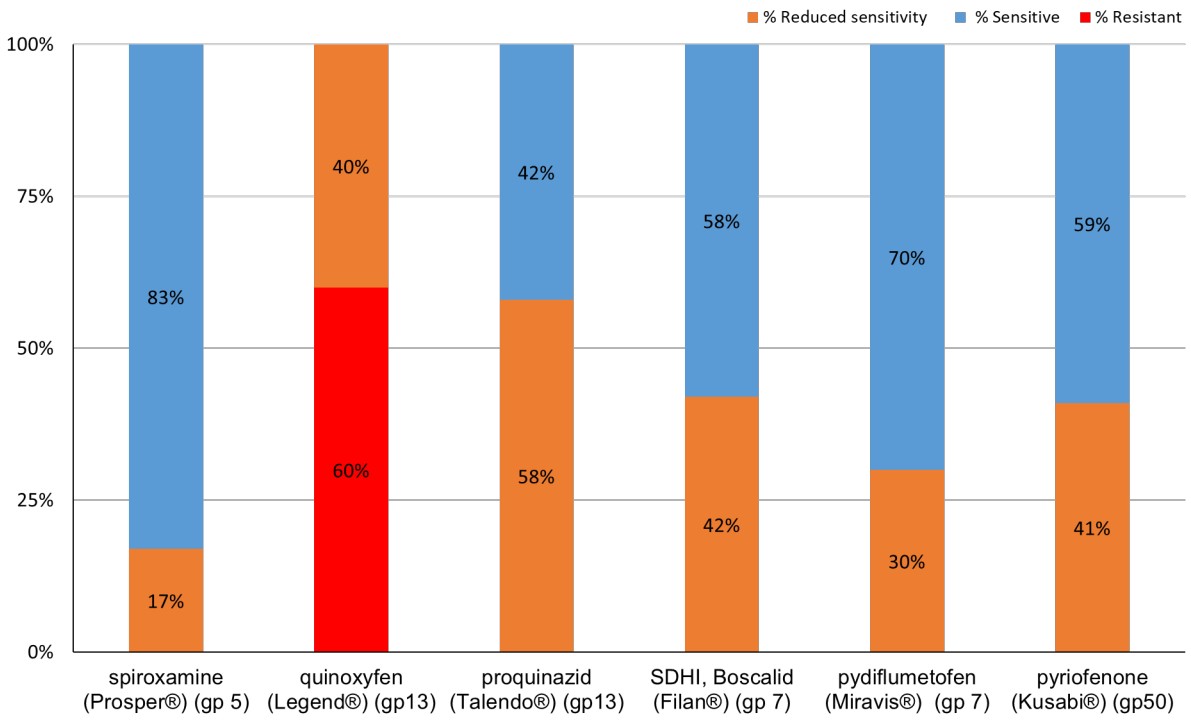


Figure 5.20. Summary of sensitivity of *Erysiphe necator* isolates to different groups of fungicides. Isolates growing more than 5% on 1 µg/mL of active ingredient are considered to have reduced sensitivity and resistant when growing at field rate o the fungicide.

5.1.3.8.2. Group 13

A. Talendo (proquinazid)

Twenty-four isolates were tested for sensitivity in a leaf disc bioassay to Talendo (proquinazid), of which three were from the unexposed sensitive group. Loss of field efficacy to Talendo was suspected for a further three isolates, two from the Hunter Valley and one from the Adelaide Hills (Table 5.30). The EC_{50} values were high and ranged between 0.7 to 3.1 µg/mL, all isolates have substantial growth at 1 and 10 µg/mL with two isolates grown on 20 µg/mL, MIC values were ≥20 indicating reduced sensitivity. Overall, 58% of the tested isolates showed reduced sensitivity (Figure 5.20). The genetic mechanism of resistance is not known, therefore genotype analyses is not possible.

Table 5.30. Results for three *Erysiphe necator* isolates with reported field failure to Talendo (proquinazid) in a leaf-disc assay.

Isolate code (region and state)	EC_{50} µg/mL	%Growth at µg/mL			MIC
		1.0	10.0	20.0	
213 (Hunter valley, NSW)	3.1	77	21	24	>20
214 (Hunter valley, NSW)	0.7	55	27	1	20
216 (Adelaide Hills, SA)	1.4	51	61	26	>20

B. Legend (quinoxyfen)

A subset of 10 isolates tested to proquinazid was selected for testing in a leaf disc assay with quinoxyfen, of which one was unexposed, #201 with EC_{50} = 0.006 µg/mL, growth at 1.0 µg/mL = 9%, growth at 10.0 µg/mL = 2%, growth at 55 µg/mL (field rate) = 0%, and MIC = 0.1 µg/mL. This likely reflects reduced sensitivity of these unexposed isolates to quinoxyfen.

For the selected isolates, the percentage of growth range from 14 to 48% at 55 µg/mL was observed for six isolates (from Great Southern, WA; Yarra Valley, Vic; Strathbogie Ranges, Vic; King Valley, Vic and Langhorne Creek, SA), with an MIC of >55; this likely indicates resistance (Table 5.31). Overall, 60% of the tested isolates showed resistance to proquinazid and 40% reduced sensitivity (Figure 5.20).

Table 5.31. Results for nine *Erysiphe necator* isolates* tested for sensitivity to Legend (quinoxyfen) using a leaf-disc assay.

Statistical parameter	EC_{50}	Mean % growth at µg/mL			MIC
		1.0	10.0	55.0 (field rate)	
Mean (sd)	0.08 (0.1)	18 (8)	11 (6)	22 (12)	22 (24)
median	0.01	14	12	18	10
range	0.0002 to 0.3	6 to 30	2 to 21	14 to 48	0.1 to >55

* Excludes the one unexposed isolate

5.1.3.8.3. Group 7

A. Filan (boscalid)

Twenty isolates, including three unexposed sensitive reference isolates, # 201, 218 and 222, (Table 5.32), were tested to for sensitivity to Filan (boscalid) in a leaf disc bioassay. The mean EC_{50} value of 0.05 µg/mL was used to calculate the RFs for the 17 tested isolates.

Table 5.32. Results of three unexposed sensitive reference *Erysiphe necator* isolates tested with Filan (boscalid) in a leaf disc assay.

Isolate code	EC_{50} µg/mL	Mean % growth at µg/mL		MIC
		1.0	10.0	
201	0.007	0	0	1
218	0.05	17	0	10
222	0.08	0	0	1
Mean	0.05			

Results showed that 58% of tested isolates sensitive to boscalid (Figure 5.20), the mean and median of EC_{50} for 17 isolates were 0.1 µg/mL, range was 0.004 to 0.06 µg/mL, % growth was 0 at 1.0 and 10.0 µg/mL, MIC 4.6 µg/mL and the mean for RF was 2.9 (Table 5.33).

Table 5.33. Results for 17 isolates of *Erysiphe necator* tested for sensitivity to Filan (boscalid) in a leaf disc assay.

Statistical parameter	EC_{50} µg/mL	Mean % growth at µg/mL		MIC	RF
		1.0	10.0		
Mean (sd)	0.1 (0.2)	14.5 (16.1)	0	4.6 (4.6)	2.9 (2.9)
median	0.1	11	0	1	2
range	0.004 to 0.6	0 to 52	0	0.1 to 10	0.2 to 12

B. Miravis (pydiflumetofen)

A total of 30 isolates were tested in a leaf-disc bioassay to the coded product containing Miravis (pydiflumetofen; 200 g/L a.i.), which was later released to market as the fungicide Miravis (Syngenta). Of the isolates tested, three were unexposed sensitive reference isolates, *viz.* # 195, 201 and 218 (Table 5.34). The mean EC_{50} was 0.02 µg/mL, which was used to calculate the RFs for the 27 tested isolates.

Table 5.34. Results for three unexposed sensitive reference isolates tested with Miravis (pydiflumetofen) in a leaf disc assay.

Isolate code	EC_{50} µg/mL	Mean % growth at µg/mL		MIC
		1.0	10.0	
195	0.009	0.1	0	0
201	0.007	1	0	0
218	0.05	10	17	0

Results showed that 70% of tested isolates were sensitive to pydiflumetofen (Figure 5.20), the mean and median EC_{50} for 27 isolates was 0.1 µg/mL, range was 0.004 to 0.6 µg/mL, %, MIC 3.2 µg/mL, growth was 9 and 0% at 1.0 and 10.0 µg/mL respectively, and the mean RF was 4.9 (Table 5.35).

Table 5.35. Results for 27 isolates of *Erysiphe necator* tested for sensitivity to Miravis (pydiflumetofen) in a leaf disc assay.

Statistical parameter	EC_{50} µg/mL	MIC	Mean % growth at µg/mL		RF
			1.0	10	
Mean (sd)	0.1	3.2	9	0.00	4.9
median	0.1	4.4	14.7	0.00	6.6
range	0.004 to 0.6	0.1 to 10	0 to 52	0.00	0.2 to 30

A total of 57 isolates, including all that were tested with Filan and Miravis, were genotyped for presence of the *SdhB* mutations H242R and H242Y and neither mutation was detected.

5.1.3.8.4. Group 50 Kusabi (pyriofenone)

Twenty-two isolates were tested in a leaf disc bioassay to pyriofenone, three isolates were unexposed sensitive reference isolates, *viz.* # 195, 201 and 222 (Table 5.36). The mean of EC_{50} value of 0.02 g/mL, which was used to calculate the RFs for the 19 tested isolates.

Table 5.36. Results of three unexposed sensitive reference Erysiphe necator isolates tested with Kusabi (pyriofenone) in a leaf disc assay.

Unexposed isolate code	EC_{50} ug/mL	Mean of % growth at ug/mL		MIC
		1	10	
195	0	0	0	1
201	0.0000004	0	0	1
222	0.085	20	0	10

Results showed that 59% of tested isolates were sensitive to Kusabi (pyriofenone) (Figure 5.20). The mean and median EC_{50} for 19 isolates was 0.1 and 0.01 µg/mL, respectively, range was 0.0 to 1 µg/mL, % growth was 13% at 1.0, and 0 at 10.0 and 100 µg/mL, MIC 5 µg/mL and the mean for RF was 3 and ranged from 0-9 (Table 5.37).

Table 5.37. Results for 19 isolates of *Erysiphe necator* tested for sensitivity to Kusabi (pyriofenone) in a leaf disc assay.

Statistical parameter	EC_{50}	MIC	Mean % growth at µg/mL			RF
			1	10	100	
mean	0.11(0.2)	5 (4.6)	13 (18)	0 (0)	0 (0)	3 (8.5)
median	0.01	1	3	0	0	0.3
range	0-1.1	0.01-10	0-62	0	0	0-9

5.1.3.8.5. Group U6 Flute (cyflufenamid)

Six isolates were tested in a leaf disc bioassay to Flute (cyflufenamid), and two of the isolates were unexposed sensitive reference isolates, *viz.* # 218 and 222. The mean of EC_{50} value of 0.09 µg/mL, which was used to calculate the RFs for the four tested isolates.

Results showed that tested isolates were sensitive to cyflufenamid, the meanand median EC_{50} for 4 isolates was 0.007 µg/mL, range was 0.006 to 0.2 µg/mL, % growth was 0% at 1.0, 10.0 and 100 µg/mL, the mean for MIC was 0.52 µg/mL and 0.73 for RF (Table 5.38).

Table 5.38. Results for 4 isolates of *Erysiphe necator* tested for sensitivity to Flute (cyflufenamid).

Statistical parameter	EC_{50}	MIC	Mean % growth at µg/mL			RF
			1	10	17.5	
Mean	0.007(0.001)	0.52(0.4)	0 (0)	0 (0)	0 (0)	0.73(0.8)
Median	0.007	0.55	0	0	0	0.32
Range	0.006-0.2	0.01-1	0	0	0	0-2.2

5.1.3.9. Whole genome sequencing

Copy number (CN) of *Cyp51* genes was estimated to range from 1 to 7 across the isolates (Table 5.39), relative to a genome-wide CN of 1. Allele proportions were calculated by examining individual read alignments (Koboldt et al. 2012) across the Y136F region. Allele proportions ranged from 100% sensitive (A allele) in En_222 to 100% resistant (T allele) in several strains. Strains En_210 (CN ~ 3), En_213 (CN ~ 7) and En_216 (CN ~ 6) are predicted to be heterozygous across the multiple copies of *Cyp51*. Further phenotyping tests are required to link the CN with sensitivity/resistance of the strain.

Table 5.39. *E. necator* whole genome sequencing results for copy number of *Cyp51* genes and allele proportions across the Y136F region.

Strain	Copy number (average)	Copy number (std dev)	*Cyp51* allele	Allele proportions (sensitive|resistance)	Total counts
En_150	2.94	0.09	T	100%	798
En_159	2.96	0.07	T	100%	960
En_164	2.44	0.07	T	100%	281
En_172	3.02	0.09	T	100%	449
En_191	2.92	0.09	T	100%	622
En_201	2.95	0.13	T	100%	449
En_210	3.17	0.09	A|T	13.4%|86.6%	95|614
En_213	6.98	0.13	A|T	27.7%|72.3%	533|1390
En_215	3.08	0.05	T	100%	391
En_216	5.93	0.07	A|T	64.2%|35.8%	395|220
En_216_1	6.04	0.07	A|T	64.7%|35.3%	401|219
En_222	0.98	0.00	A	100%	209

5.1.4. Discussion

Whilst powdery mildew (PM) samples were obtained from different regions, some areas remain less represented than others e.g. only one sample was obtained from Coonawarra (SA), Goulburn Valley and Geelong (Vic), and East Coast (Tas). Some regions are not represented at all, e.g. Granite Belt (Qld), Pyrenees (Vic), Clare Valley (SA) and most Tasmanian regions. Regions that were well represented tended to be from South Australia, where the project team was based, and regions with high disease pressure e.g. the Hunter Valley and Yarra Valleys. Some regions with low disease pressure e.g. McLaren Vale and Coonawarra, were also less represented. Whilst the number of samples from the Adelaide Plains is the highest, this reflects the collection of samples from home gardens and the Waite campus experiments, where several of these samples formed the basis of the 'sensitive unexposed reference' group of isolates.

Powdery mildew cultures were maintained using the Self-Maintained System for Grapevine Powdery Mildew (SMSGPM) for six months, with minimal system maintenance. The system has potential for ongoing use as it proved to be practical space saving, less laborious, easy to handle around the laboratory as every unit was detachable and it does not require expensive laboratory consumables (e.g. petri dishes, agar, antibiotics) as well as other laboratory instruments to prepare the media. Although stability of mutation frequency has not been monitored in this system after culturing the isolates for six months, the likelihood of cross-contamination among isolates is low as cultures are fully enclosed in each unit. In addition, there was no presence of unwanted fungi (e.g. saprophytes) and bacteria than when using detached leaves. The system is cost-effective at ~$25/unit, especially compared to the costs of ongoing maintenance using detached leaves. However, a major drawback was nutrient deficiency after a few months, resulting in plant death after six months. Several tests with various dilutions of liquid fertilisers, including one designed for use in hydroponic systems (Uni-one 4-3-6 Uber hydroponic), did not solve the problem. On examination of dead plants and

the root chamber, a slimy, white, muddy substance was noted inside the plastic tubing. This material might block the root system or have a toxic effect on the plants. One hypothesis is that the material might be due to a reaction between the fertiliser and the tubing. Further investigation is required to identify the cause of plant death including experimenting several nutrient regimes and using different plastic material especially for the irrigation tubes. In an unrelated project, an alternative sub-culturing system was trialed for fungicide resistance in powdery mildew on wheat and proved to be useful and practical, as the wheat required less time to maintain.

Maintenance of PM cultures using the mini-greenhouse system was successful and provided a good alternative to the detached leaf culture method, as well as providing a back-up for critical isolates and was suitable for use in several experiments (Sections 6.1.2.2 and 9.2). The system has been running for more than two years with no major problems. Cross-contamination among isolates has not been found for up to six months, as few isolates had changes in frequency of G143A or Y136F. These changes might be attributed to the initial prototype design of the mini-greenhouses where the plastic covers were poor quality and easily torn, hence there were some gaps in the covers, which might have allowed cross-contamination to occur. Also, the ventilation holes were not sealed properly. Improvements have been made to the system by replacing the plastic cover with PVC clear covers with specialised ventilation vents built in. Monitoring frequency of mutations will continue and it is recommended that this system be used in the future to maintain PM isolates and conduct experiments.

The whole leaf assay is a useful tool for high throughput phenotype screening, especially using a discriminatory dose, but EC_{50} values are less reliable than those obtained from the leaf-disc assays. This is due to the physical limit of growth being around 15 mm diameter, limiting the range of results or response variables that could be obtained. Although only a small number of isolates were tested, the relationship of both EC_{50} values and growth results at 1.0 and 10.0 µg/mL with the % G143A mutation was better using data from the leaf disc assays than the whole leaf assays. , However, the whole leaf assay was a quick and efficient method to carry out resistance testing, and MIC values and relative growth at each concentration still gave valuable information and an indication about whether an isolate is sensitive or resistant.

The outcome of testing three genetically different isolates with three DMI fungicides, both commercial product and technical grade, was that there appeared to be little difference in the EC_{50} values between the product and technical grade for each isolate. As for the previously reported DMI results (all carried out with commercial product), there was no obvious relationship between Y136F% and the EC_{50} value for the technical grade active. In this small study, the impact of the carriers, solvents, wetting agents, spreaders and stickers in the commercial product did not appear to affect efficacy (McGrath, 2016).

For Group 11 QoIs, leaf disc, whole leaf and spore germination assays were useful to detect and monitor the sensitivity status of powdery mildew isolates phenotypically, however they are time consuming and labour intensive (Kunova et al. 2021). Reduced sensitivity was recorded in Australian isolates of *E. necator* for pyraclostrobin, azoxystrobin and trifloxystrobin. In New York, USA, the frequent use of these fungicides eventually led to the detection of fungicide resistant *E. necator* sub-populations five years after introduction of QoIs (Baudoin et al. 2008). Reports of QoI-resistance spread to other grape-growing regions in the eastern United States and France (Baudoin et al. 2008, Dufour et al. 2011). In Australia, resistance of *E. necator* to QoIs was first reported 10 years after introduction to Australia (Wicks et al. 2013). The current study has detected 62% of isolates with reduced sensitivity to QoIs, indicating resistance development.

Generally, there are positive relationships between EC_{50}, MIC and the frequency of G143A and it is a good indicator for resistance. Sequencing of PCR amplicons and phenotyping of isolates also revealed that these assays accurately detected each allele (100% agreement) and there was an absolute agreement between the presence/absence of the G143A mutation and resistance to QoIs in the *E. necator* samples (Miles et al., 2021). The current study showed 92% agreement between phenotyping and genotyping when isolates were tested against QoI fungicides. Field failure was observed and was strongly associated with high levels (>95%) of G143A. Cross-resistance has been shown between all fungicides within the QoI group with FRAC considering the QoIs as having high risk of resistance development (FRAC, 2021). As QoI resistance is qualitative, our data fit the expected bimodal distribution for sensitive and resistant isolates. Testing a greater number of isolates, genetically and phenotypically, will further strengthen the differences between the sensitive and resistant populations. Development of high-throughput molecular tests will assist in achieving greater monitoring of fungicide resistance in Australian vineyards.

Phenotypically, the majority of isolates tested were sensitive to Group 3 DMI fungicides, with only a few displaying possible low level loss of sensitivity which ranged between 8.7 and 50% depending on the fungicide. However, 87% of the tested isolates had the Y136F mutant, which indicated genetic resistance. Irrespective of the phenotypic method and statistical assessment used to classify 'resistance', there was no clear relationship to genotype, using either Sanger or NGS to detect and quantify the Y136F mutation. Many studies have reported that resistance to DMIs depends on several genetic factors such as *Cyp51* gene copy number (Rallos and Baudoin, 2016) and other mutants e.g. A1119C (Kunova et al. 2021). This study found that most *E. necator* isolates had at least two copies of *Cyp51* and up to seven copies, with the copy number being associated with reduced sensitivity. Therefore, investigating these tools to determine sensitivity of *E. necator* is useful. However, it is also useful to monitor changes in sensitivity of populations or samples from the same region over time using both phenotyping and genotyping. Either Sanger or NGS can be used to detect the Y136F mutation, but only NGS can quantify the mutant. While not equating to phenotype data, these types of results can give an early warning of the 'gateway' or precursor mutation for a loss of efficacy to DMIs, for example penconazole reduced sensitivity. This encourages strict adherence to resistance management strategies including fungicide rotation both within and outside the DMI group of fungicides.

There are inconsistencies in the literature regarding measurement of resistance to DMIs. Delye et al. (1997) classified isolates collected from France and Germany as resistant when the MIC was ≥1.0 µg/mL. Using this classification, all of our isolates, including the unexposed population would be classified as resistant. Steden et al. (1994) with isolates from France, Portugal, Germany and Switzerland, reported EC_{50} values for the unexposed group ranged from 0.01 to 1.0, and 0.01 to 3.0 µg/mL for the exposed group. Similarly, the ranges for our two populations overlapped, not revealing a significant difference between the two populations indicating a lack of resistance. Also, 0.5 µg/mL was the largest EC_{50} value for our data, which is six-fold smaller than reported by Steden et al. (1994) for the exposed group and two-fold smaller than the upper end of their unexposed group.

Resistance of *E. necator* to DMIs is reported worldwide, in France and Portugal (Steva et al. 1988), New York, USA (Erickson and Wilcox, 1997), California, USA (Gubler et al. 1994, Ypema et al. 1997), Europe and India (Delye, 1997), Ontario, Canada (Northover and Homeyer, 2001), mid-Atlantic USA states (Colcol et al. 2012), as well as Australia (Savocchia et al. 2004). The poor relationship between phenotype and genotype is consistent with the qualitative or multigenic nature of DMI resistance (Brent, 2007). In addition, differences have been reported between two populations of genetically different groups (group A & B) of *E. necator (Brewer et al. 2011)*, in terms of abundance and life cycle traits e.g. aggressiveness, infection efficiency, number of spores produced, sexual vs. asexual reproduction are reported in France, Italy and Australia (Dufour et al. 2011). In France, DMI resistant isolates mainly belong to genetic group B (Dufour and Corio-Costet, 2013) and this group has double resistance in grape powdery

mildew i.e. DMI and QoI. In Australia, two genetically distinct groups, A and B (or I and III), were originally identified because of their marked differentiation with anonymous, dominant, multilocus genetic markers (Gadoury et al. 2012), however any differences in resistance status between groups A and B in Australia are not known.

Increased usage of DMIs results in decrease sensitivity of *E. necator* over time and reduced field efficacy. Y136F is highly correlated to the resistance of *E. necator* to DMIs (Delye et al. 1997, Vielba-Fernández et al. 2020).Rallos and Baudoin (2016) found that some isolates possessed both alleles mutant (Y136F) and wild type (Y136), which demonstrates gene duplication or increased gene copy number and possibly a requirement for at least one mutant copy of *Cyp51* for resistance.

For the Group 5 Amines (morpholines), most of the *E. necator* isolates were sensitive to spiroxamine (Prosper) and only 17% had reduced sensitivity (based on the % of fungal growth). The resistance mechanism is unknown for this group so no genetic analysis could be performed. Currently, the only fungicide registered worldwide against grapevine powdery mildew is spiroxamine (Kunova et al. 2021). Miller and Gubler (2004) evaluated 36 Californian isolates of *E. necator* and reported a mean EC_{50} of 0.365 µg/mL, data in this study showed that Australian isolates had 0.2 µg/mL EC_{50} value indicating that they were sensitive to spiroxamine compared to Californian isolates. Morpholine fungicides showed cross-resistance between fungicides, but no cross-resistance to DMIs or other fungicide groups was reported. Similarly to DMI, resistance to amines is presumed to be multigenic and of quantitative type. Regular monitoring in Europe reported a stable situation with low resistance factors towards amines and only small regional fluctuations close to the baseline (FRAC, 2021). The molecular basis of the reduced sensitivity in *E. necator* and other pathogens to amines has not yet been elucidated. However, phenotyping is required to continue monitoring for sensitivity shifts.

For the Group 13 azanaphthalenes (proquinazid and quinoxyfen), 58% of *E. necator* isolates had reduced sensitivity and three of them were reported with field failure and had high growth rate at 20 µg/mL of proquinazid (Talendo). The resistance was expected as the isolates came from regions with high disease pressure and high application of fungicides. However, testing with field rate has not been performed to determine the field rate resistance. Currently only proquinazid, which was approved in 2010, is registered for use in Australian agriculture. The exact mode of action of azanaphthalenes is not known, but the studies from barley powdery mildew suggest that they interfere with the appressorium development and perception of host-derived signals required for correct differentiation of the germinating spore (Kunova et al. 2021).

Quinoxyfen resistance was detected in six isolates (one from WA and SA and four from Vic), which grew at the field rate concentration of Legend. Quinoxyfen-resistant *E. necator* was detected in one vineyard in Virginia, USA experiencing difficulties with powdery mildew control (Colcol and Baudoin, 2016). Quinoxyfen resistance has medium risk of development, and cross-resistance was found in *E. necator* but not in *Blumeria graminis* (FRAC, 2021). As the mechanism of resistance is unknown, phenotyping is necessary to monitor resistance in Australia.

Erysiphe necator isolates tested against SDHIs showed reduced sensitivity to boscalid and pydiflumetofen, however no mutation has been detected in all isolates. In 2014, a single isolate from a single vineyard in Italy with decreased sensitivity to fluxapyroxad was detected (Cherrad et al. 2018) and then spread to French populations of grapevine powdery mildew. Two mutations in the SdhB subunit were identified: H242R/Y and I244V, and one in SdhC, G169D/S (Cherrad et al. 2018). The H242R mutation confers resistance to boscalid and it has no or little impact on the effectiveness of fluopyram and fluxapyroxad (Cherrad et al. 2018, Kunova et al. 2021).

Colcol and Baudoin (2016) tested 160 isolates with boscalid (group 7) and found the MIC for these was ≤ 0.3 µg/mL and all were considered sensitive. Wong et al. (2003) reported baseline sensitivities to boscalid for 33 isolates of *E. necator* obtained from California; the mean EC_{50} was 0.02 µg/mL and the range from 0.039 to 0.052 µg/mL. The EC_{50} for 10 isolates tested in our study was greater than the highest value found by (Wong et al. 2003), however, their study was carried out using hyphal growth on fungicide treated leaf discs rather than the same *in vitro* disc assay used in this study and by Colcol and Baudoin (2016). Differences in methodology make comparing results difficult and not always accurate. Although, Colcol and Baudoin (2016) consider that boscalid resistant isolates of *E. necator* have not been found anywhere, resistance has been detected for other pathogens including *Botrytis cinerea* and *Podosphaera xanthii,* the cause of powdery mildew on cucurbits (Colcol and Baudoin 2016). Two isolates, from Wagga Wagga and Mornington Peninsula, may have a decrease in sensitivity to group 7 fungicides as their MIC was 1.0 µg/mL, and the EC_{50} of 146 (0.36 µg/mL), which was just above the threshold of 0.3 µg/mL determined by Colcol and Baudoin (2016). However, these isolates with higher EC_{50} and MIC values still fall within the normal distribution for sensitive isolates. These results could be used in future studies to assist in determining resistance factors. However, of the isolates in this study, many may have been exposed to group 7 fungicides in the past and most have been exposed to other synthetic fungicides. Four were considered unexposed, however, three of these were obtained from vineyards that were located close to vineyards that use synthetic fungicides. It is important that to determine baseline sensitivity levels, isolates have never been previously exposed to the fungicide group in question.

Testing results showed that 59% of *E. necator* isolates showed reduced sensitivity to Group 50 pyriofenone in this study. Cross-resistance with metrafenone (Vivando) was reported by (Kunova et al. 2016). However, based on the parameters used by Kunova et al. (2016) all tested isolates (in this study) were sensitive, but continuous monitoring is required.

Generally, *E. necator* populations have shown reduced sensitivity to major fungicide groups including QoIs, DMIs, Spiroxamine, proquinazid and quinoxyfen, boscalid and pydiflumetofen and pyriofenone. No resistance was detected for all groups except group 13, and further monitoring is required to identify shifts in the sensitivity.

5.2. *Botrytis cinerea*

5.2.1. Introduction

Botrytis bunch rot (BBR) is caused by the generalist necrotrophic fungal pathogen *Botrytis cinerea*. Along with cultural practices, the main approach to control *B. cinerea* in vineyards is through the routine application of fungicides (Elad et al. 2016). Due to the high risk nature of resistance development in *B. cinerea*, regular monitoring of *B. cinerea* resistance frequency is critical to provide current information for resistance management strategies. Phenotyping of single spore *B. cinerea* isolates is a simple and non-ambiguous method accepted by the international scientific community for the monitoring of resistance frequencies. In this study, 456 single spore *B. cinerea* isolates were sampled from 48 vineyards in WA, SA, Vic, and NSW. These isolates were tested phenotypically for the presence of resistance on discriminatory concentrations (DCs) of three fungicides currently critical to *B. cinerea* control programs in Australia, fenhexamid (FRAC group 17), fludioxinil (FRAC group 12) and pyrimethanil (FRAC group 9). Previous studies in Australia have characterised resistance to FRAC group 9, 12 and 17 in *Botrytis* from grapes (Harper et al. 2021, Sergeeva et al. 2002). Genotypic analysis on a subset of resistant isolates was completed to identify genotypes associated with group 9 and group 17 resistance. Mutations in *Pos5* or *Mdl1* (*Pos5* - V273I/L, P293S, P319A, G408R/V, L412F/S/V, *Mdl1* – E407K) and *Erg27* (F412C/I/S/V) have been previously associated with resistance to group 9 and 17 fungicides, respectively (Fillinger et al. 2008, Grabke et al. 2013, Mosbach et al. 2017, Harper et al. 2021).

5.2.2. Methods

5.2.2.1. Phenotyping

A previous report established DCs for group 9, 12 and 17 fungicides to characterise resistance in *B. cinerea* isolates from wine grapes (Harper et al. 2021). The sampling and phenotyping protocols used in this report are based on protocols described in Harper et al. 2021. Briefly, swabs inoculated with actively grown *Botrytis* from wine grapes were swabbed onto potato dextrose agar (PDA) and any apparent *B. cinerea* was sub-cultured onto additional PDA plates. The resulting plates were exposed to a 12h dark/light cycle under near-UV light to induce sporulation, and single conidia were collected using a sterile 25G hypodermic needle. The resulting isolates were stored as mycelial agar plugs at 4°C. During the 2017–2022 seasons, a total of 456 single spore isolates across 48 vineyards were collected from WA, SA, Vic, and NSW. To phenotype each isolate, each isolate was initially placed on yeast soluble starch agar (YSSA) or YSSA minus yeast extract for testing against group 12/17 or group 9 fungicides, respectively (Fillinger et al. 2008). Two 4 mm plugs were taken from the edge of the actively growing colonies and placed on 0.4 µg/mL pyrimethanil, 0.1 µg/mL fludioxonil and 1 µg/mL fenhexamid. Plates were incubated for three days in the dark, and then scored on their ability to grow on each fungicide concentration.

5.2.2.2. Genotyping

In this report, the genotyping techniques included, qPCR, Sanger sequencing and cleaved amplified polymorphic sequence (CAPS) testing. For qPCR, primer details and annealing temperatures are described in Table 5.40. The assays were designed from the B05.10 *Pos5* sequence (*B. cinerea* B05.10 chromosome 10; CP009814) and *Erg27* (AY220532), respectively. All amplification primers were ordered from Macrogen Inc. (South Korea). The qPCR reaction mixture was as follows; 10 µL 2X SensiFAST™ probe no-rox mastermix (Bioline, U.K.), 0.25 µL of each probe (5 µM each), 1 µL of primer mixture (5 µM of each primer), 4 µL of DNA and 5.5 µL of water The qPCR cycle protocol was as follows, 95°C for 5 min, followed by 40 cycles of 95°C for 10s and 63 - 64°C for 30s (Table 5.40). All assays were optimised and tested with replicates in either a magnetic induction cycler (MIC) qPCR instrument (Bio Molecular Systems, Australia) or CFX96 (Bio-Rad laboratories, U.S.A.)

thermocycler. To establish the conditions that allowed probe specificity in each assay, a range of annealing temperatures was tested using the above reaction mixture and qPCR cycle parameters. To investigate primer efficiencies, standard curves were constructed by plotting control DNA dilutions against Cq values (Figure 5.22). To extract control DNA, isolates Bc-7 (WT) and Bc-396 (L412F, F412S) were sporulated by sub-culturing onto potato dextrose agar (PDA), and then exposed to near-UV light (12h light cycle) for 7–10 days (Harper et al. 2019). Conidia were harvested by flooding petri plates with dH_20, were filtered in syringes containing glass wool and were then washed twice with dH_20 (Harper et al. 2019a). The harvested conidia were freeze dried and DNA was extracted from the conidial biomass using the BioSprint 15 instrument and BioSprint DNA Plant kit (QIAGEN) according to the manufacturer's instructions (Harper et al. 2019a) To screen resistant isolates for the L412F and/or F412S genotype, the qPCR method used DNA template extracted using a quick extraction method adapted from Dodhia et al. (2021). To extract DNA, isolates were first sporulated Approximately 0.2 cm^3 of sporulated biomass was scraped off and placed in a 1.7 mL tubes containing 400 µL of quick extraction buffer (0.5 % w/v SDS, 5 % w/v $NaCl_2$). The mixture was hand ground for approximately 30 s with a micro-pestle and then diluted 100-fold in TE buffer (pH 8.0). qPCR analysis was carried out as described above to determine the presence/absence of each genotype. For Sanger sequencing, *Pos5*, *Mdl1* and *Erg27* 'hot spot' regions were amplified using PCR and subsequent products were sent to Macrogen Korea for sequencing. DNA extraction was based on a quick extraction method described above. PCR amplification of *Pos5*, *Mdl1* and *Erg27* was undertaken as described by Harper et al. (2019), with primers and annealing temperatures described in Table 5.41. A cleaved amplified polymorphic sequence (CAPS) based method using the *Mbo* II enzyme was used to rapidly test isolates for the presence or absence of non-synonymous changes at residue 412 in the *Erg27* gene. The WT *Erg27* 412 amplicon contains a *Mbo* II site, while the site is absent in a mutant amplicon (F412C/I/S/V). If the F412S qPCR assay failed, the PCR product from the failed reaction was digested with *Mbo* II.

Table 5.40. Primer and annealing temperature details for allele specific qPCR assays to characterise L412F and F412S genotypes.

Assay name (mutation target, gene, FRAC group)	Primer name	Sequence (5'-3')	Annealing temperature (°C)
BcL412Fv2 (L412F, *Pos5*, 9)	BcL412-HEX	5HEX/TGAATGG+GT+T+G+CTGAAGTT/3IABkFQ/	64
	BcL412F-FAM2	FAM/CTTGAATGG+GT+T+T+CTGAAGTTCAA/3IABkFQ	
	Bcpos5-412-F2	CAAGGCGAGGAAATGGTAAA	
	Bcpos5-412-R2	CATTCAATCGATCTCGGACA	
BcF412Sv2 (F412S, *Erg27*, 17)	BcF412-HEX	5HEX/ACAGATTGATC+T+T+CTACCTTGTAAG/3IABkFQ	63
	BcF412-FAM2	56-FAM/AGATTGATC+T+C+CTACCTTGT/3IABkFQ	
	Bcerg27-412-F2	ATCATGCCCTTGAATTTTGT	
	Bcerg27-412-R2	AGTGTAAGGCTTGATGGTAT	

Table 5.41. Primers used for amplification of *Pos5* and *Erg27* residue 412 regions.

gene	primer name	Purpose	primer sequence (5′-3′)	product size (bp)	annealing temperature (°C)	extension time (s)	reference
Pos5	pos5 2F	Sanger sequencing	GTACTCTAGGGTTCTTGG	1031	58	60	Harper et al 2022
	pos5 5R		AAATAGCAGGGTTTCTGGG				
Mdl1	mdl1 2F	Sanger sequencing	GGAGAACGAATTGTAGCC	1140			Harper et al 2022
	Mdl1 5R		TGTATCCAGACCTTCAGG				
Erg27	erg27 F3	Sanger sequencing, *Mbo* II CAPS	ACCAGCAGCAAAATTGATAA	198		20	This study
	erg27 R3		GGAGATAATGCTACCCAGA				

5.2.3. Results

5.2.3.1. Phenotyping

Phenotypic results from the DC agar screen are shown in Figure 5.21 and Table 5.42. Frequencies on a per MOA basis were 10.1, 5.9 and 5.9% for group 9, 12 and 17, respectively (Figure 5.21). Seventy-one (15.5%) out of the 456 isolates screened exhibited resistance to at least one fungicide (Table 5.42). The total number of resistant isolates across the wine regions in WA, SA, Vic, and NSW, ranged from 3.8 – 34.4, 0 – 100, 0 – 68.4 and 0 – 25%, respectively. The three most common phenotypes were single resistances to FRAC group 9, 12 and multi-resistance to 9 and 17. The remaining phenotypes: single resistance to group 17 and multi-resistance to 9 and 12, and 9, 12 and 17 were uncommon (*n* = 8). Out of 71 resistant isolates, 26 were resistant to more than one fungicide simultaneously (multi- resistance). No multi-resistance to group 12 and 17 was identified.

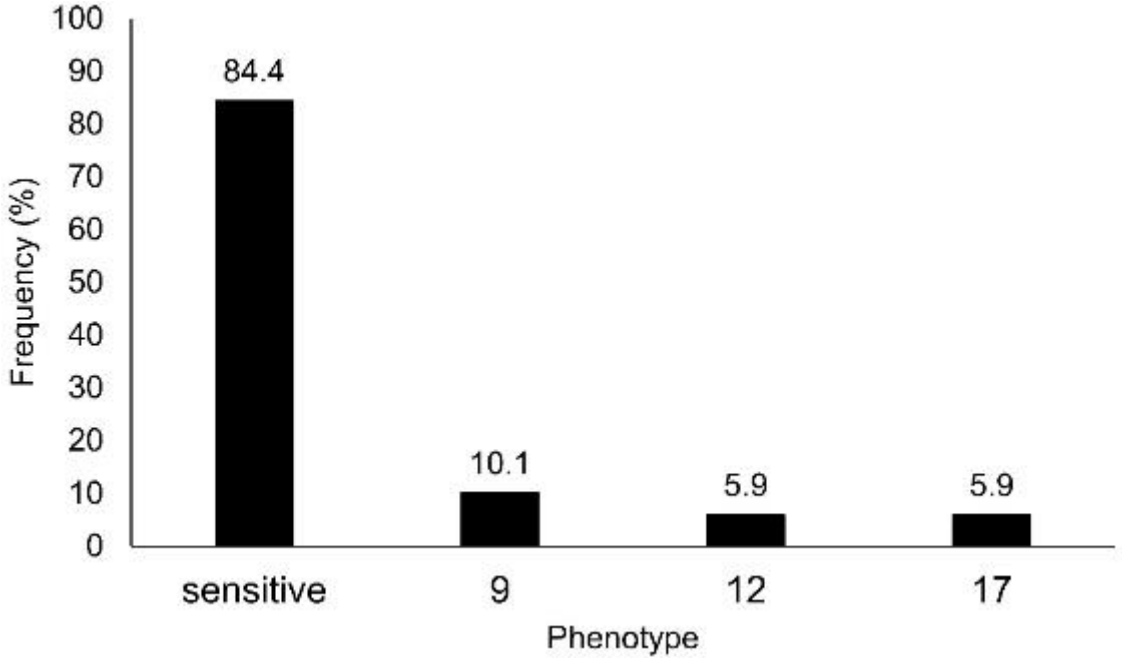


Figure 5.21. Resistance frequency on a per MOA basis for FRAC group 9, 12 and 17. Percentages are indicated above each column.

Table 5.42. Resistance frequency for pyrimethanil (9), fludioxinil (12) and fenhexamid (17) for 456 *B. cinerea* isolates sampled from WA, SA, Vic, and NSW during the 2017 – 2022 seasons.

	Wine region	Vineyards sampled	Isolates tested	Sensitive	9	12	17	9 + 17	9 + 12	9 + 12 + 17	No. of resistant isolates (%)
WA	Margaret River	4	106	102	0	1	2	1	0	0	4 (3.8)
	Great Southern	1	11	10	1	0	0	0	0	0	1 (9.1)
	Pemberton	4	64	42	6	4	0	12	0	0	22 (34.4)
SA	Langhorne Creek	1	2	2	0	0	0	0	0	0	0
	Adelaide Hills	1	2	2	0	0	0	0	0	0	0
	Padthaway	1	2	0	2	0	0	0	0	0	2 (100)
	Coonawarra	1	23	23	0	0	0	0	0	0	0
	Barossa Valley	3	7	7	0	0	0	0	0	0	0
Vic	King Valley	13	95	71	8	16	0	0	0	0	24 (25.2)
	Alpine Valleys	2	19	6	1	1	1	5	2	3	13 (68.4)
	Rutherglen	1	6	6	0	0	0	0	0	0	0
NSW	Hunter Valley	6	37	37	0	0	0	0	0	0	0
	Tumbarumba	1	8	6	0	0	0	2	0	0	2 (25)
	Riverina	9	74	71	2	0	0	1	0	0	3 (4.1)
	Total (%)	48	456	385	20	22	3	21	2	3	71 (15.5)

5.2.3.2. Genotyping

Standard curves for the two assays are shown in Figure 5.22. At each respective annealing temperature, only the target DNA was amplified for each probe (data not shown). Thirty-seven isolates resistant to group 9 and 18 isolates resistant to group 17 were genotyped (Table 5.43). The V273I (*Pos5*) change was identified in isolates from Padthaway and Alpine Valleys. The *Pos5* changes, P293S and P319A were identified in isolates from Padthaway (SA) and Alpine valleys (Vic), respectively. The L412F (*Pos5*) change was present across all wine regions except Padthaway. The L412V (*Pos5*) change was found in Padthaway. Several resistant isolates from the King Valley and Alpine Valleys were WT in the targeted regions of *Pos5* and *Mdl1*. The F412S (*Erg27*) change was identified from isolates originating from Margaret River, Pemberton, Alpine valleys, Tumbarumba and the Riverina. The additional changes F412I and F412V were identified in isolates from Pemberton and Margaret River, respectively.

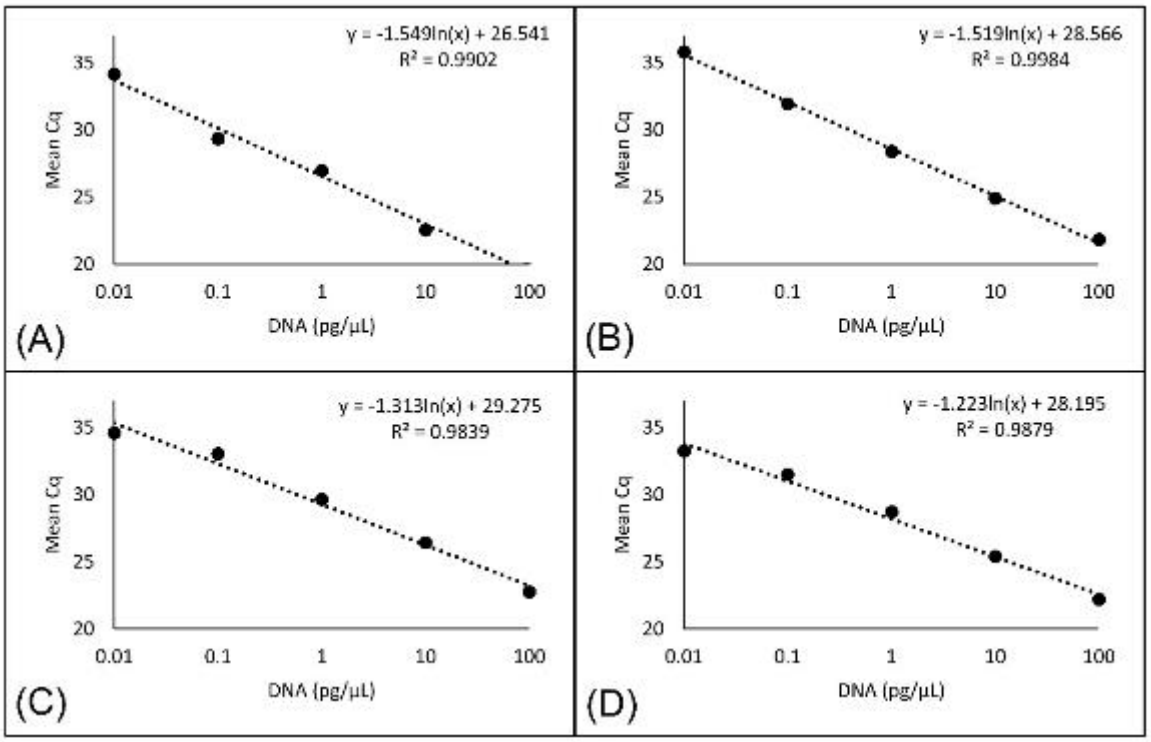


Figure 5.22. Standard curves for *B. cinerea Pos5* and *Erg27* (group 17) duplex assays against WT and mutant DNA. A = BcL412Fv2 with WT DNA, B = BcL412Fv2 with L412F DNA, C = BcF412Sv2 with WT DNA, D = BcF412Sv2 with F412S DNA

Table 5.43. *Pos5* and *Erg27* genotypes characterised in all resistant isolates across each wine region. Numbers indicate the number of isolates which a particular genotype. WA = Western Australia, SA = South Australia, Vic = Victoria, NSW = New South Wales, * = Sanger sequencing, ** = qPCR, * = *Mbo* II CAPS, - = not tested.**

			Margaret River (WA)	Great Southern (WA)	Pemberton (WA)	Padthaway (SA)	King Valley (Vic)	Alpine Valleys (Vic)	Tumbarumba (NSW)	Riverina (NSW)	Total
group 9		isolates tested	1	1	18	2	8	11	2	3	46
	Pos5	V273I	-	-	-	2	-	2	-	-	4
		P293S	-	-	-	-	-	1	-	-	1
		P319A	-	-	-	-	-	1	-	-	1
		L412F**	1	1	14	-	2	5	2	2	27
		L412V	-	-	1	-	-	-	-	-	1
	Mdl1	E407K	-	-	3	-	3	-	-	-	6
		G408R	-	-	-	-	-	-	-	1	1
	Pos5/Mdl 1	WT*	-	-	-	-	3	2	-	-	5
group 17		isolates tested	3	-	12	-	-	9	2	1	27
	Erg27	F412I*	-	-	1	-	-	-	-	-	1
		F412S**	1	-	5	-	-	5	2	1	14
		F412V*	1	-	-	-	-	-	-	-	1
		F412C/I/S/V***	1	-	6	-	-	4	-	-	11

5.2.4. Discussion

In this report, resistance screening of a population of 456 *B. cinerea* isolates showed resistance to pyrimethanil (group 9), fludioxonil (group 12) and fenhexamid (group 17), at frequencies of 10.1, 5.9 and 5.9%, respectively. These frequencies are either similar or lower than previous wine grape reports from Germany, France and New Zealand (Leroch et al. 2011, Walker et al. 2013, Beresford et al. 2017). However, these frequencies are higher than reported in wine grape studies from Italy (Campia et al. 2017, Toffolatti et al. 2020). A previous Australian wine grape study from 1998 – 2001 (*N* = 910), showed a group 9 frequency range between 0–14% across the seasons sampled (Sergeeva et al. 2002). A more recent Australian wine grape report with isolates collected between 2013 – 2016 (*N* = 725) reported resistance frequencies to group 9, 12 and 17 fungicides of 7.7, 6.2 and 2.1%, respectively (Harper et al. 2021). Similarly, the frequencies in this report are also low, but with group 9 and 17 frequencies higher than reported in Harper et al. (2021).

The low overall frequency observed in this study could be due to any number of reasons. The recommended two sprays per season limit for group 9, 12 and 17 fungicides in Australian vineyards could have a significant role in maintaining low frequencies (Harper et al. 2021). Additionally, potential fitness costs in isolates resistant to group 12 or 17 could contribute to suppressing frequencies (Billard et al. 2011, Ren et al. 2016, Saito et al. 2013, Zhao et al. 2010, Ziogas et al. 2005). Low sample size (e.g., SA, *n* = 36), could impact the adequate estimation of resistance frequencies.

Some intensely sampled wine regions (e.g. Pemberton and King Valley) are showing higher frequencies than the overall frequency (Table 5.42). These regions are a priority for further monitoring. Additional monitoring is required in all wine regions to better assess the resistance frequency for these key compounds in wine grape production. Of major interest, is the potential emergence of isolates in Australian vineyards with medium to high resistance (MR and HR) to the PP fungicide fludioxonil. Currently the resistance factor for fludioxonil resistance in Australian grape isolates is very low (RF = 2), so these have been described as low resistance (LR) isolates (Harper et al. 2021). However, in Chile, China and the USA, fludioxonil resistant isolates with RF values between 8 - 88 have been described (Fernández-Ortuño et al. 2014, Latorre and Torres, 2012, Ren et al. 2016, Sang et al. 2018). Thus, monitoring of resistance factors of fludioxonil resistant isolates is also needed to see whether highly resistant fludioxonil *B. cinerea* populations will emerge in Australian vineyards.

In this report, the L412F change was widely associated (59%) with group 9 resistance and was reported in Harper et al. (2021). Mosbach et al. (2017) characterised the L412F change as the most common genotype (68%) across a collection of grape and strawberry isolates. This is the first report of the P293S (*Pos5*), E407K (*Mdl1*) and G408R (*Mdl1*) changes in Australian vineyards. These mutations have been previously reported in strawberries and grapes (Mosbach et al. 2017). In this report, isolates with mutations simultaneously in *Pos5* and *Mdl1* were not found. Five isolates were found to have no changes in the *Pos5* and *Mdl1* regions that were characterised in this study. Mosbach et al. (2017) also described group 9 resistant isolates without changes in these genes. Harper et al 2021 previously reported the F412S genotype in Australian vineyards. In this study, F412S was found across several wine regions. This is the first report describing the additional changes F412I/V in Australian vineyards. These additional *Pos5*, *Mdl1* and *Erg27* genotypes provide further targets for the development of molecular assays for high throughput detection within the laboratory or in a rapid in-field detection pipeline.

5.3. *Plasmopara viticola*

5.3.1. Introduction

Plasmopara viticola is an obligate host-specific biotroph that causes grapevine downy mildew disease in grapevines. First reported in Australia in 1917, *P. viticola* spread rapidly across eastern Australia in 1918-21, and then later to Tasmania in 1959 and Western Australian 1997 (Emmett, 1992, Taylor et al. 2019). Chemical control is the most effective measure to protect grapes from downy mildew disease. However, resistance to single-site fungicides is one of the main issues in downy mildew management (FRAC pathogen risk list, www.frac.info; (Maddalena et al. 2020). There are several international reports of resistance to different fungicide mode of action (MOA) groups such as Quinone outside Inhibitor (QoI) fungicides, Carboxylic Acid Amide (CAA), fungicides (Quinone outside Inhibitor, and stigmatellin binding type (QoSI). Phenylamide (PA) and carboxylic acidamide (CAA) fungicides. Fungicide resistance has been detected in several vineyards of WA, VIC and NSW (Hall et al. 2017).

The aim of this study was to measure the sensitivity of Australian isolates of *P. viticola* collected from all winegrape growing states to the fungicides from the four MOA groups (QoI, CAA, QoSI and PA) used in Australia to control downy mildew.

5.3.2. Material and methods

5.3.2.1. Propagation of glasshouse-grown grapevines

To provide a constant supply of disease-free leaves for the mass production of *P. viticola* sporangia, grapevines, *Vitis vinifera* cv. Sultana, was propagated from rooted cuttings and grown in a greenhouse at the Waite Campus, Urrbrae, South Australia. Initially, two-bud cuttings were inserted into holes made in styrofoam trays. The trays were floated on water inside 50 L plastic tubs. After four weeks, once shoots had formed from the buds and the cuttings had developed roots, they were transferred into 4.5 L pots containing a mixture of potting media comprising two parts bio grow (van Schaik's, South Australia), one part cocopeat and one part University of California (UC) mix. The plants were fertilised fortnightly with a completely water soluble fertiliser (1:200 Moeco 30® (NPK 12%:3%:8%; Moeco Pty Ltd, Australia) or Manutec soluble foliage feeder (NPK 25%:4.5%:12%; Fertool Pty Ltd, Australia). During the autumn and winter months, active shoot growth was maintained by providing a 1 h photoperiod, from 00.00 to 01.00 h (330 $\mu E\ s^{-1}m^{-2}$ from cool white fluorescent bulbs) positioned above the plants. The plants were watered as required and sprayed with either Microthiol Disperss (800 g/kg sulfur, Nurfarm) or EcoCarb (Eco-Fungicide 940 g/kg potassium bicarbonate, Organic Crop Protection, NSW, Australia) to control powdery mildew when required. The predatory mite, *Phytoseiulus persimilis* (Biological Services, Riverland, South Australia) was used to control two-spotted spider mite (*Tetranychus urticae*) alternating with Eco-Oil (850 g/L Emulsifiable Botanic Oil, Organic Crop Protection, NSW, Australia) as required. Flying insects, including, white flies and aphids were controlled by sticky aphid and white fly traps (Bugs for Bugs, Mundubbera, Queensland).

5.3.2.2. Collection of downy mildew infected grapevine material

Diseased leaves were collected from commercial vineyards in South Australia (SA), New South Wales (NSW), Victoria (Vic), Western Australia (WA), Tasmania (Tas) and Queensland (Qld) between 2017 and 2022. Samples were mostly collected by agronomists who sampled from disease hot-spots, particularly where they considered that decreased fungicide sensitivity may be an issue. Samples were collected at random to be representative of an area or vineyard. Sample collection, storage and transport protocols were amended from those used in SAR 1204 (Hall et al. 2017), which improved sample viability on arrival at the laboratory in the current project. The improvement included adding several paper towel layers between leaves to maintain the freshness of the leave and transporting the sample as quick as possible to the laboratory using refrigerated iceboxes. A protocol has been developed and documented (Appendix 1) in this project and was sent to prospective collaborators offering to send samples.

5.3.2.3. Isolation and establishment of *P. viticola* isolates

Upon sample receival, symptomatic leaves were divided into three sub-samples. Two sub-samples were used for isolation of *P. viticola* and the third was stored at 4°C as backup material. The two isolation methods are described below:

A. Petri-dish method

Infected leaves were floated with the underside facing upwards on the surface of sterile reverse osmosis (SRO) water contained in a 190 mm diameter glass petri dish. The leaves were sprayed with a fine mist of SRO water and the petri dish lids replaced. Dishes were then incubated overnight in the dark at room temperature to encourage production of sporangia (Figure 5.23). Sporangia (containing zoospores) were retrieved by placing the leaves in a 50 mL sterile tube containing 5-10 mL of SRO water and shaken for 30 seconds to make a sporangial suspension comprising a mixed population of *P. viticola*. The suspensions were placed in a 30 mL atomiser ready for inoculation.



Figure 5.23. Petri dish method used for culturing *Plasmopara viticola*, with a grapevine leaf (cv. Sultana) floating upside down on the surface of sterile reverse osmosis water contained in a glass Petri dish. Sporangia of *P. viticola* are visible on the leaf surface.

B. Bag test procedure

The bag test procedure, widely used for rapid *P. viticola* isolation, was described by Department of Primary Industries and Regional Development's Agriculture and Food, WA (https://www.agric.wa.gov.au/spring/bag-test-downy-mildew-grapes). SRO water was poured into a sealable plastic bag, shaken and the bag was emptied until only a few droplets remained. An infected leaf was placed abaxial side up inside the bag so the lesion(s) faced upwards. Air was blown in by mouth, to expand the bag and prevent any lesions/lesion surface from touching the bag. It was then sealed to maintain high humidity. Bags were placed inside a closed drawer to provide darkness for 24 h at room temperature (Figure 5.24). The following day, leaves were removed and placed inside 50 mL disinfested tubes with 5-10 mL of SRO water and shaken for 30 seconds to make a mixed population sporangial suspension of *P. viticola*.

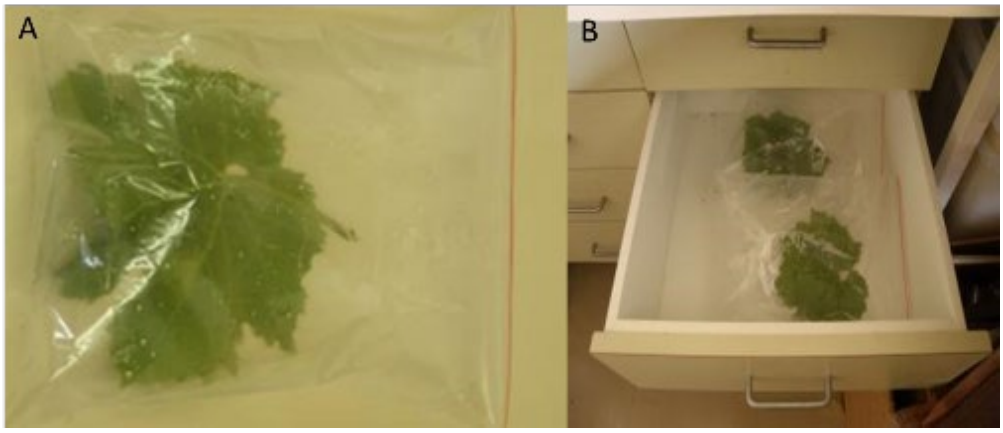


Figure 5.24. Bag test procedure to induce sporulation of *Plasmopara viticola* (A) infected leaf inside a sealed plastic bag that is (B) kept in the dark in a drawer at room temperature for 24 hours.

5.3.2.4. Inoculation and maintenance of *P. viticola* isolates

Two techniques were used to culture *P. viticola* in the laboratory. The first one was the petri dish method as described in section 5.3.2.3 using fully expanded leaves (cv. Sultana) taken from the third to fifth node. For the second method, fully expanded Sultana leaves were placed abaxial side up on water agar (WA) (16% agar) in plastic petri dishes 150 mm diameter, amended with 25 μg/mL Pimaricin (Sigma Chemical Co., St Louis) and 100 μg/mL benzimidazole (Sigma Chemical Co.) (Figure 5.25) (Aoki et al. 2013). For both methods, leaves were inoculated with a *P. viticola* sporangial suspension using an atomiser. Inoculated leaves were placed inside a cupboard or covered with a towel to provide darkness for 24 h at room temperature. The following day lids were removed until the leaves were dry, and then placed back. The plates were incubated on the laboratory bench for up to 10 days with sporulation of *P. viticola* visible between four and seven days. These infected leaves were used to prepare bulk inoculum for sensitivity tests. Following evaluation of both techniques, the water agar method provided more consistent infection than the floating water method, and was more practical, so was used in subsequent experiments. *P. viticola* spores from infected field leaf samples were collected and suspended in SRO water, adjusted to 1 x 10^6 sporangia mL, placed in 1.5 mL Eppendorf tubes and stored at -20°C until required, as described in (Hall et al. 2017).

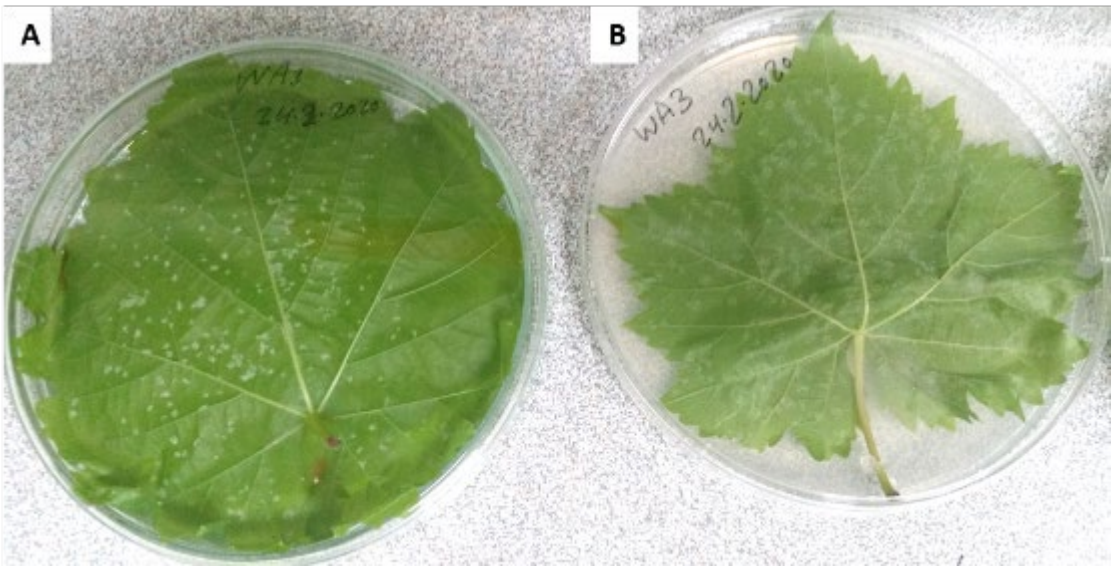


Figure 5.25. Two techniques used to culture the samples from *Plasmopara viticola* on petri dishes; (A) leaf floating on top of sterile reverse osmosis water and (B) leaf placed on top of water agar.

5.3.2.5. Fungicide sensitivity discriminatory dose bioassays

Preparation and application of fungicides

The following commercial products were used in the fungicide sensitivity bioassays: Cabrio® (pyraclostrobin 250 g/L, Nufarm Australia); Ridomil® Gold 450 SL (metalaxyl-M 480 g/L, Syngenta, Australia); Revus® (mandipropamid 250 g/L, Syngenta Australia) and Acrobat® (dimethomorph 500 g/L, BASF, Australia). Technical grade ametoctradin (Sigma Aldrich, Australia) was also tested to represent one active ingredient of Zampro® (300 g/L ametoctradin 225 g/L dimethomorph), ametoctradin was not available as commercial fungicide product therefore technical grade ametoctradin was used. Ametoctradin was stored at 4°C until use in the bioassays. It was dissolved in 1 mL of acetone and diluted with SRO to the desired concentrations. The fungicides were tested at concentrations ranging from 0 to 10 µg a.i./mL and field rate (depending on the fungicide and the year of testing) using SRO water with 0.1% Tween 20 surfactant added immediately prior to each assay (Fontaine et al. 2019). Based on preliminary testing and previous work (SAR 1204; Hall et al. 2017), discriminatory doses were used (Gisi et al. 2007, Wicks et al. 2005), therefore EC_{50} values could not be calculated.

Three fungicide application methods were used in the bioassays; A and B were used in SAR 1204 (Hall et al. 2017) and C was used for the first time in this project:

A. Floating on a fungicide suspension.

Detached leaves were surface-disinfested in 0.5% sodium hypochlorite (White King® bleach) and 0.1% Tween 20 for 60 sec and rinsed three times, for 2 min each time, in SRO water. Leaves were blotted dry with sterile paper towel and 9 mm discs were excised from leaves using a cork borer. Ten leaf discs were placed abaxial side up and floated on the surface of the pre-prepared fungicide suspensions contained in 150 mm glass petri dishes. Inoculation by *P. viticola* was carried out directly on to the surface of the floating discs by pipetting a 10 µL droplet of spore suspension onto each leaf disc. SRO water was used as the non-inoculated controls.

B. Dipping in a fungicide suspension

Leaf discs were placed into sterile beakers containing fungicide suspensions of each concentration, agitated for 1 h (Wong and Wilcox, 2000), then dried in a fume cabinet. Ten leaf discs were placed abaxial surface upwards on to 1.5% water agar (WA) amended with 25 µg/µL pimaricin (Sigma Chemical Co.) and 50 mg/L streptomycin (Sigma Chemical Co.) contained in 60 mm petri dishes. Inoculation was conducted as described in Section 5.3.2.6.

C. Spraying with a fungicide suspension

Detached leaves were sprayed until runoff on both sides with the different fungicides at each concentration. The fungicide droplets were allowed to dry on the leaves before discs were excised using a sterile 9 mm diameter cork borer. Discs were placed, abaxial surface upwards, on WA, then the discs were inoculated by *P. viticola* as described in Section 5.3.2.6.

5.3.2.6. Fungicide sensitivity bioassay

After fungicide application, spore suspensions were obtained as above (see Section 5.3.2.4) and adjusted to between 10^5 and 10^6 spores/mL using a haemocytometer and sprayed using an atomiser. Ten discs were used for each treatment with five discs in each of two 60 mm diameter petri dishes. Each dish was considered a replicate and each disc was a pseudo-replicate. Plates were randomised within plastic trays (Figure 5.26). The discs were incubated overnight at room temperature in darkness, then the droplets of spore suspension were allowed to dry in a laminar flow cabinet. Plates containing the discs were incubated at room temperature in the dark until sporulation was observed at between 5 to 7 days. Leaf discs were visually assessed by measuring the percentage area of *P. viticola* growth on each disc. Following assessment, the infected leaf discs were stored at -20°C or freeze-dried until required, except for those from fungicide spray application method (method 3) where the leaf discs were washed with 2 mL SRO water to collect spores then the suspensions were stored at -20°C.

Growth analyses: The sensitivity of downy isolates was evaluated based on the percentage area of growth on leaf discs compared to the control. An isolate is considered to have reduced sensitivity if substantial growth occurs on leaf discs treated with 1 µg/mL and above of active ingredient of fungicide/s (Fontaine et al, 2019). Based on several pilot studies, a growth percentage threshold of >5% was required to be considered substantial growth. The minimal inhibition concentration (MIC) was also determined using single discriminatory dose as per (Sun et al. 2010, Wicks and Hall, 1990, Wicks et al. 2005).

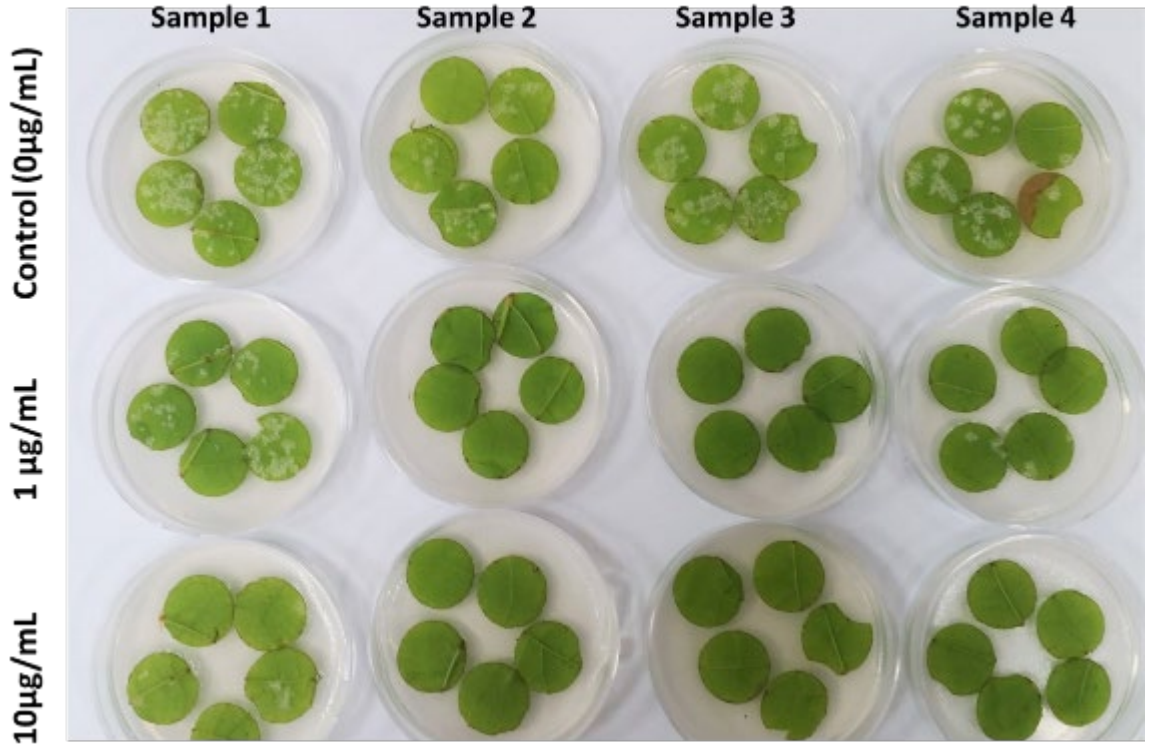


Figure 5.26. The experimental set up for the fungicide sensitivity bioassay for downy mildew

5.3.2.7. Genotyping of *P. viticola* isolates

Sporangia and mycelial solution was collected from each isolate by adding SRO water onto infected leaves and brushing or shaking the leaves inside 50 mL sterile Falcon tubes. Samples were sequenced for cesA3 G1105s region (CAA target) and the *CytB* G143A region (QoI target). For metalaxyl treatment, spores were collected from the control (SRO water), 1 µg/mL and 10 µg/mL and sent for whole genome sequencing to identify the mutation/s if known.

For high-throughput sequencing analysis of resistance amplicons, DNA was amplified using primer pairs specific for either the *CytB* or *Ces3A* locus (Table 5.44) and which surrounded the known resistance mutation hotspots. To increase the sensitivity of the detection, a nested PCR approach was used in which a second amplification step was performed using additional gene-specific primers, to increase the yield of the PCR product. A final PCR step was performed to anneal sequences to each amplicon that were necessary for the Illumina sequencing process.

Following PCR, individual amplicons were pooled at approximate equimolar ratios and sequenced using Illumina chemistry at 2 x 150 bp read length (Ramaciotti Centre for Genomics, NSW, Australia).

Raw sequencing data were processed using an analytical pipeline consisting of quality trimming (Bolger et al. 2014), adaptor trimming (Martin, 2011) and paired-end read merging (Magoč and Salzberg, 2011), with both Illumina and in-line barcodes used to uniquely identify reads that originated from each biological sample. Merged reads were then aligned to a reference DNA sequence for the relevant target locus using Novoalign and the proportion of alleles at each base pair in the amplicon recorded from these read alignments (Koboldt et al. 2012).

Table 5.44. Primers used for HTS amplicon sequencing

Primer	locus	Sequence
Pv_cytB_G143A_292F	*CytB*	TTTAGGGGTTTGTATTACGG
Pv_cytB_G143A_628R	*CytB*	ATGGATTATTTGAACCTACCTC
Pv_cytB_G143A_331F_N701	*CytB*	CCTACACGACGCTCTTCCGATCTTAAGGCGAGAAGCTTTA TGGTGTTCAGG
Pv_cytB_G143A_567R_N701	*CytB*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAGG CGATACAAATGGAAAGGTGAAATG
Pv_ces3A_G1105S_3234F	*Ces3A*	AATTGGTGGCATTGATTCG
Pv_ces3A_G1105S_3607R	*Ces3A*	CACAATTGCATGTCTTTCTCG
Pv_ces3A_G1105S_3340F_N701	*Ces3A*	CCTACACGACGCTCTTCCGATCTTAAGGCGACATGGTCA AGATGAGTATCACG
Pv_ces3A_G1105S_3534R_N701	*Ces3A*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAGG CGAGCAGCATCCCCAGTTTCG

5.3.3. Results

5.3.3.1. Collection of downy mildew infected grapevine samples

In the previous project SAR 1204, 64 samples of downy mildew were received from around Australia between 2013 and 2016, with a further 124 samples received between 2017 and 2022 in the current project (Table 5.45). In project SAR 1204, isolates of *P. viticola* were recovered from 39% of all samples received, and recovery improved to 66% of all samples received from 2017 to 2022.

5.3.3.2. Isolation of *P. viticola*

The petri dish method was useful to induce sporulation, however the dishes containing water were hard to handle resulting in water spilling on the leaves if the dishes were moved. This resulted in tissue degradation, contamination by other microorganisms and less successful isolation of *P. viticola*.

The bag test procedure was simple and easy to carry out, and resulted in abundant sporulation of *P. viticola* with minimal tissue degradation and contamination. It was chosen as the preferred method of isolation for *P. viticola*.

5.3.3.3. Phenotype testing

Three methods of applying the fungicide suspension to leaf discs were evaluated at separate times; A. floating, B. dipping suspension (SAR 1204; Hall et al. 2017) and C. spraying. Hall et al. (2017) reported that the dipping method produced the most consistent results so was chosen for all their subsequent bioassays. Spraying was more practical, less wasteful of fungicide solutions and appeared to provide more consistent results (data not shown), and was more representative of field fungicide application than the other two methods. Isolates were considered to have reduced sensitivity when they grew on MIC of 1 µg, and to be resistant when they grew on the field rate of active ingredient.

Table 5.45. Downy mildew samples received from 2013 to 2022, the number of isolates successfully established and the percentage of isolate recovery.

Year	State	Region	No. samples received	No. isolates established	Recovery (%)
2013-2016	NSW	Hunter Valley	30	12	40
		Wagga Wagga	2	2	100
	Tas	Tamar Valley	5	4	80
		Kayena	1	0	0
	Vic	Yarra Valley	5	3	60
		King Valley	3	0	0
	WA	Margaret River	8	5	63
	SA	Adelaide Hills	2	0	0
		Limestone Coast	6	0	0
		Southern Vales	2	0	0
Total (SAR 1204)			**64**	**25**	**39**
2017	NSW	Hunter Valley	15	0	0
	SA	Clare Valley	2	1	50
		McLaren Vale	1	1	100
	Tas	North/Tas	3	0	0
2018	Vic	King Valley	6	6	100
		Yarra Valley	6	6	100
	SA	Adelaide Hills	2	2	100
2019-2020	WA	Swan Valley	4	3	75
	SA	Adelaide Hills	1	1	100
	Vic	Yarra Valley	9	9	100
2020-2021	NSW	Hunter Valley	19	15	78
	Vic	King Valley	6	6	100
	Qld	Mareeba	5	5	100
2021-2022	NSW	Barooga	5	4	80
		Griffith	20	10	50
		Southern Highlands	2	2	100
	SA	Adelaide Hills	8	6	75
	Tas	Pipers River	4	0	0
	Vic	Yarra Valley	6	5	83
Total 2017-22			**124**	**82**	**66**

Metalaxyl (Group 4, Phenylamide (PA) fungicide)

Figure 5.27 shows bioassay results for samples collected between 2017 and 2022. Reduced sensitivity to metalaxyl has been detected in samples from Qld, NSW, and Vic, and resistance to metalaxyl has been detected in NSW and Vic. However, all samples from SA and WA were sensitive to the fungicide. For NSW, three of four samples from Barooga were resistant to metalaxyl with the fourth sample showing reduced sensitivity, five and four of the 15 samples from Hunter valley showed reduced sensitivity and resistance, respectively. All six samples from Griffith were sensitive. For Qld, two of the four samples collected showed reduced sensitivity. For Vic, all 10 samples from King Valley showed reduced sensitivity and one was resistant, and nine and three samples from Yarra Valley showed reduced sensitivity and resistance, respectively. In SA (seven samples) and WA (one sample), all samples were sensitive.

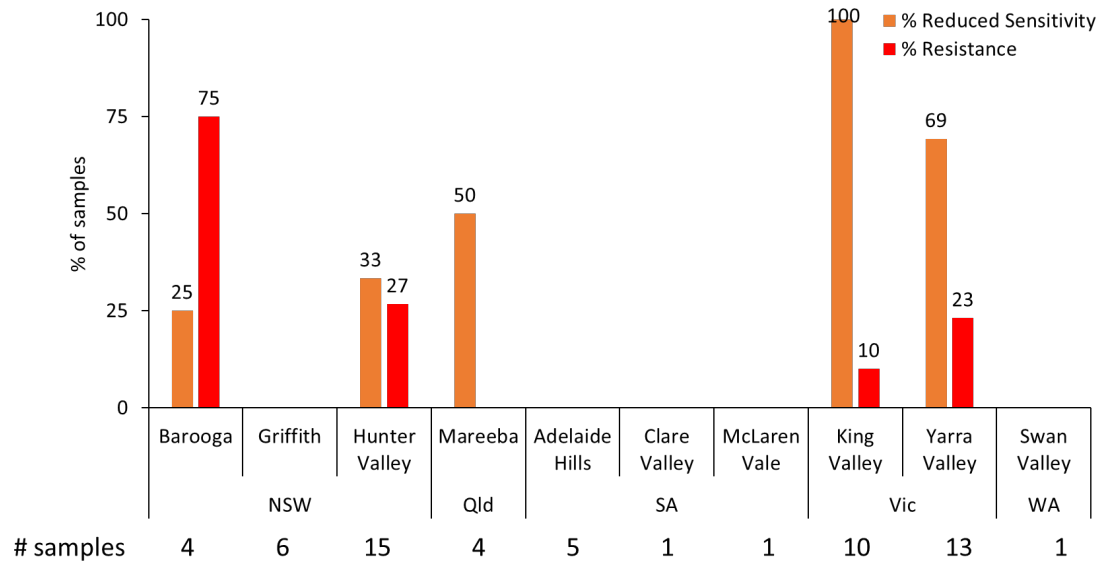


Figure 5.27. Sensitivity of *Plasmopara viticola* samples, collected from different states and regions in Australia between 2017 and 2022, to metalaxyl (Ridomil® Gold, group 4) fungicide. Sensitivity was determined based on growth of the pathogen on grape leaf discs. Samples were considered to have reduced sensitivity when they grew at the minimal inhibition concentration (MIC) of 1 µg a.i. metalaxyl/mL and resistant when they grew at field rate of 50 µg a.i./mL. # samples indicates the number of samples collected from each region.

Mandipropamid (Group 40, Carboxylic acid amide (CAA) fungicide)

Forty-seven samples, from different states around Australia, were tested against mandipropamid during 2020 to 2022 and only one sample from Yarra Valley Vic showed reduced sensitivity with minimal growth at 1 µg/mL on leaf discs. Prior to 2020 no samples were tested with mandipropamid.

Dimethomorph (Group 40, CAA fungicide)

Between 2020 and 2022, 48 samples from four states were tested against dimethomorph. For NSW, all four samples from Barooga, three of six samples from Griffith and two of 15 samples from Hunter Valley had reduced sensitivity to dimethomorph, and four of the 12 samples from Yarra Valley also had reduced sensitivity (Figure 5.28). However, no resistance was detected in any region.

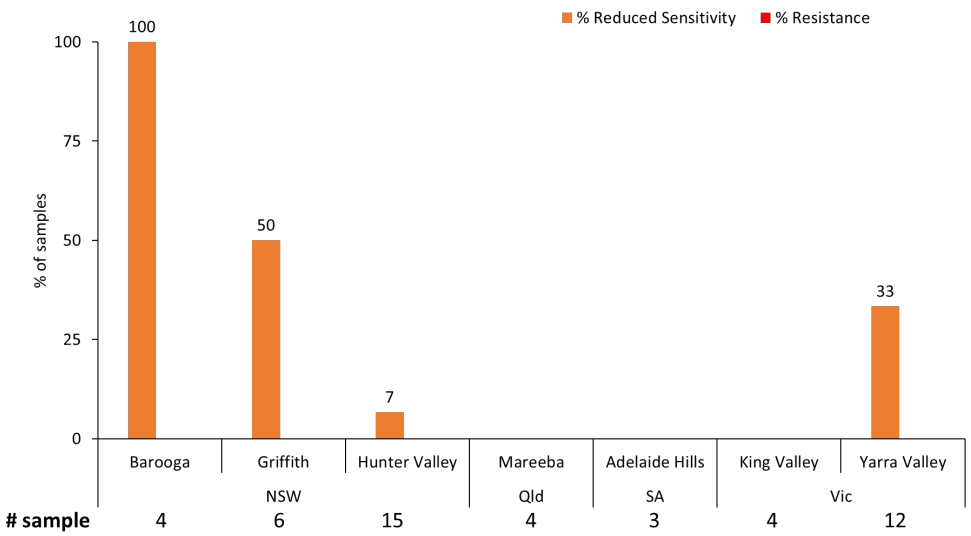


Figure 5.28. Sensitivity of *Plasmopara viticola* samples, collected from different states and regions in Australia between 2020 and 2022, to dimethomorph (Acrobat® group 40) fungicide. Sensitivity was determined based on the growth of the pathogen on grape leaf discs. Samples were considered to have reduced sensitivity when they grew on minimal inhibition concentration (MIC) of 1 µg a.i. dimethomorph/mL and resistant when they grew at a field rate of 180 µg a.i./mL. # samples indicates the number of samples collected from each region.

Pyraclostrobin (Group 11, Quinone outside Inhibitor (QoI) fungicide)

Between 2017 and 2022, 66 samples were tested with pyraclostrobin and 26 had reduced sensitivity and five were resistant. The highest incidence of reduced sensitivity and resistance were in samples from Yarra Valley with 79 and 16%, respectively (Figure 5.29), followed by samples from Hunter Valley with 47 and 7%, respectively. Of the six samples from Griffith, two had reduced sensitivity and one was resistant, however all samples from other regions were sensitive to pyraclostrobin (Figure 5.29).

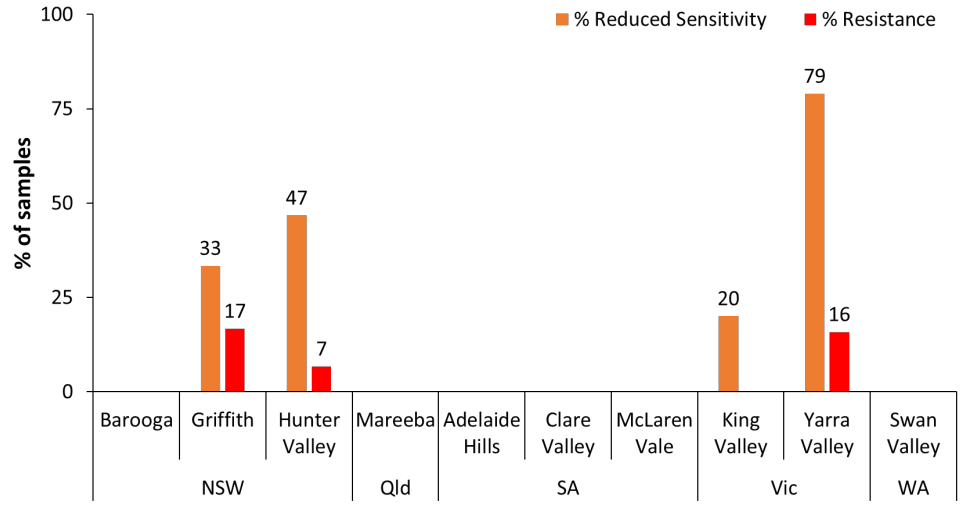


Figure 5.29. Sensitivity of *Plasmopara viticola* samples, collected from different states and regions in Australia between 2017 and 2022, to pyraclostrobin (Cabrio®) (gp 11) fungicide. Sensitivity was determined based on the growth of the pathogen on grape leaf discs. Samples were considered to have reduced sensitivity when they grew on minimal inhibition concentration (MIC) of 1 µg a.i. pyraclostrobin/mL and resistant when they grew on a field rate of 100 µg a.i./mL. # samples indicates the number of samples collected from each region.

Ametoctradin (Group 45, Quinone outside inhibitor stigmatellin binding type (QoSI) fungicide)

Ametoctradin (technical grade) was only tested for a small number of samples in 2020. Six of the 15 samples from Hunter Valley and two of the eight samples from Yarra Valley had reduced sensitivity, however no resistance was detected in any samples (Figure 5.30).

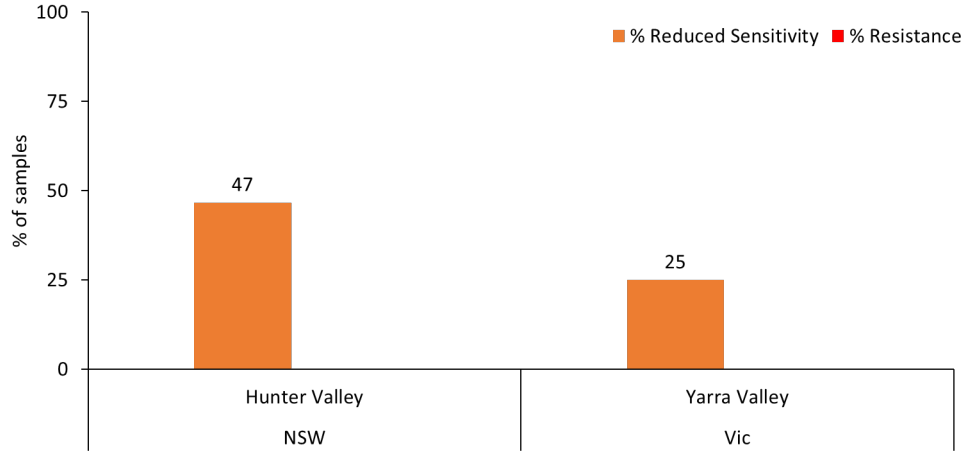


Figure 5.30. Sensitivity of *Plasmopara viticola* samples, collected from two regions in Australia in 2020, to ametoctradin (gp 45) (part of Zampro®) fungicide. Sensitivity was based on the growth of the pathogen on grape leaf discs. Samples were considered to have reduced sensitivity when they grew on minimal inhibition concentration (MIC) of 1 µg a.i. ametoctradin/mL and resistant when they grew at a field rate of 100 µg a.i./mL. # samples indicates the number of samples collected from each region.

5.3.3.4. Genotype testing

From 2019 to 2022, 65 samples were sequenced to identify the G143A mutation in the *CytB* gene for QoI (gp11) resistance and the G11055 mutant in the PvCesA3 gene for CAA (gp 40) resistance (Figure 5.31). Thirty-four samples were detected with the mutation (G143A) with ranging frequency from 1.1 to 100%. The highest number of samples with the G143A mutation came from NSW (20) and Vic (13), with only one sample from Qld, and no mutation was identified in SA or WA. The G11055 mutant was not detected in any sample.

For pyraclostrobin, there was 90% agreement between phenotyping (47%) and genotyping (52%) (Figure 5.32), indicating that molecular detection of the G143A mutation is correlated to the phenotyping bioassay.

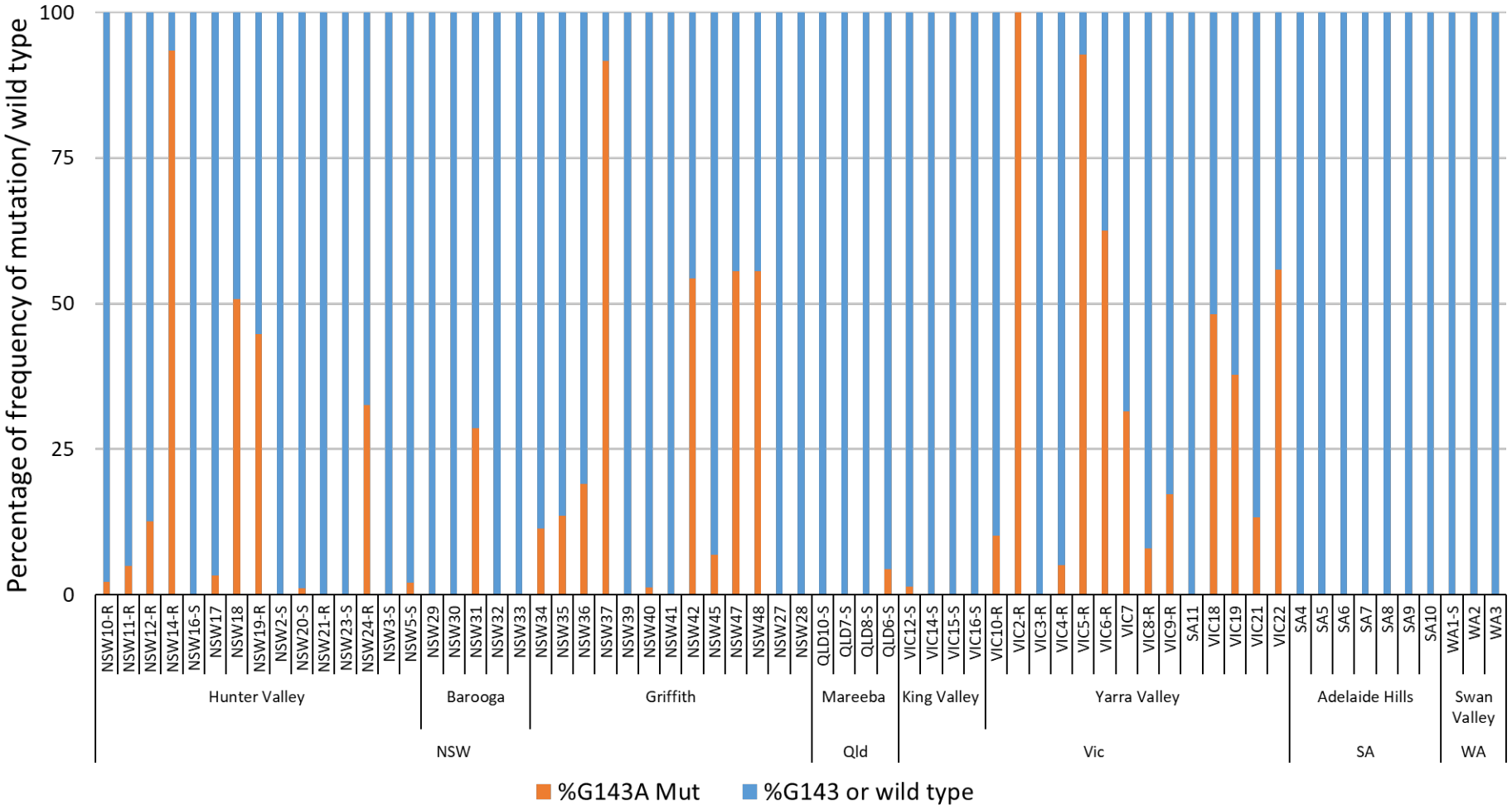


Figure 5.31. Genetic analysis of the *CytB* (G143A mutant) in *Plasmopara viticola* samples collected from different regions around Australia from 2019 to 2022. Next generation sequencing was used to measure the frequency of the G143A mutation and wild type (G143) in each sample.

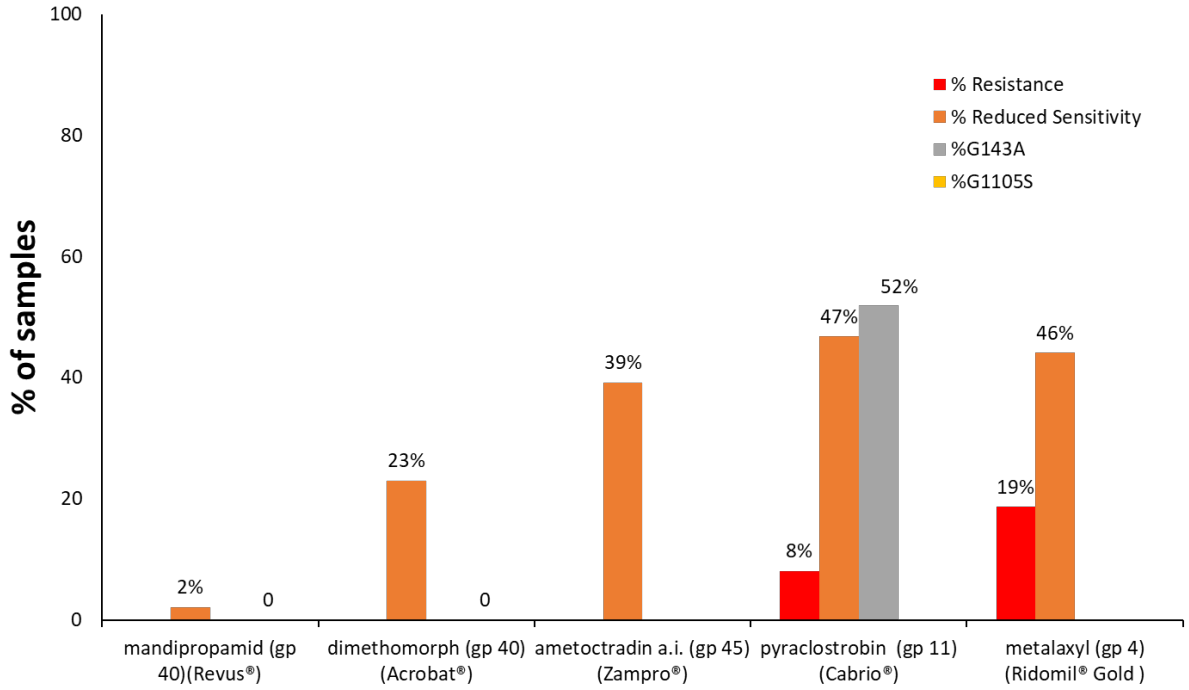


Figure 5.32. Summary of phenotyping and genotyping results of *Plasmopara viticola* samples to five fungicides from four activity groups between 2017 and 2022. Samples were phenotyped for resistance (red) and reduced sensitivity (orange) and genotyped using next generation sequencing for frequency of G143A (grey) and frequency of G1105S (yellow). # samples indicates the number of samples tested for each fungicide.

5.3.4. Discussion

Over the course of this project, modifications to the transport and isolation methods resulted in improved recovery of *P. viticola* from 39% during 2013-2016 (SAR 1204) to 66% in the current project 2017-2022. It is recommended that multiple layers of paper towels and sealed plastic bags in a cool box be used for transportation and a bag test was shown to be a reliable and simple method to isolate the pathogen.

Spray application of fungicides provided consistent results and is considered more reflective of field fungicide application than the other two methods previously used (dipping and floating in fungicide solutions). Also, floating the discs on the fungicide suspension may overexpose *P. viticola* to the fungicide which could result in over-dosing and not be representative of field application. Dipping the discs in the fungicide solution was also more time consuming than spraying. Therefore, it is recommended that spray application of fungicides should be used in all future phenotyping tests.

Fungicide sensitivity was determined for 82 samples of *P. viticola* collected from Australian vineyards to fungicides from four modes of actions. Decreased phenotypic sensitivity was detected in each of the four groups, with detection of the mutation, G143A, found in association with resistance in the QoI fungicide group (Figure 5.32). However, the G1105S mutation has not been detected in any samples.

Metalaxyl had the highest percentage of reduced sensitivity and resistance. The majority of isolates obtained from the King and Yarra Valleys, Vic, had high levels of decreased sensitivity to metalaxyl, growing at 1 and 10 µg/mL. In previous work (SAR 1204), 33% of isolates from the Yarra Valley showed decreased sensitivity to metalaxyl. Although, there appeared to be an increase in the levels of resistance found, particularly in the Yarra Valley, this may reflect

the selection of samples based on poor disease control or field failure. High levels of decreased sensitivity have been found previously in the Hunter Valley (SAR 1204) where 50% of the samples were either resistant or had reduced sensitivity. The sensitivity of *P. viticola* to metalaxyl was originally reported in Australia, when growth of 15 isolates collected from vineyards in SA, NSW, Vic and Qld, were completely inhibited by 1 µg/mL of metalaxyl (Wicks et al. 1987). Resistance was reported in the Hunter Valley region of NSW in 2005 (Wicks et al. 2005). Between 2013 and 2016, 19 isolates were tested against metalaxyl and 67% and 33% of isolates from the Hunter and the Yarra Valley showed reduced sensitivity respectively (SAR 1204; Hall et al. 2017). There was no decreased sensitivity detected in any samples from SA and WA regions, which most likely reflects the lower disease pressure due to drier climatic conditions that are less conducive for infection than in the wetter regions such as the King, Yarra and Hunter Valleys.

Previous research applied concentrations of 1 and/or 10 ug/mL as discriminatory doses to classify resistance (Fourie 2004, Wicks et al. 2005), even though the label rate is equivalent to 48 µg/mL. Using the previous testing methods where leaf discs were floated or dipped in fungicide suspensions, it was not possible to use concentrations higher than 10 µg/mL due to phytotoxicity. However, adopting the spray application method, *P. viticola* can be exposed to field rate concentrations of fungicide, which results in a more accurate estimation of sensitivity status. Wicks et al. (2005) reported resistance to metalaxyl using only 10 µg/mL, whereas Fourie (2004) reported that 92% of 813 samples tested in South Africa were resistant at 100 µg/mL. The minimum inhibition concentration (MIC) is determined using single discriminatory doses and has been widely used to test fungicide resistance in *P. viticola* (Sun et al. 2010, Wicks and Hall, 1990, Wicks et al. 2005). MIC is extensively used in fungicide resistance studies (Giraud et al. 2013). The use of label rate is more representative to field application and so was routinely used in this project.

The mechanism of resistance to metalaxyl is unknown, therefore genotype analysis cannot be carried out. However, research funded by Wine Australia is being undertaken by AWRI, aiming to identify the mutation(s).

Isolates of *P. viticola* were tested for phenotypic sensitivity to two CAA fungicides, mandipropamid and dimethomorph, and then genotyped using Next Generation Sequencing (NGS) to quantify the G1105S mutation responsible for resistance in the CAAs (Blum et al. 2010). Only one isolate from the Yarra Valley grew on 1 µg/mL mandipropamid, with only 8% mean area of growth. All isolates lacked the G1105S mutation, indicating genotypic sensitivity. Further testing is required to confirm whether this isolate has reduced sensitivity. This result might also reflect the mixed population of the sprorangial suspension used in testing. The use of single spore isolates is ideal when testing fungicide sensitivity, however, it is difficult and time consuming to obtain single spore isolates of this oomycete organism. In the previous project (SAR 1204), of the 24 samples tested, a slightly high EC_{50} of 4.95 µg/mL was found for only one isolate from Wagga Wagga (Hall et al. 2017). This may indicate reduced sensitivity to mandipropamid (Gisi et al. 2007), however, genotype analysis was not carried out which would have confirmed if this isolate was showing reduced sensitivity. Revus (a.i. 250g/L mandipropamid) is a relatively new fungicide in Australia (registered in 2014) and these results may suggest a possible emergence of reduced sensitivity to this fungicide, therefore it is important to continue monitoring isolates. Gisi et al. (2007) and Toffolatti et al. (2018) reported cross-resistance between mandipropamid, dimethomorph and other CAA fungicides in *P. viticola*. Resistance to CAA fungicides has been reported for *P. viticola* in a number of countries overseas (FRAC info, 2015), and more specifically to mandipropamid (Aoki et al. 2011, Nanni et al. 2016, Toffolatti et al. 2018). Gisi et al. (2007) used up to 300 µg/mL of four CAA fungicides: mandipropamid, dimethomorph, iprovalicarb and benthiavalicarb and reported 50% inhibition of growth for 41 isolates when the concentration was 300 µg/mL, indicating that they were resistant to these fungicides. Genotype testing, in this study, showed that all tested isolates do not carry the mutant G1105S which is responsible for resistance in mandipropamid. Aoki et al. (2011), Aoki et al. (2013), Blum et al. (2010) confirmed the presence of G1105S mutant in all samples collected from Japan and Blum et al. (2010) found

strong correlation between this mutant and the resistant phenotype which suggested it is responsible for resistance to CAA fungicides. The current study, which involved both phenotyping and genotyping, showed that 98% of the tested samples were sensitive to mandipropamid, with only one isolate having minimal growth, confirming the genetic analysis. It will be important to continue monitoring for development of resistance to mandipropamid.

Reduced sensitivity to dimethomorph was detected in 23% of samples and given that cross-resistance has been demonstrated between dimethomorph and mandipropamid (Gisi et al. (2007) and Toffolatti et al. (2018) further stressing the importance of continued monitoring for reduced sensitivity to mandipropamid. Populations of *P. viticola* resistant to dimethomorph were first reported in France in 1994, and since then has been increasing (Nanni et al. 2016). Wicks and Hall (1990) conducted a study to evaluate the efficacy of dimethomorph against downy mildew of grapevines using discriminatory dose and they found that *P. viticola* isolates were sensitive to the fungicide. In addition, Sun et al. (2010) found that 307 isolates of *P. viticola* from China were completely inhibited by a single discriminatory dose of 1.6 µg/mL of dimethomorph. However, in this study decreased sensitivity was detected at 10 µg/mL for one sample from Hunter Valley/NSW and 11 samples grown at 1 µg/mL, therefore it is important to continue monitoring for dimethomorph resistance.

With regard to pyraclostrobin, in the current study, 47% of isolates tested were detected with reduced sensitivity (fungal growth on 1 µg/mL and above) and 8% resistant (fungal growth on label rate), compared to none of the isolates tested in the previous study (2013-2016) having reduced sensitivity to pyraclostrobin (Hall et al. 2017). However, the highest discriminatory dose used in the test of the previous project was 0.1 µg/mL, which might explain the reason for not detecting reduced sensitivity. Isolates are now considered as having reduced sensitivity when the pathogen grows on 1 µg/mL and above. Resistance has been reported from when 3 µg/mL (Giraud et al. 2013) and 390 µg/mL (Gauthier, 2014). Baseline sensitivities to pyraclostrobin of up to 0.94 µg/mL EC_{50} were reported by (Wong and Wilcox, 2000).

Cross-resistance has been reported between all fungicides in the QoI group, and they are at high risk of *P. viticola* developing resistance to them according to FRAC 2016. High levels of resistance to pyraclostrobin (EC_{50} up to 25 µg/mL) have been reported in the USA (Baudoin et al. 2008). Giraud et al. (2013) reported reduced sensitivity of *P. viticola* isolates (with MIC of 3 µg/mL pyraclostrobin) in Luxembourg. It is necessary to continue monitoring the spread of resistance across Australian viticultural regions. The three isolates detected with the G143A mutation grew on leaf discs treated with 10 µg/mL pyraclostrobin, indicating resistance had developed. However, some isolates that grew on 10 µg/mL pyraclostrobin, had a low frequency of G143A, suggesting that 10 µg/mL might not be the MIC dose to identify resistance. The fact that 8% of the samples were resistant at the field rate, has led to the adoption of label rate for routine testing since 2020. The highest incidence of resistance was recorded in Griffith (17%) and Yarra Valley (16%), so continued monitoring is required for these regions.

Reduced sensitivity to ametoctradin was detected in samples from the Hunter and Yarra Valleys, with 39% of the samples growing on 1 µg/mL. Fontaine (2019) only detected resistance to ametoctradin in low frequency in France. Target-site resistance to ametoctradin was first detected in 2015 and is likely caused by a single point mutation in the cytochrome b gene, leading to the S34L substitution (Fontaine et al., 2019).

Results from the current study showed that there were a few samples with multiple resistance to different groups of fungicides. For example, a sample from Yarra Valley showed reduced sensitivity to dimethomorph, ametoctradin, pyraclostrobin and metalaxyl. This indicates a high risk of this *P. viticola* population developing multi MOA resistance and warrants further investigation by intensive monitoring of the vineyard to determine the extent of the resistant population. Elimination could then be attempted by removing certain active ingredients from the spray program and using multi-site fungicides before it spreads to other vineyards. There was also cross-reduced sensitivity in several samples between pyraclostrobin and metalaxyl, so these vineyards should also continue to be monitored closely.

Genotyping and phenotyping results were closely aligned for the G143A mutation detection and QoI resistance, so molecular detection should be used in future for detection of QoI resistance in *P. viticola*. This mutation will make an ideal candidate for high-throughput and in-field detection technology, which is planned for future research. Although no mutant has been detected for CAA fungicides in Australia, continued monitoring is required for this group. Furthermore, phenotyping for metalaxyl resistance is still crucial in the future until the resistance mechanism is identified and linked to the phenotype.

6. Relationships between phenotype, genotype and field efficacy

6.1. Powdery mildew

6.1.1. Introduction

Understanding the relationships between phenotype, genotype and field efficacy can be relatively straightforward when the mechanism of resistance is known and the pathogen can be cultured on agar media (e.g. *Botrytis cinerea*; (Harper et al. 2021). However, for some fungicides the mechanism of resistance is not known e.g. groups 5, 7 and 13 for *Erysiphe necator,* so resistance tests rely on phenotype assays, which for an obligate biotroph poses challenges such as maintaining the cultures, bioassay on detached leaves and leaf breakdown during bioassays and sub-culturing. The assessment of field efficacy of a fungicide also has challenges, in that the apparent breakdown of efficacy may not be due to fungicide resistance, but other issues, such as poor calibration, malfunction of spray equipment, inadequate spray coverage or canopy penetration. Another challenge for fungicide resistance research is the inability to induce resistance or introduce resistant *E. necator* populations into vineyards, so that the path of resistance development can be followed in response to the application of particular fungicides. In this study, several controlled environment approaches were used, including shadehouse, laboratory and greenhouse experiments to understand QoI and DMI resistance development and the relationships between phenotypic and genotypic results, and the link to field efficacy and fitness of resistance.

As QoI resistance is qualitative (i.e. 'all or nothing') the presence of the mutation G143A in *E. necator* should confer complete resistance, which was expected to be reflected in the phenotypic tests results, i.e. high EC_{50}, MIC and RF values. However, in the previous study (SAR 1204) there was a poor correlation between the phenotypic resistance parameters and the quantification of G143A (Hall et al. 2017). Although, poor correlations between phenotype and genotype were also observed for the DMI fungicides in *E. necator* in the previous study, this phenomenon is generally consistent with the quantitative nature of resistance in the DMIs (Kunova et al. 2021). Factors such as *Cyp51* gene copy number (Rallos and Baudoin, 2016) also influence resistance to DMIs. As a result of the unexpected results with QoI fungicides, an extensive review of phenotype and genotype methods was undertaken at the beginning of the current study. Some changes were made to the phenotype testing protocols, as described in sections 5.1.1.4, which improved time efficiency, however the lack of phenotype/genotype correlation remained. Furthermore, a review of the genotype methods previously developed in SAR 1204 (Hall et al. 2017) led to proposed changes in the primers used to detect G143A based on published primers and with input from collaborators, Dr Walt Mahafee, Tara Nield (USDA) and Dr Gert Stammler (BASF, Germany) (Section 5.1.1.7). Genotype analysis was repeated using new updated primers on isolates still available in the culture collection, and results showed good relationships between phenotype and genotype for the QoI fungicides (section 5.1.3.6.2).

Quantification of a resistance allele e.g. G143A, Y136F, can range between 0 and 100% (section 5.1.3.6.2 and 0). Cultures at the extremes of the percentage scale were mostly phenotypically sensitive or resistant. However, the frequency of resistance allele required to cause a change in phenotype parameters of EC_{50} and MIC values and an increase in disease severity (i.e. the beginning of field failure) is still unknown. Furthermore, it is still not clear if low frequency is a warning sign that resistance may be developing, even when disease control is still achieved.

Fitness can be defined as the overall ability of an organism to thrive and reproduce in a given environment (Pringle and Taylor, 2002), which in this case is the exposure of a fungus to a fungicide. If a fungal cell is 'fitter' than its sensitive counterpart, then it will survive and reproduce successfully, effectively outcompeting its sensitive sibling. However, a resistant cell containing a mutation that confers resistance to a particular fungicide, may not be as fit as its sensitive counterpart i.e. contains no mutation, as the particular mutation may also adversely

affect another essential cell function. When this is the case, the resistant cell may be less fit (i.e. may not grow, reproduce or survive) than the sensitive genotype cell. This results in a lack of persistence of the resistant cell lines in the fungal population. This is termed a 'fitness cost' or 'penalty'. Maintaining resistance to fungicides may be associated with a fitness cost (Hawkins and Fraaije, 2018). For example, if there is no fitness cost on the pathogen, withdrawal of the fungicide from the spray program will not affect the proportion of resistant cells in the population resulting in persistence of resistance. Here, fungicides from different chemical groups will be needed to control disease. Conversely, if there is sufficient fitness cost or penalty associated with resistance, withdrawal of the target fungicide may result in a decline in frequency of resistance to the point that the fungicide could be reintroduced for occasional use (Frenkel, 2014). The dynamic shifting of resistance/sensitivity of a pathogen population depends on the prevalence of fit or unfit individuals in the population (Rallos et al. 2014). Understanding this resistance/sensitivity shift impacts on fungicide selection in a spray program.

For QoI fungicides, little to no fitness costs associated with resistance have been frequently reported for several pathogens e.g. *Magnaporthe grisea* (Avila-Adame and Koller, 2003), *Alternaria alternata* and *Phytophthora nicotianae* (Mikaberidze and McDonald, 2015). Currently, little is known about fitness cost and resistance stability in *E. necator* to the QoI and DMI fungicides. Only one study, by Rallos et al. (2014), investigated the fitness of *E. necator* containing G143A (the mutation that confers resistance to the QoIs), and found that frequency of G143A tended to decrease in one growing season after withdrawal of QoIs, probably due to spore migration and mixing of populations with natural background inoculum. However, after a second season in the absence of QoIs, G143A persisted at high frequency in the population, and furthermore, G143A persisted in the population for four years. As QoI resistance persists, QoI fungicides are unable to be reintroduced into the spray program without adversely affecting disease control.

For DMI fungicides, complete loss of efficacy has not yet been reported, possibly associated with a fitness cost that has been reported for some pathogens and/or the quantitative nature of resistance (Chen et al. 2012). There have been no reports of fitness costs in isolates with mutations in *Cyp51* and *CytB* (Klosowski et al. 2016).

Measuring the fitness cost in fungal pathogens can be conducted with experiments in the laboratory using detached leaves or in the greenhouse using vines in pots, however field experiments are costly in terms of resources and labour. Generally, a mark–release–recapture experimental design has been shown to work well to measure competitive fitness for three different cereal pathogens (Zhan and McDonald, 2013). Next Generation Sequence analyses of entire genomes has been used to identify mutations associated with resistance (Anderson, 2005). Currently, little is known regarding fitness compromise and resistance stability in *E. necator*. Rallos et al. (2014) found that frequency of G143A tended to decrease in one growing season, probably due to spore migration and mixing of populations with natural background inoculum. In the second season, QoI resistance persisted at high frequency for four weeks. Resistant populations were also found to persist in one vineyard without QoI application for four consecutive years (Rallos et al. 2014).

The aim of this study was to improve understanding of the relationship between phenotype, genotype and field failure for QoI and DMI fungicides by investigating:

1. the effect of consecutive fungicide application on frequency of G143A and Y136F and disease severity.
2. the fitness of isolates with varying frequencies of G143A and Y136F.

6.1.2. Methods

6.1.2.1. Effect of consecutive fungicide spraying on mutant frequency and disease severity

Rooted cuttings of cv. Cabernet Sauvignon were grown in 25 cm pots in a mix of ½ Bio gro® (van Schaiks, South Australia), ¼ cocopeat and ¼ University of California (UC) potting mix (Dimond, 1958) for four months in a controlled environment room, then transferred to a shadehouse, located at the Waite Campus, Urrbrae, SA in November 2017. Plants were watered regularly and fertilised with slow-release pellets (Nutiricote, Yates, Australia) every four months and a Manutec foliage feeder (a water-soluble fertiliser, NPK 25:4.5:12, Australia) applied every three weeks. Plants were watered as required and vines were pruned during dormancy. Natural infection by *E. necator* was allowed to develop, with infection first obvious in early December in each year. Plants were placed into groups of four and kept on benches that were separated approx. 4 m from one another and 1.8m between the lines (Figure 6.1). Each group of four was assigned a fungicide treatment, i.e. Cabrio, Mycloss, Microthiol, Topas, Domark and Talendo plus no spray control (Table 6.1). In the following season (2018/19), more vines were added and assigned with fungicide treatments as follows: Cabrio (half rate), Legend, Propser, Digger, Flute, Vivando and Kusabi (Table 6.1). In the final growing season of the trial (2021-2022), several treatments were removed from the trial as data were not proving to be useful due to cross-contaminations i.e. Cabrio half rate, Prosper, Legend, Talendo, Flute, Kusabi and Vivando. Fungicides were sprayed until just before run-off, at label rates (except for Cabrio half rate) at 2-4 weekly intervals during each growing season using a 2 L hand-held pump sprayer. Infestations of blister mite, *Colomerus vitis*, were controlled by spraying with Eco-oil at 2.5 mL/L during summer 2018 and autumn 2019, and from winter 2019, with Vertimec® Pro (abamectin 18 g/L, Syngenta) at 150 µL/L.

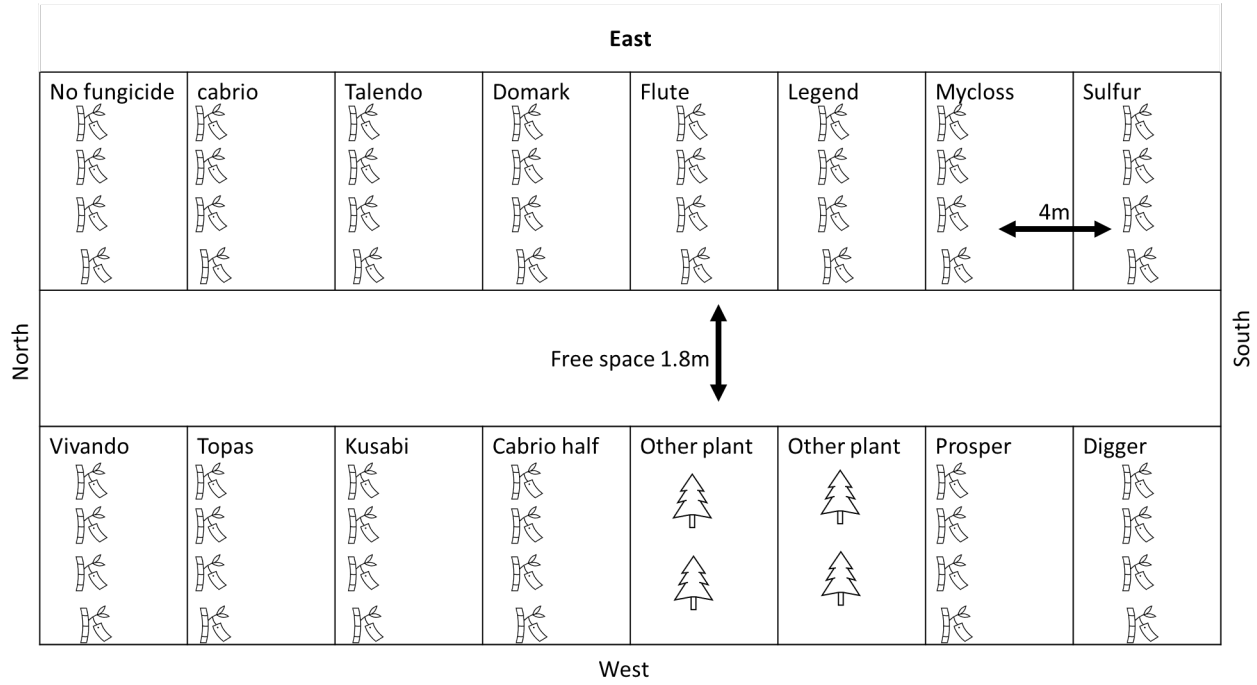


Figure 6.1. Diagram for shadehouse experiment set up.

Table 6.1. List of fungicides used to spray natural infection of *Erysiphe necator* in the shadehouse trial from 2017 to 2022

FRAC group	product	Active ingredient	Rate applied/100 L	Years of application
3	Digger	difenoconazole	25mL	2018 to 2022
	Domark	tetraconazole	30 mL	2017 to 2022
	Topas	penconazole	12.5 mL	2017 to 2022
	Mycloss	myclobutanil	16 mL	2017 to 2022
5	Prosper	spiroxamine	60 mL	2018 to 2021
11	Cabrio	pyraclostrobin	50 mL	2017 to 2022
	Cabrio half-rate	pyraclostrobin	25 mL	2018 to 2021
13	Legend	quinoxyfen	15 mL	2018 to 2021
	Talendo	proquinazid	25 mL	2017 to 2021
M2	Microthiol disperss	sulfur	400 g	2017 to 2022
U6	Flute	cyflufenamid	35 mL	2018 to 2021
50	Kusabi	periofenone	30 mL	2018 to 2021
	Vivando	metrafenone	25 mL	2018 to 2021
Control	No fungicide	NA	NA	2017 to 2022

The percentage of disease severity was assessed immediately prior to each spray using a rating scale from 0 to 100%, based on the proportion of infected leaves on each plant.

Isolates of *E. necator* were obtained from leaves of each treatment, immediately prior to each fungicide spray time, by rubbing the sporulating surface of infected leaves onto the upper surface of new leaves contained in petri dishes (5.1.1.3). These new leaves had been prepared the day prior to spraying. Single spore isolates were then established from each fungicide treatment and were phenotyped for sensitivity using leaf disc assays (5.1.1.4.1).

In addition to the establishment of isolates from each fungicide treatment, fungal material (spores and hyphae) was also collected for NGS analyses to quantify the G143A and Y136F mutations. The first collection of spores occurred in mid-Dec 2017, then subsequently collected immediately prior to each spray during each season. Spores and hyphae were collected by brushing sporulating lesions with sable hair paintbrushes (previously disinfested with 100% followed by 70% ethanol for 10 mins each, then washed in SDW several times and dried in a laminar flow cabinet). At collection time, a paintbrush was first dipped in sterile SDW to moisten the bristles to aid spore pick-up, then spores were deposited to ~ 500 µL sterile Milli-Q (SMQ) water contained in 1.5 mL sterile Eppendorf tubes (two tubes per fungicide treatment). For each fungicide treatment, spores were collected from lesions on each of the four plants and bulked together into the two tubes. Tubes were frozen at -20°C until processing.

6.1.2.2. Fitness of isolates with mutations

Phase I

Ten *E. necator* isolates, five with high and five with low frequencies of G143A (determined by NGS) were used for the experiment (Table 6.2). NGS data were obtained from previous sequencing results of other experiments and used to make the initial frequency mix. Isolates were grown on detached leaves (as previously described in 5.1.1.3) over a period of four weeks to provide sufficient inoculum.

Table 6.2. The frequency of G143A for individual isolates used to make sensitive and resistant spore suspensions for phase I of the fitness experiment on detached leaves.

Isolate	% G143A*	Status	Isolate	% G143A*	Status
158	100	Resistant	193	1	Sensitive
159	100		194	9	
164	100		195	0	
178	100		201	0	
210	100		215	0	

*data obtained from previous NGS sequencing results from other experiments,

On the day of inoculation, two spore suspensions (10^6 spores/mL), *viz.* sensitive (S) and resistant (R) were prepared by mixing the spores of all sensitive (S) isolates (193, 194, 195, 201 and 215) and all resistant (R) isolates (158, 159, 164, 178 and 210). Spores were collected from infected leaves using a sterile paintbrush and suspended in 30 mL of SMQ. Subsequently, five x 10 mL spore suspensions referred to as treatments 100% R, 100% S, 90% R, 80% R, 50% R, 20% R and 5% R were prepared by mixing various ratios of the S and R spore suspensions (Table 6.3).

Table 6.3. The ratio of sensitive:resistant (S:R) mixture used to study the fitness of resistance to QoIs and the frequency percentage of G143A mutant.

Treatment	S:R ratio	% G143A*
100% Resistant (R) mix	0:100	90
100% Sensitive (S) mix	100:0	45
90% R	10:90	88
80% R	20:80	75
50% R	50:50	60
20% R	80:20	55
5% R	95:5	45

*data obtained from previous NGS sequencing results from other experiments, however isolates were sequenced again after the conclusion of this experiment.

Six replicate grapevine leaves per treatment were surface disinfested with bleach and placed on water agar (5.1.1.4.1). They were sprayed with ~9 mL of each spore suspension treatment using a hand-held atomiser sprayer until just before run-off. The petri dishes were left open in a laminar flow cabinet until the leaves were dry, then the lids were replaced. The remaining 1 mL of each treatment spore suspension was put into 1 mL tube, frozen at -20°C prior to analysis by NGS to quantify the frequency of G143A in each treatment (section 5.1.1.7).

The inoculated leaves were incubated at 22°C, for 14 h photoperiod. Every four weeks, spores were removed from leaves for each treatment using a disinfested sable hair paintbrush and suspended in 10 mL of SMQ water (Figure 6.2). Six fresh surface-disinfested leaves were spray inoculated with each treatment as described previously and 1 mL frozen for analysis by NGS (Figure 6.2). This process was repeated a further four times.

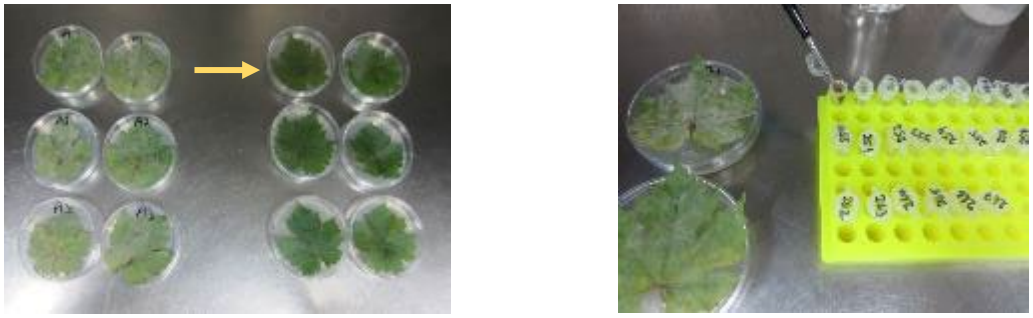


Figure 6.2. Spores from leaves infected with various treatments of powdery mildew were transferred on to fresh leaves every four weeks by spraying with spore suspensions. One mL of each spore suspension was retained for next generation sequencing to determine the frequency of the G143A mutation.

Phase II

Four resistant and four sensitive isolates were selected based on the frequency of G143A (Table 6.4). Although, the aim of this experiment was to determine the fitness of *E. necator* containing G143A, the use of NGS allowed Y136F to be analysed at the same time. Two experiments were carried out in parallel on both detached leaves that were prepared, inoculated and sub-cultured as previously described (section 6.1.2.2) and on potted plants grown in mini-greenhouses on benches in a greenhouse (Section 5.1.1.3.3).

Sensitive isolates (201, 217, 218 and 222) and resistant isolates (164, 213, 215 and 216) (Table 6.4) were grown on detached leaves as previously described (section 5.1.1.3). Spores were harvested using a cyclone separator powered by a vacuum pump, which was attached to a 2 mL Eppendorf tube with 4 mm PVC tube (Figure 6.3). Each 2 mL Eppendorf tube contained 500 µL SMQ water with 0.05% Tween 20 in which the spores were deposited, resulting in two spore suspension mixes, one containing sensitive (S) and the other resistant (R) isolates. A pilot experiment was conducted to test the viability of the spores after using the cyclone spore collector, which showed no effect on spore viability (data not shown). Subsequently, five 10 mL spore suspension mixtures (2×10^5 spores/mL), referred to as 100% R, 100% S, 75% R, 50% R and 25% R, were prepared from the S and R spore suspensions (Figure 6.3). The initial inoculation on detached leaves and leaves on potted plants was carried out as described in Section 6.1.2.2. A 1 mL aliquot of each treatment spore suspension was frozen and subjected to NGS to quantify G143A and Y136F.

Table 6.4. List of isolates used in Phase 2 fitness study with isolate information including the resistance status, isolate number, frequency of G143A and Y136F.

Isolate	% G143A*	% Y136F*	Status	Isolate	% G143A*	% Y136F*	Status
164	100	100	Resistant	218	0	0	Sensitive
215	100	100		201	0.1	100	
216	100	38		222	0	0	
213	100	72		217	0	100	

*data obtained from previous NGS sequencing results from other experiments, however isolates were sequenced again after the conclusion of this experiment.

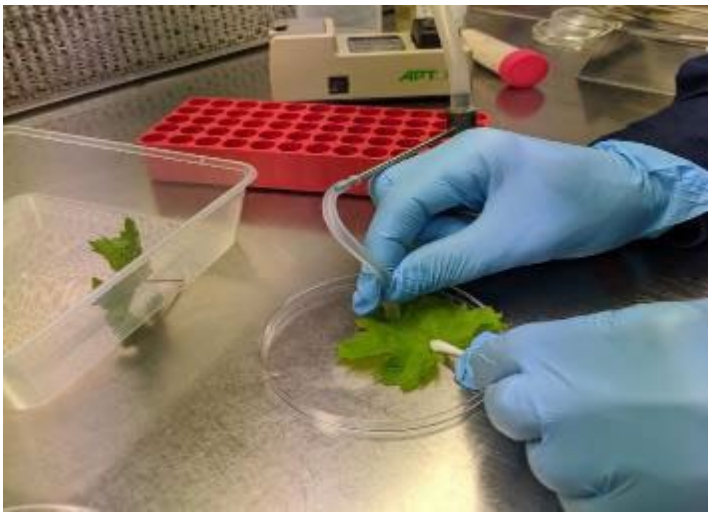


Figure 6.3. Spore collection using cyclone separator connected to vacuum pump.

Table 6.5. The ratio of sensitive:resistant (S:R) mixture of isolates used in the fitness experiment, number of isolate and resistance status and volume used to make the mixtures.

Treatments	S:R ratio	%G143A*	% Y136F*
100% Resistant (R)	0:100	47.3	77.1
100% Sensitive (S)	100:0	0	78.1
75% R	25:75	29.7	78
50% R	50:50	18	74.8
25% R	75:25	7.4	83.5

*data obtained from previous NGS sequencing results at the beginning of the experiments.

Detached leaves

After the mixtures were made, 1 mL aliquots from each mixture were removed for mutant detection using NGS analysis to determine the baseline frequency of the G143A mutant. For detached leaves, fresh leaves (section 6.1.2.2) were sprayed with the mixtures until run-off. The petri dishes were left open in a laminar flow cabinet until the leaves were dry, then the lids were replaced. The dishes with leaves were kept in the incubator at 21-22°C 12h dark and 12 light and sub-cultured every three weeks. Spores were collected every month for mutant analysis using NGS.

Potted plants

Potted plants were grown in mini-greenhouses (Section 5.1.1.3.3), which were placed on benches in a large greenhouse (20-25°C), for six months. The mini-greenhouses were initially tested for spore exclusion and remained without powdery infection for two weeks. Plants inside the mini-greenhouses were sprayed with the mixtures (Table 6.5) until run-off and droplets were air dried as the ventilation holes allowed air circulation. Sub-culturing was conducted using a stepped pruning method; the plant was visually divided into three sections (top, middle and bottom) and once the infection was established in the top section, the old infected leaves were removed from the middle and bottom sections of the plant to minimise mixing the new with the old generations of powdery mildew (Figure 6.4 B). After 2-3 weeks, the plants generated new leaves in the middle and bottom sections and the infection moved to new leaves, so then the top part was removed and so on. Sub-culturing was conducted every three weeks inside each mini greenhouse by rubbing the infected leaves with the fresh leaves, spores were collected from tagged leaves every month for mutant analysis using NGS. Plants were maintained free from insects and mites as described above.

Spore collection

The spores were collected, once each month for six months. For the mini-greenhouse, 20 mL of SMQ water was added to a plastic bag containing 10-15 infected leaves then shaken vigorously for 2 minutes. The resulting spore solution was collected and sent for NGS analysis or stored at -20°C until required. For the detached leaves, 5 mL of SMQ water was added into a 50 mL tube and one leaf was placed inside and shaken for 1 minute, then removed and a second leaf added and so on until 3-4 leaves were used for each sample. The resulting spore solution was collected and sent for mutant analysis using NGS or stored in -20°C until required. For the potted plant experiment, NGS was not performed in November and December 2019 as the first inoculation was not successful, so plants were re-inoculated.



Figure 6.4. A. Mini-greenhouses used for the fitness experiment, B. Sections of vine labelled for stepped pruning method for sub-culturing of isolate mixtures of *Erysiphe necator.*

6.1.3. Results

6.1.3.1. Effect of consecutive fungicide application on mutant frequency and disease severity

QoIs

Consecutive treatment with Cabrio resulted in a build-up of disease from 2019 to 2022, after little to no disease occurred in 2017 to 2018 (Figure 6.5). By the 2021/22 season, Cabrio (label rate) treatment resulted in the highest in disease severity, similar to the control treatment, while consecutive sulfur applications resulted in the least disease. Cabrio (half rate) was generally similar to the full label rate.

The mean of NGS results from across the years revealed the highest frequency of G143A was 65 and 38% for Cabrio (gp 11) label and half rates, respectively, followed by Kusabi (30%) from gp 50 (Figure 6.6). G143A was detected in all treatments except the control (no fungicide), most likely due to cross contamination between treatments (Figure 6.1). A medium range of G143A frequency was detected for fungicides from gp 3; Topas (19%), Domark (22%), Digger (20%) and gp 5 Prosper (25%). A low range of G143A frequency was detected for Mycloss (3%) from gp 3, Talendo (6%) and Legend (1%) from gp 13, Vivando (2%) gp 50, Microthiol (sulfur) (4%) from gp M2 and Flute (1%) gp U6, (Figure 6.6).

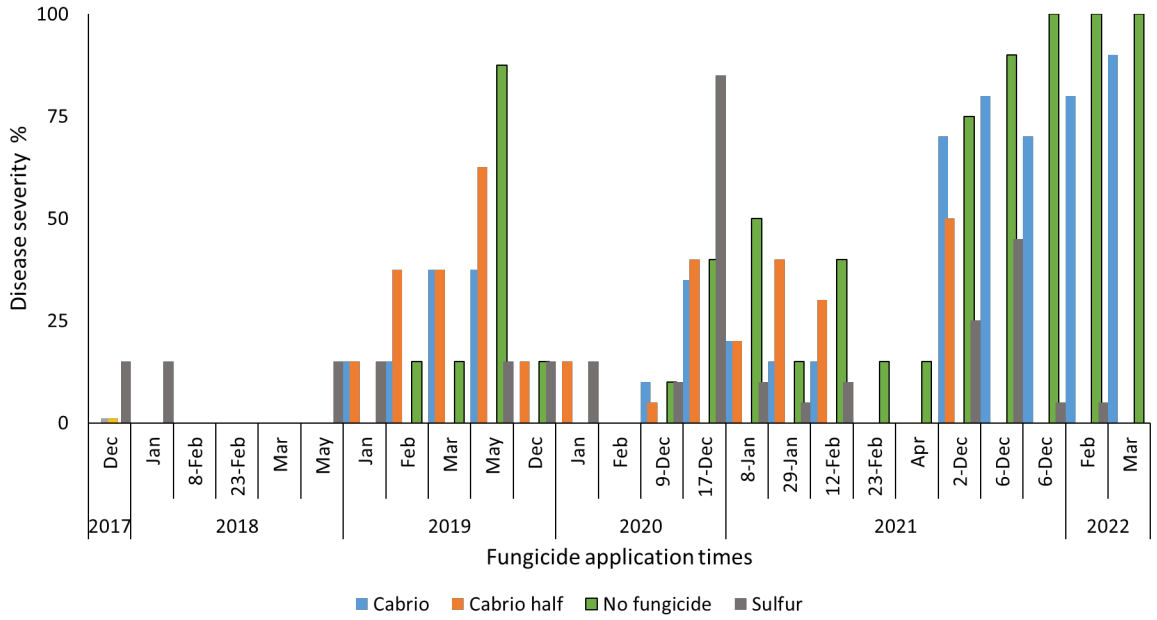


Figure 6.5. Disease severity percentage of powdery mildew on potted vines, cv. Cabernet Sauvignon, grown in a shadehouse from 2017 to 2022 following label rate spray applications of Cabrio (light blue), Cabrio (at half rate; orange) and two controls of either no fungicide (green) or sulfur (grey).

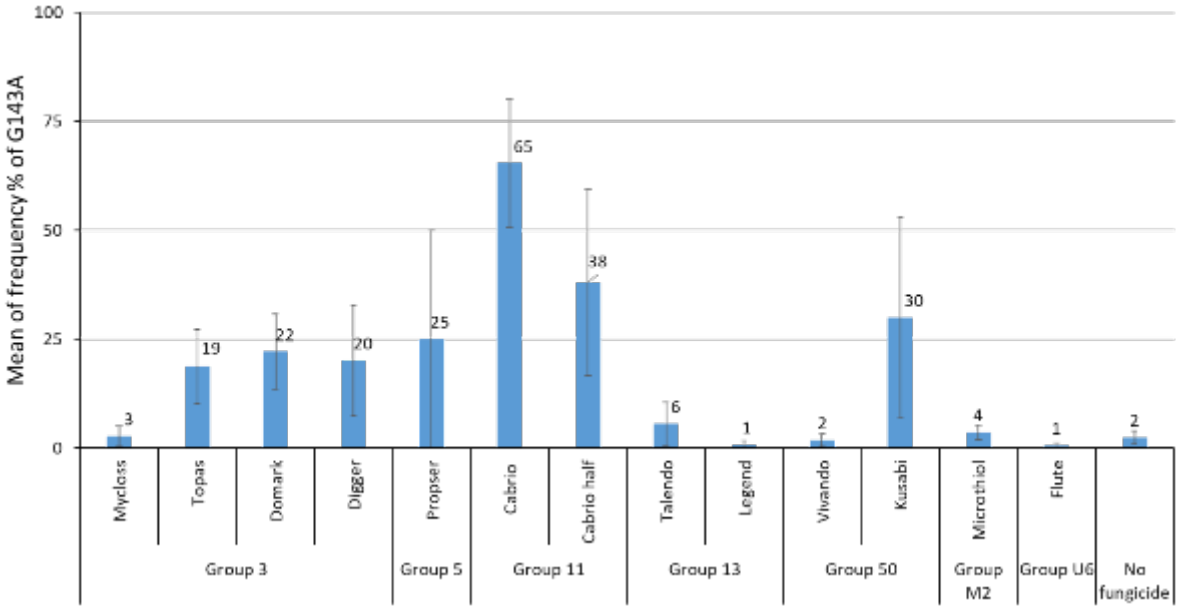


Figure 6.6. Mean frequency percentage of G143A over five years, using NGS analysis of *E. necator* on potted vines, cv. Cabernet Sauvignon, grown in a shadehouse in 2017 to 2022 with consecutive spray applications of different fungicides from different groups.

DMIs

Consecutive DMI treatments resulted in increased disease severity over the duration of the trial from 2017 to 2022 (Figure 6.7). Repeated applications of Mycloss, Topas and Domark failed to control powdery mildew in the last two seasons, resulting in high disease severity (65, 75 and 70%, respectively), however repeated Digger and sulfur treatments resulted in low disease severity of 5 and 0%, respectively compared with control (no fungicide) 100% by March 2022.

Y136F was detected in all treatments including the control (no fungicide) (Figure 6.8). The highest frequency of Y136F was detected in Mycloss treatment (82%) while the lowest was at Vivando (32%). These results indicated that treatments were cross-contaminated with different populations of *E. necator.*

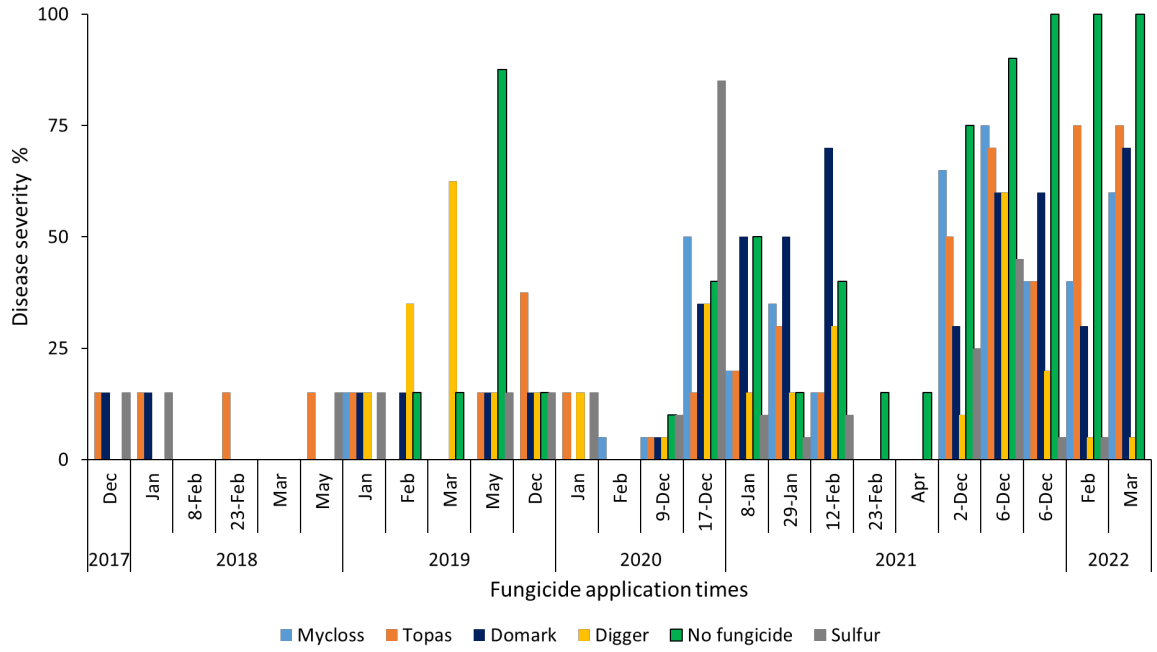


Figure 6.7. Disease severity percentage of powdery mildew on potted vines, cv. Cabernet Sauvignon, grown in a shadehouse from 2017 to 2022 following label rate spray applications of DMIs; Mycloss (light blue), Topas (orange), Domark (dark blue), Digger (yellow) and two controls of either no fungicide (green) or sulfur (grey).

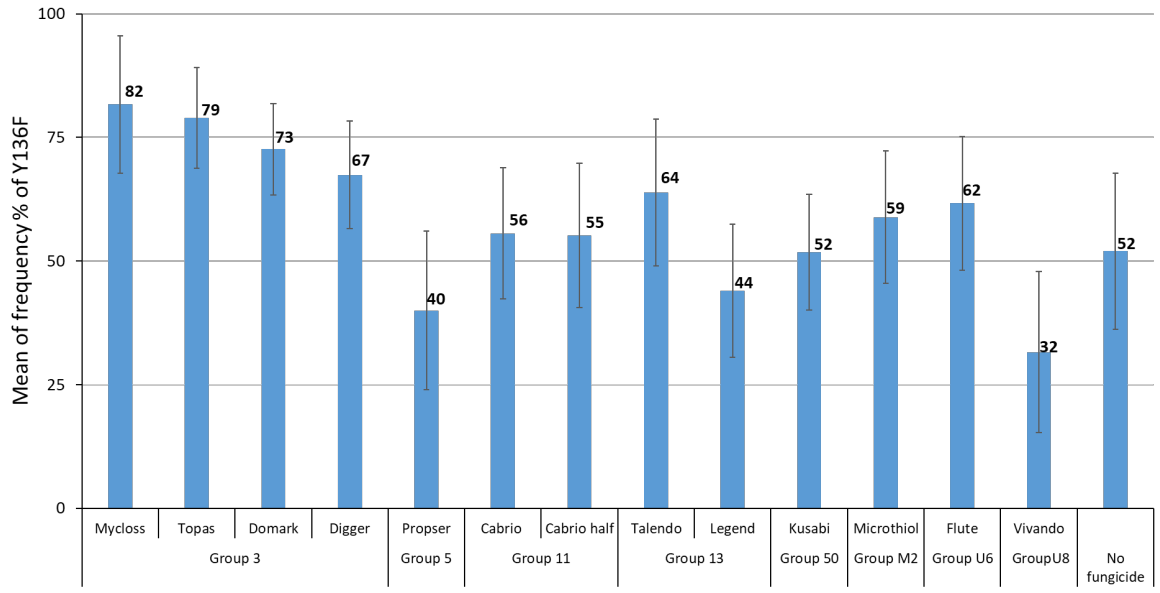


Figure 6.8. Mean frequency percentage of Y136F of five years, using NGS of *E. necator* on potted vines, cv. Cabernet Sauvignon, grown in a shadehouse from 2017 to 2022 and following consecutive spray applications of different fungicides from different groups.

Other fungicide groups

Consecutive applications of fungicides from other groups resulted in increased disease severity from 2017 to 2022 (Figure 6.9). Talendo, Legend and Kusabi treatment had the highest disease severity compared to severity with consecutive sulfur applications. Consecutive applications of Vivando and Prosper were more effective for control of powdery mildew by 2022. No genetic analysis was conducted for these fungicides as the resistance mechanisms are unknown.

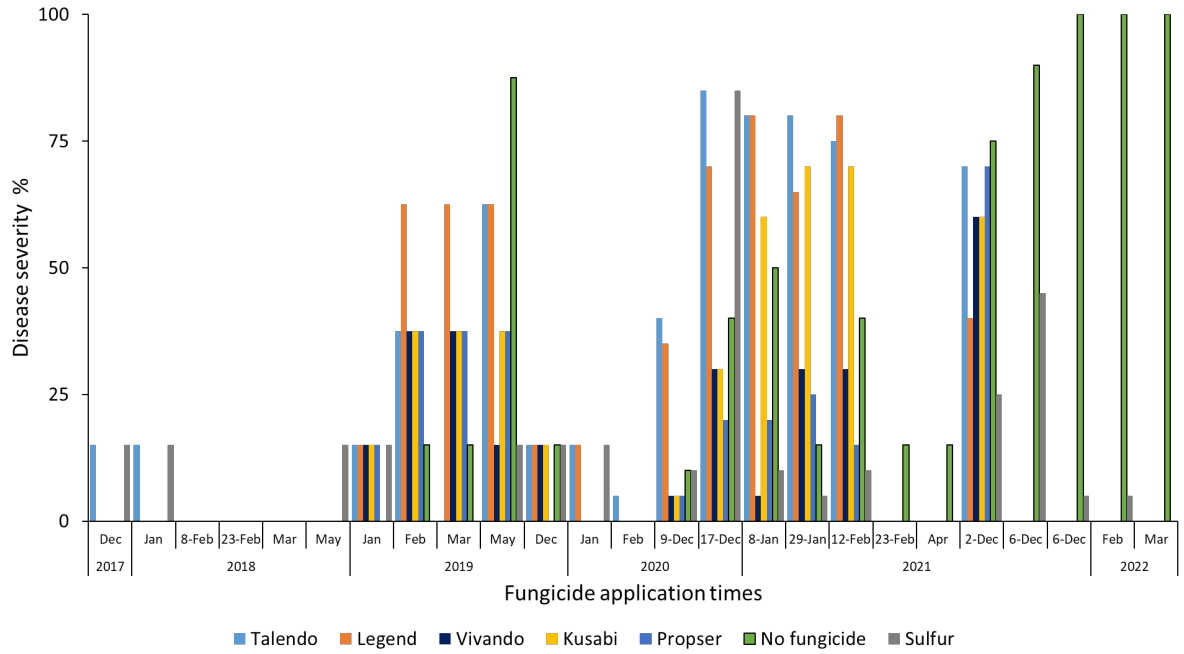


Figure 6.9. Disease severity percentage of powdery mildew on potted vines, cv. Cabernet Sauvignon, grown in a shadehouse from 2017 to 2022 following label rate spray applications of different fungicides from different groups; Talendo (group 13; light blue), Legend (group 13; orange), Vivando (group U8; dark blue), Kassbi (group 50; yellow) and two controls of either no fungicide (green) or sulfur (grey).

6.1.3.2. Fitness of isolates with mutations

Phase I

The mean frequency of G143A for each treatment over six months is shown in Figure 6.10. Due to an update of the primers for G143A, sequencing was repeated on the isolates after the experiment was concluded (section 5.1.1.7). Results confirmed the initial frequencies of G143A, except for isolate 215, which was detected with 100% G143A, rather than the initial 0%, consequently increasing the %G143A in the sensitive mixture. Therefore, treatments 20% R and 5% R were not reflective of a population containing low frequencies of G143A and explain the higher starting frequencies of G143A than expected. The initial frequencies of G143A in 95% R, 80% R and 50% R treatments were withing the expected range (88, 75 and 60%, respectively).

Over the first three months of the experiment G143A frequency decreased for all treatments, then in the following two months the frequency increased back to similar levels as at the beginning of the experiment (Figure 6.10).

One of the aims of this experiment was to determine if the method would be suitable to evaluate fitness and competitive ability of spores with G143A. The method worked well, with

little variability in results and showed that G143A is fit, therefore further experiments on detached leaves and potted plants were undertaken.

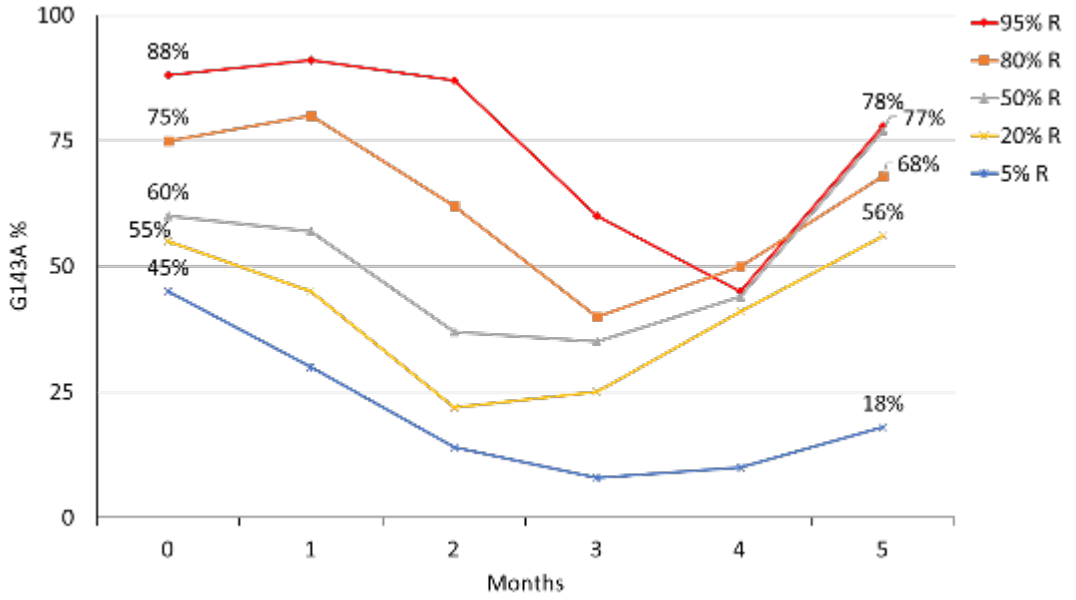


Figure 6.10. Mean frequency of the G143A mutant in five mixtures of resistant and susceptible *E. necator* populations over 5 months of subculturing on detached leaves. % R represents the frequency of G143A at the beginning of the experiment (Table 6.3).

Phase II

QoI mutant G143A

Powdery mildew disease was well established inside mini-greenhouses and disease severity increased rapidly from 1-2 months reaching 100% (Figure 6.11). Inoculation was not successful in the first two weeks, therefore a second inoculation was conducted to establish the infection.

The G143A mutant was generally fit and competed with the wild type in both detached leaf and potted plant experiments (Figure 6.12). In both experiments, the initial frequency of G143A for the 100% R mixture was 47%, and not 100%, due to the inclusion of isolate 164 which was subsequently detected to be 0% not 100% G143A with the updated primer. The G143A frequency of S mix remained 0% for 6 months during the experiments of detached leaves and potted plants (Figure 6.12).

In the detached leaf experiment, the 100% R mix increased from 47% to 98% over the first month and reached 100% by 3 months and remained this way until the end of the experiment at six months (Figure 6.12 A). On the other hand, the S mix started with 0% G143A and remained 0% for the six months. For the 75% R and 25% R mixtures, cultures followed the same trend as the R mix over the experimental period. However, for the 50% R mixture, the frequency of G143A increased in the first month and then decreased to 0% over the experimental period.

In the potted plant experiment, generally the frequency of G143A was less than detached leaf experiment, 100% R mix started at 47% G143A and reached to 92% by four months before dropping back to 53% after six months (Figure 6.12 B). For the other three mixtures; 75% R, 50% R and 25% R the trend was similar to 100% R mix, with frequency peaking after four months before dropping back at six months, but the values of G143A frequency were higher than the 100% R mix. The G143A frequency of S mix remained 0% for duration of the six month experiment (Figure 6.12).

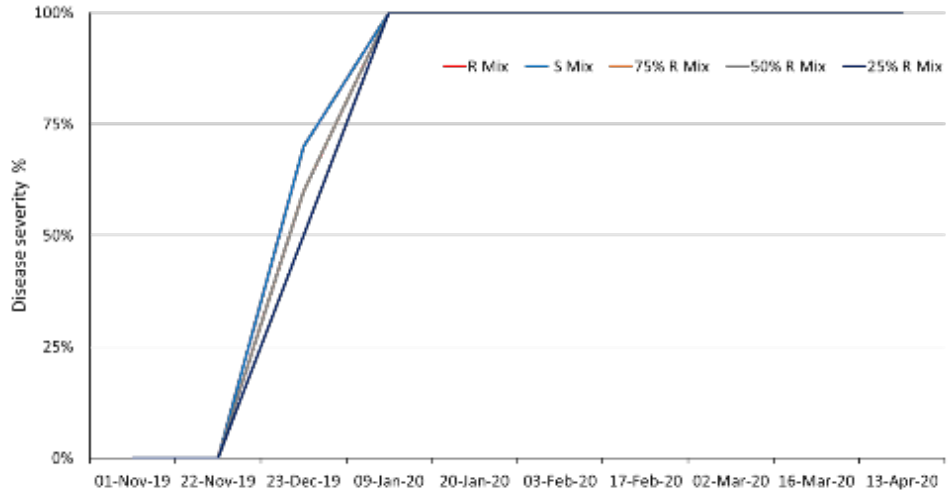


Figure 6.11. The disease severity of *E. necator* on potted plants in mini-greenhouses which were inoculated with different sensitive:resistant mixtures of *Erysiphe necator* populations.

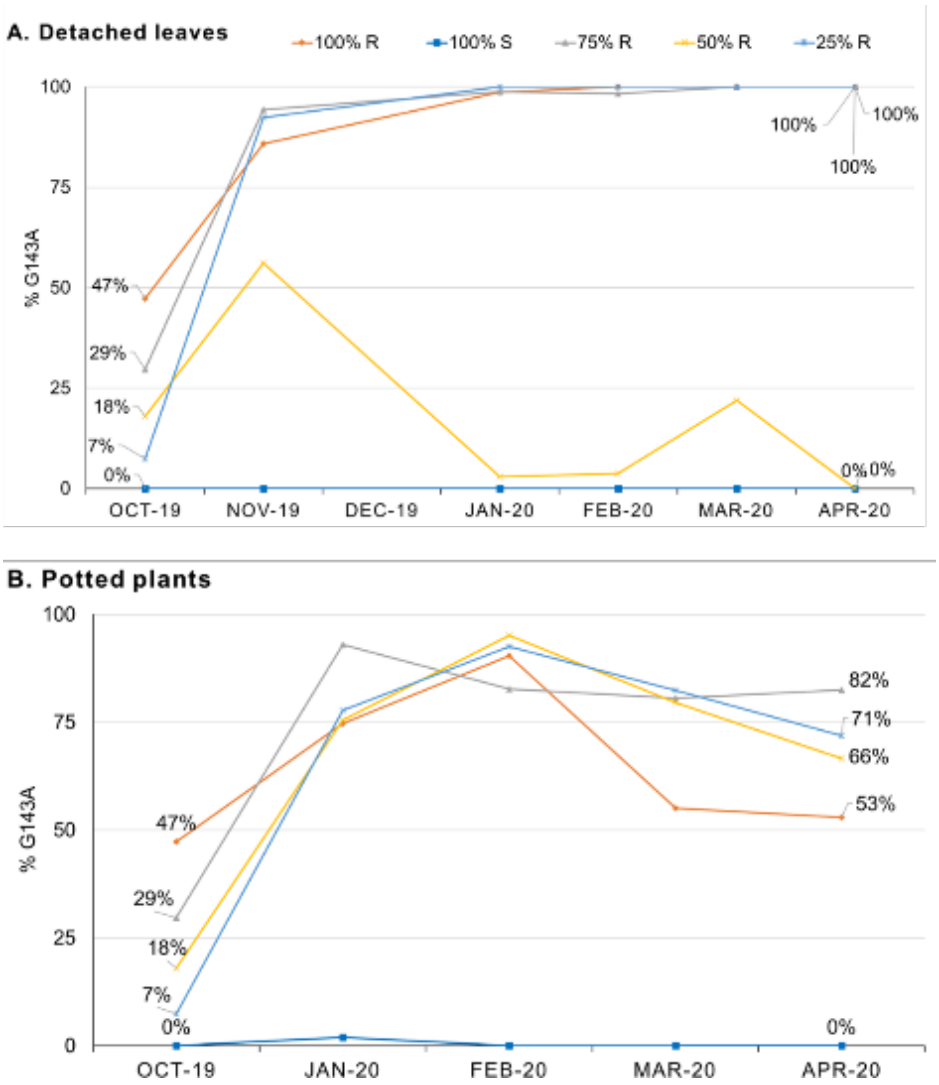


Figure 6.12. Frequency of G143A mutant in five sensitive:resistant mixtures of *Erysiphe necator* populations over 6 months of sub-culturing on A. detached canes or B. potted plants phase II.

DMI mutant Y136F

The Y136F mutant was fit and competed with the wild type in both detached leaf and potted plant experiments (Figure 6.13). According to NGS analysis, the frequency of Y136F in isolates 201 and 217 was 100% (Table 6.4), even though these isolates were considered sensitive to DMI fungicides as they both came from organic gardens. The initial frequency of Y136F for the 100% R mix was 76% and confirmed as 77% when sequenced again at the end of the experiment. For the 100% S mix, the initial frequency was 50% however when sequenced again it was 78% (Figure 6.13), indicating that the S mix was not a truly sensitive mixture of *E. necator*, possibly due to the isolates 201 and 217.

In detached leaves, the frequency of Y136F of the 100% R, 75% R and 25% R was stable ranging between 74% to 70%, however the 50% R mix decreased to 8% at three months before it increased again to 44% at five months, and then dropped back to 2%. Although the 100% S mix started with high frequency of Y136F (78%), it dropped sharply after one month (20%) then 2% after three months, and then to 0% at four months onwards (Figure 6.13 A).

In the potted plant experiment, Y136F frequency of 100% R, 75% R and 25% R was stable for the duration of the six month experiment (Figure 6.13 B). The 50% R mix gradually decreased from 74 % to 44% and the 100% S mix decreased sharply from 78% to 7% after four months then increased up to 21% by six months (Figure 6.13 B).

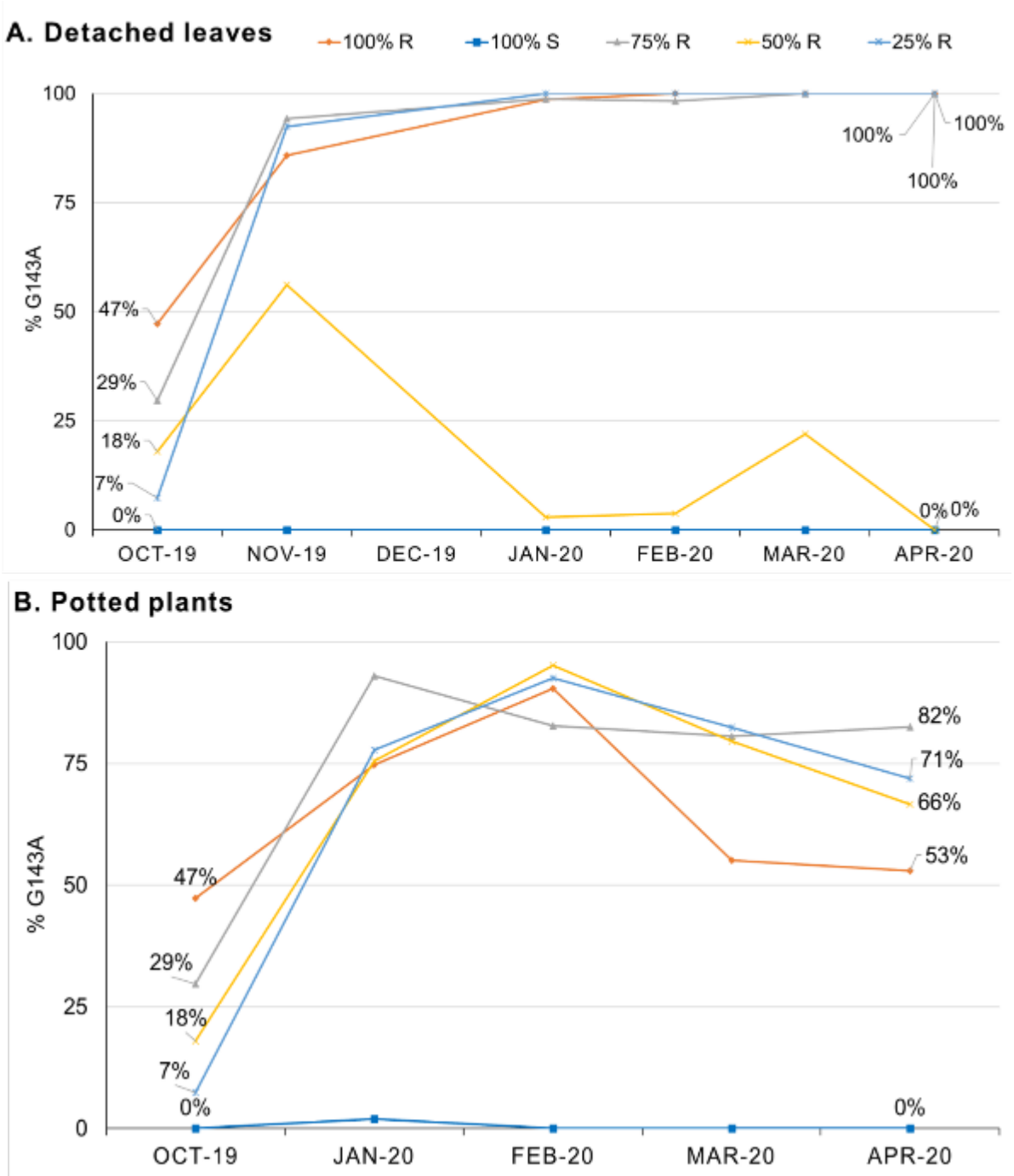


Figure 6.13. Frequency of Y136F mutant in five mixtures of *Erysiphe necator* populations over six months of sub-culturing on A. detached leaves or B. potted plants.

6.1.4. Discussion

Consecutive spray applications for prolonged periods are not recommended as industry practice, nor should sprays be applied after disease is observed. This approach was used for shadehouse experiments to demonstrate how quickly resistance can develop. However, these results also show how the application of a multi-site fungicide (sulfur) can result in the maintenance of stable and low levels of the G143A mutation in the population and provide effective disease control. FRAC (2018) recommends inclusion of multisite 'mode of action' fungicides in spray programs to decrease the risk of fungicide resistance development. Not

applying QoI fungicide sprays also results in a similar outcome in terms of controlling the frequency of G143A, likely by not adding any selection pressure, however, this results in poor disease control and so is not a practical solution to manage the levels of resistance alleles in a fungal population. Also, using half the field rate of Cabrio has resulted in more disease and consequently increased the frequency of G143A. The purpose of adding half rate treatment of Cabrio was to investigate the effect on resistance development.

Detection of G143A in most treatments suggested that cross-contamination of *E. necator* populations may have occurred between treatments as there were no physical barriers and enough distance between treatment. Powdery mildew in early season and when controlled effectively usually spreads over several metres (Magarey, 2010). Given that vines were sprayed every 3-4 weeks (which allows the fungus to grow and reproduce during this 3-4 weeks) and only few metres apart from each other, the likelihood of local cross-contamination was high. However, the control treatment had detected with low frequency of mutant fungus, perhaps due to its location in the north-east corner of the shadehouse (Figure 6.1), and the prevailing wind direction was south-west (BOM, 2022). In addition, frequent applications of QoIs (Cabrio) caused an increase in the frequency of G143A mutant throughout the duration of the trial, providing ample G143A inoculum. This practice is not recommended as it has been reported that over the time the sensitive population (wild type) will be eliminated and replaced with a resistant population (Leadbeater, 2012).

The 3-4 week gap between fungicide applications may also have contributed to failure of controlling the disease, as it is typically recommended to have two-week intervals between sprays (Magarey, 2010). Considering the life cycle of *E. necator* is 5-7 days (Magarey, 2010), the amount of disease build up between sprays might have exacerbated the cross-contamination of treatments with the G143A population.

Similarly, with DMIs, many consecutive applications over five years resulted in an increase in disease severity over the time and a lack of efficacy by five years when DMI fungicides were applied. There are reports that Y136F is highly correlated to the resistance of *E. necator* to DMIs (Delye et al. 1997, Vielba-Fernández et al. 2020), particularly when put under heavy pressure by consecutive sprays, as was the case in the shadehouse trial. Rallos and Baudoin (2016) found that some isolates possessed both alleles, mutant (Y136F) and wild type (Y136), which demonstrates gene duplication or increased gene copy number and possibly a requirement for at least one mutant copy of *Cyp51* for resistance.

The current research has struggled to find Y136 haplotypes (wild type) throughout the life of this project. This is clearly a consequence of the selection pressure placed by DMI on the *E. necator* populations. This selection pressure has likely driven the replacement of Y136 haplotypes by F136 due to a slight increase in resistance to DMIs conferred by the latter. This phenomenon has been observed for other powdery mildew pathogens such as *Blumeria graminis* f.sp. *hordei*, *B. graminis* f.sp. *tritici* and *Podosphaera xanthii*. However, for these three mildews, the emergence of Y136F was the first step towards the selection of other mutations that conferred higher levels of resistance against DMIs. The evolution of additional mutations in other closely related powdery mildew pathogens implies that new resistance mutations could quickly emerge in *E. necator* populations if DMI use is not managed according to existing anti-fungicide resistance guidelines.

The other groups of fungicides, when applied consecutively over a long period of time, also lost efficacy over time.

Results from both fitness experiments showed that the G143A mutant was fit for at least six months under laboratory and glasshouse conditions However, the data revealed some irregular fluctuation in frequency of G143A, which concurs with Rallos et al. (2014), who reported that frequency increased in detached leaves but decreased in the field in 1st season, followed by an increase in the 2nd season. The authors showed, that even after more than 50 consecutive transfers on grape leaves (free from fungicide), the frequency of the mutant

remained stable. Therefore, the G143A-carrying mitochondria in the resistant fungal cell are not lost through repeated sub-culturing, probably because they have become homoplasmic in most isolates (Rallos et al. 2014). Also, this mutant is not harmful to the pathogen and has not affected the growth of *E. necator*. Our results confirm that resistance to QoI fungicide, represented by the presence of the G143A mutant, are stable and fit, so withdrawing these fungicides from the spraying program may not have any impact on the reducing QoI resistance.

The occurrence of double resistance to DMI and QoI in *E. necator* has been reported in Europe (Dufour et al. 2011). In addition, Luo and Schnabel (2008) found that DMI resistant isolates of *Monilinia fructicola* were predisposed to accelerated development of QoI resistance, although the mechanism was not investigated. These phenomena can limit the use of DMIs and QoIs as rotational or tank mix partners and the durability of QoI resistance in this population was probably further enhanced by DMI-selection of double mutants. Rallos et al. (2014) report that there was no evidence that the QoI and DMI resistances are linked but the results suggest that even chance combinations of resistance can affect selection.

Also, from observation on detached leaves, the growth of susceptible isolate 164 was vigorous and produced abundant conidia, potentially leading to it dominating the mixed culture further reducing the frequency of G143A.

In this study, the fitness of the G143A mutant depended on the ratio of mutant: wild type, if high (i.e. 75%) the mutant was fit and if low (i.e. 25%) the wild type was fitter. Rallos et al. (2014) found that the G143A mutant was fit in both laboratory and field experiments.

The mini-greenhouse system was an effective method of evaluating the fitness of mutations, and prevented cross-contamination while maintaining optimal conditions for *E. necator* and the grapevine to grow. It also reduced the risk of escape of mutant populations into the environment. This system will be employed for future research on fungicide resistance.

6.2. Botrytis bunch rot

6.2.1. Introduction

Fungicide resistant isolates can exhibit fitness penalties due to the presence of mutations in genes linked with resistance (Hawkins and Fraaije, 2018). Further understanding of this phenomenon could assist resistance management in determining how long resistant isolates may persist once selection is removed (Hawkins and Fraaije, 2018). In *Botrytis cinerea*, fitness penalties have been identified in field isolates resistant to succinate dehydrogenase inhibitors (SDHIs), ketoreductase inhibitors (KRIs) or phenylpyrroles (PPs) (Li et al. 2014, Saito et al. 2013, Veloukas et al. 2011, Zhao et al. 2010). In other *B. cinerea* studies, fitness penalties have been absent in group 2, 7, 9 and 11 field resistant isolates (Bardas et al. 2008, Beever and Brien, 1983, Cui et al. 2004, Fernández-Ortuño et al. 2013, Grabke et al. 2014, Kim and Xiao, 2011, Oshima et al. 2006). In this study, the presence of pathogenic fitness penalties in Australian *B. cinerea* isolates collected from grapes was investigated in isolates resistant to group 2, 9 and 12 fungicides. Chip digital PCR (cdPCR) and droplet digital PCR (ddPCR) were utilised to measure relative biomass levels of mutant and wild-type (WT) isolates in *in vitro* and *in planta* experiments. Relatively lower levels of mutant biomass compared to the WT could indicate the presence of a pathogenic fitness penalty in the mutant. WT and mutant isolates were also co-inoculated to see whether there are competitive differences between WT and mutant isolates. Single point mutations (SNPs) linked with resistance to each of these fungicides were used as molecular targets in this analysis.

6.2.2. Methods

6.2.2.1. Detached berry assay (DBA)

The *in vitro* DBA method was developed and optimised in 2017 and has been described by Harper et al. (2019). Briefly, conidia were harvested for each isolate, and were diluted in water and Tween 20 (0.03%) to 5 X 10^3 conidia/mL. Berries (cv. Cabernet sauvignon) were harvested from an experimental vineyard at Curtin University (Perth, Australia) and disinfested with sodium hypochlorite (0.625 %) and ethanol (10 %) for 5 min. The berries were rinsed five times in distilled water, air dried, and placed in 90 mm petri dishes. A 10 µL aliquot (containing approx. 50 conidia) of each conidial solution was placed on a wound site on each berry. In combination treatments, the conidial solution contained 5 X 10^3 conidia/mL of each isolate. The petri dishes were placed on damp tissue paper in plastic trays and wrapped in cling film to produce a humid environment. Trays were incubated at room temperature under white light 12 h light/dark conditions. The experimental details for the various treatments across the three DBA experiments (2018–2020) are shown in Table 6.6. Three infected berries were combined, and freeze dried, as one biological replicate for subsequent DNA extractions and dPCR analysis. For dPCR analysis, each treatment and time point had at least two biological replicates assessed.

Table 6.6. Experimental details for each detached berry assay (DBA) experiment across 2018 – 2020. R = resistance, Azo = azoxystrobin, Fen = fenhexamid, Flu = fludioxinil, Ipr = iprodione, Pyr = pyrimethanil, cdPCR = chip digital PCR, ddPCR = droplet digital PCR.

DBA experiment	1	2	3
Season	2018	2019	2020
Target/gene	I365S/*Os1*	L412F/*Pos5*	D616G/*Mrr1*
Fungicide group	2	9	12
WT isolate/s for inoculations	Bc-7	Bc-7, Bc-410, Bc-486	Bc-7, Bc-410
Mutant isolate/s (resistant phenotype) for inoculations	Bc-179 (Azo^R, Ipr^R)	Bc-132 (Ipr^R, Pyr^R), Bc-396 (Azo^R, Fen^R, Ipr^R, Pyr^R) Bc-618 (Pyr^R)	Bc-128(Ipr^R, Flu^R, Pyr^R) Bc-391 (Azo^R, Flu^R, Ipr^R, Pyr^R)
Sampling time points	Day 4 and 7	Day 3 and 6	Day 3 and 6
dPCR used for analysis	cdPCR	cdPCR	ddPCR

6.2.2.2. Vineyard experiments

Experimental vineyards in South Australia (SA) and at Curtin University (WA) were used to carry out *in planta* experiments. Table 6.7 provides details for each vineyard experiment and WA plot layouts are shown in Figure 6.14. Preliminary experiments at Curtin University were conducted in 2017 to establish the inoculation method for subsequent seasons. Spores for inoculations were harvested for each isolate as described by Harper et al. (2019). Briefly, isolates were cultured on PDA and then exposed to near-UV to allow for sporulation. After sporulation, plates were washed with dH_20, and the conidia pelleted and washed twice in dH_20 by centrifuging at 3220 g for 10 min. Conidial density was adjusted to 10^6 conidia/mL using a haemocytometer. In each vineyard, bunches were inoculated at flowering (80 – 100% capfall) by spraying with a 10^6 conidia/mL (0.5% gelatin) solution. Combination treatments had a 1:1 mixture of 10^6 conidia/mL of each isolate. Control bunches included non-inoculated bunches (experiment 2) or spraying with 0.5% gelatin or water only (experiment 4 – 5) (Figure 6.14). In the SA experiments, harvested bunches were placed into plastic trays lined with moistened 100% natural fibre cloths covered with paper towels. Trays containing 6 - 8 bunches per tray were enclosed in plastic bags and incubated for 10 - 14 days at room temperature to induce sporulation. Infected material was harvested from each bunch, with some material combined from bunches from the same treatment. The samples were then freeze dried and sent to Curtin University for analysis. In the WA experiments, four bunches per plant were inoculated for 3–5 plants per treatment (Figure 6.14). Inoculated bunches were covered overnight with zip lock bags containing approximately 10 mL of water, to produce a humid environment conducive to conidial germination. Post-veraison, sugar levels were measured using a hydrometer. Once sugar levels reached approximately 28 %, intermittent misting was carried out for 2– 4 weeks until disease was well established (Table 6.7). When infected material was harvested, a total of nine berries from one bunch were collected as one biological replicate and were then freeze dried for subsequent DNA extraction. In all treatments, except the non-inoculated control (0.5 % gelatin), non-infected material was not harvested if no expressing *Botrytis* was visible. In all vineyard experiments, nine infected berries were considered a biological replicate for DNA extraction, with at least five replicates taken for each treatment. If there were less than nine infected berries, non-infected berries were added to make the biological replicate. For dPCR analysis, each treatment had at least five biological replicates assessed.

Table 6.7. Details of treatments for each vineyard experiment across 2018 - 2020. R = resistance,

Azo = azoxystrobin, Flu = fludioxinil, Ipr = iprodione, Pyr = pyrimethanil. cdPCR = chip digital PCR. ddPCR = droplet digital PCR.

Experiment	1	2	3	4	5
Location	SA	WA	SA	WA	WA
Season	2018	2018	2019	2019	2020
WT isolate for inoculations	Bc-385	Bc-7	Bc-385	Bc-7	Bc-7
Mutant isolate for inoculations (resistant phenotype)	Bc-401 (IprR, FluR)	Bc-179 (AzoR, IprR)	Bc-400 (AzoR, IprR, PyrR)	Bc-618 (PyrR)	Bc-128 (IprR, PyrR)
molecular target/gene	I365S/*Os1*	I365S/*Os1*	L412F/*Pos5*	L412F/*Pos5*	D616G/*Mrr1*
Fungicide/s applied	Iprodione (Rovral®)	Iprodione (Iprodione 500SC)	Pyrimethanil (Scala®)	Pyrimethanil (Scala®)	Pyrimethanil + Fludioxinil (Switch®)
Dose rate	400ml/100L (2X rate)	200ml/100L (2X rate)	200ml/100L (2X rate)	200ml/100L (1X rate)	80g/100L (1X rate)
Dose application time	6 weeks before harvest	4 days post commencement of misting	6h after inoculations	24h before inoculations	24h before and 4d post-inoculations (2nd spray required due to rain event)
Number of misting days	N/A	25	N/A	14	18
Harvest time point	veraison	25 days post commencement of misting	veraison	14 days post commencement of misting	18 days post commencement of misting
dPCR method	cdPCR	cdPCR	cdPCR	cdPCR	ddPCR

A

row A	row B	row C	row D
cab sav	mus	cab sav	cab sav
1	11	21	34
2	12	22	35
3	13	23	36
4	14	24	37
5	15	25	38
6	16	26	39
7	17	27	40
8	18	28	41
9	19	29	42
10	20	30	43
		31	44
		32	45
		33	46
			47



N

B

vine	row	experiment 2 (2018)	experiment 4 (2019)	experiment 5 (2020)
5	A	Bc-7 + Bc-179	Bc-7 + FG	Bc-7 + FG
6	A	Bc-7	Bc-7 + Bc-618	Bc-128
7	A	Bc-179	Bc-7 + Bc-618 + FG	Bc-7
8	A	Bc-7 + FG	Bc-7	Bc-128 + FG
9	A	Bc-179 + FG	Bc-618 + FG	Bc-7 + Bc-128 + FG
10	A	Bc-7 + Bc-179 + FG	Bc-618	Bc-7 + Bc-128
21	C			
22	C			
23	C		Bc-7 + Bc-618 + FG	0.5% gelatin
24	C	Bc-7	Bc-7 + FG	Bc-128
25	C	Bc-7 + Bc-179	Bc-618 + FG	Bc-128 + FG
26	C	Bc-179	Bc-7	Bc-7 + Bc-128
27	C	Bc-179 + FG	Bc-7 + Bc-618	Bc-7 + Bc-128 + FG
28	C	Bc-7 + FG	Bc-618	Bc-7 + FG
29	C	Bc-7 + Bc-179 + FG	0.5% gelatin	Bc-7
30	C	Bc-179	Bc-7 + FG	Bc-7
31	C	Bc-7 + Bc-179	Bc-618 + FG	Bc-7 + FG
32	C	Bc-7	Bc-7	Bc-128 + FG
33	C		Bc-7 + Bc-618	Bc-7 + Bc-128
34	D		Bc-7 + Bc-618 + FG	Bc-7 + Bc-128 + FG
35	D		Bc-618	Bc-128
36	D		Bc-618 + FG	Bc-7
37	D		0.5% gelatin	Bc-128
38	D		Bc-7	Bc-128 + FG
39	D		Bc-618 + FG	0.5% gelatin
40	D		Bc-7 + FG	Bc-7 + Bc-128
41	D	Bc-7 + Bc-179	Bc-7 + Bc-618 + FG	Bc-7 + Bc-128 + FG
42	D	Bc-7	Bc-618	Bc-7 + FG
43	D	Bc-179	Bc-618 + FG	Bc-128
44	D	Bc-7 + Bc-179 + FG	Bc-7 + Bc-618	Bc-7 + FG
45	D	Bc-7 + FG	Bc-7 + FG	Bc-7
46	D	Bc-179 + FG	Bc-618	Bc-7 + Bc-128
47	D	Bc-7 + Bc-179	0.5% gelatin	0.5% gelatin
48	D	Bc-7	Bc-7 + Bc-618 + FG	Bc-7 + Bc-128 + FG
49	D	Bc-179	Bc-7	Bc-128 + FG

Figure 6.14. Curtin University vineyard row and vine layout (A), with numbers indicating individual vines. List of each treatment for each experiment (B). Grey boxes indicate vines that were not inoculated. cab sav = cabernet sauvignon, mus = muscat, + FG = fungicide treatment.

6.2.2.3. DNA extraction and dPCR analysis

To extract total DNA for the DBA and vineyard experiments, each biological replicate was ground three times in liquid nitrogen using a mortar and pestle, with all seeds removed between the first and second grinding steps. DNA was extracted using a CTAB method (Porebski et al. 1997), with modifications. For the extraction buffer, 3% CTAB (w/v) was used with the addition of 1% (w/v) polyvinyl pyrrolidine (PVP). For the vineyard samples, extraction buffer and chloroform volumes were doubled due to the large amount of starting material (nine berries). For ethanol precipitation, 3 M NaAc (pH 5.2) was used instead of 5 M NaCl, and samples were centrifuged for 20 min at 3900 g. Pellets were re-suspended in 500 µL of TE buffer (pH 8). DNA was visualised via gel electrophoresis to ensure integrity and absence of

RNA. DNA samples were diluted (10 – 20-fold) in TE buffer and concentrations measured using a Quantas™ fluorometer (Promega, U.S.A). CdPCR and ddPCR were both utilised in this study. All assay sets were designed in the Custom Taqman® Assay Design Tool (Thermo Fisher Scientific, U.S.A) and included 10 µM and 36 µM of each probe and primer (40X), respectively (Table 6.6). The primer sequences and dPCR annealing temperatures are shown in Table 6.8. For initial optimisation purposes, pure gDNA was first extracted from isolates Bc-7 (WT), Bc-128 (D616G), Bc-179 (I365S), and Bc-396 (L412F) as described in Section 5. The gDNA was used to optimise annealing temperatures for all probe sets in cdPCR, to ensure separation of VIC, FAM, and the no-reaction clusters. CdPCR was carried out as described by Zulak et al. (2018). The cdPCR reaction mixture included 0.3 µL probe set, 8.5 µL buffer (Bioline, U.K.), 2-75 ng of diluted DNA, with the addition of water up to a total reaction volume of 17 µL. The reaction mix was loaded on to a QuantStudio™ 3D Digital PCR chip and cycled on Geneamp 9700 thermocycler (Applied Biosystems, U.S.A). The PCR cycle was as follows; 96°C for 10 min, 39 cycles at 50°C or 58°C for 2 min and 98°C for 30s, followed by 50°C or 58°C for 2 min, then 22°C hold. Chip reading and data analysis were carried out as described by Zulak et al. (2018). The ddPCR reaction mixture included 0.4 µL probe set, 11 µL ddPCR probe supermix (Bio-Rad, U.S.A), 1 – 13 ng of diluted DNA (TE buffer), with the addition of water up to a total reaction volume of 22 µL. Each reaction was then loaded into a 96 well microtiter plate and plates were then sealed in a PX1 PCR plate sealer (Bio-Rad Laboratories) at 180°C for 5 s. Sealed sample plates were placed in a QX200 Auto droplet generator (Bio-Rad Laboratories, U.S.A) for droplet generation. Droplet plates were sealed as described above and cycled on a C1000 Touch™ Thermal cycler (Bio-Rad Laboratories, U.S.A) with the following cycle conditions: 95°C for 10 min, then 39 cycles of 94°C for 30 s and 50°C annealing for 1 min, followed by 98°C for 10 min, then 4°C hold.

Table 6.8. Details for each assay used in dPCR.

Assay name (Target, Gene, FRAC group)	Primer name	Sequence (5′-3′)	Annealing temperature (°C)
BcI365S (I365S, *Os1*, 2)	Bcbos1-I365-VIC	VIC/CGCCTTCGATTTCT/MGB-NFQ	58 (cdPCR)
	Bcbos1-S365-FAM	FAM/CGCCTTCGTTTTCT/MGB-NFQ	
	Bcbos1-365-F	GCAAGAGATGTAGGAACTGAAGGT	
	Bcbos1-365-R	GCCATAGCGTTGACGTTCACTA	
BcL412F (L412F, *Pos5*, 9)	Bcpos5-L412-VIC	VIC/CTTGAATGGGTTGCTGAAG/MGB-NFQ	50 (cdPCR and ddPCR)
	Bcpos5-F412-FAM1	FAM/CTTGAATGGGTTTCTGAAGT/MGB-NFQ	
	Bcpos5-412-F1	AAAGATGACGACGGTTGGGTTG	
	Bcpos5-412-R2	TCGCCAAAAGGATGGTTGA	
BcD616G (D616G, *Mrr1*, 12)	Bcmrr1-D616-VIC	VIC/ATTCGTTGCAGATACAAA/MGB-NFQ	50 (ddPCR)
	Bcmrr1-G616-FAM	FAM/TTCGTTGCAGGTACAAA/MGB-NFQ	
	Bcmrr1-412-F1	GGAGGGCATTGAGTTTCACTACAA	
	Bcmrr1-412-R2	GGTCGCCAAATCATCCCATCT	

Droplet plates were then placed in a QX200 droplet reader (Bio-Rad Laboratories, U.S.A) for droplet analysis. All ddPCR data was manually edited and analysed using QuantaSoft™ Analysis Pro 1.0 software (Bio-Rad Laboratories, U.S.A). All copy numbers from cdPCR and ddPCR were then normalised according to amount of input DNA to allow for comparisons. All statistical analyses were conducted in SPSS software (IBM). To compare mean copy numbers between treatments, values were analysed using a student's t-test (P = 0.05).

6.2.3. Results

6.2.3.1. Detached berry assays (DBA)

For all DBA experiments, relative biomass levels of sensitive or resistant isolates were established based on WT or mutant copy numbers of each resistance associated gene. In experiment 1 (2018) and 2 (2019), no significant differences in biomass were found when comparing WT and mutant isolate treatments independently (Figure **6.15** and Figure **6.16**) and in co-inoculations (Figure **6.15**). In experiment 3 (2020), there were no significant differences in biomass when comparing mean WT and mutant isolate treatments independently (Figure **6.17**). In the Bc-7 + Bc-128 and Bc-7 + 391 co- inoculation treatments, WT biomass was significantly greater (P < 0.05) than mutant levels. In the Bc- 410 + Bc-128 and Bc-410 + Bc-391 co-inoculation treatments, WT biomass was not significantly different (P > 0.05) and significantly lower (P < 0.05) compared to mutant levels, respectively (Figure **6.17**).

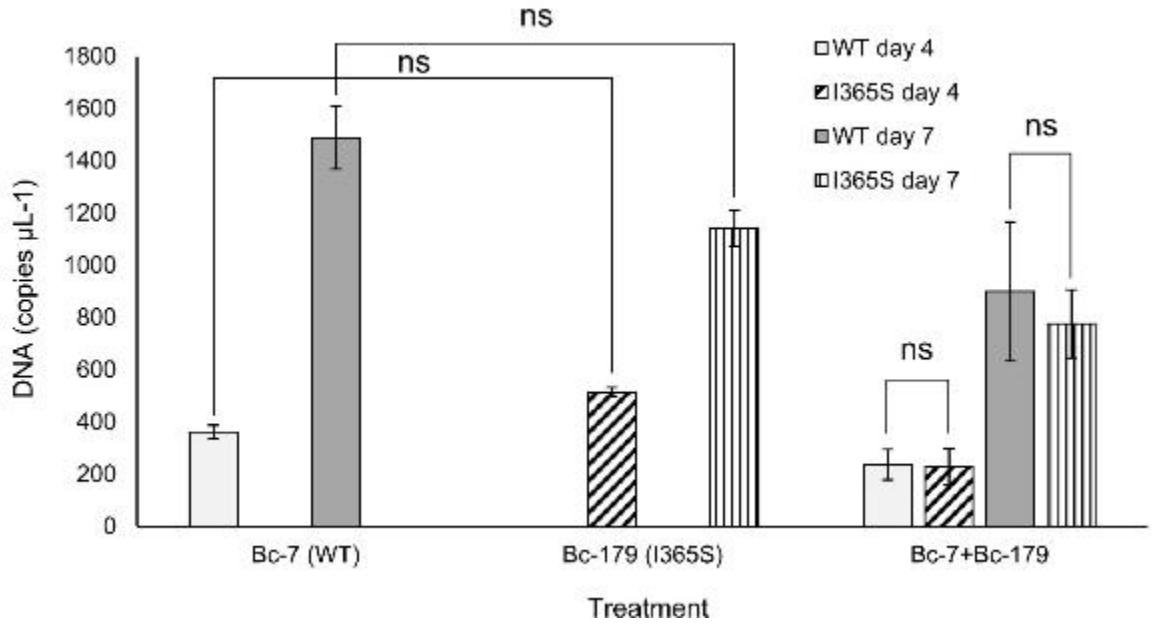


Figure 6.15. DBA Experiment 1 (2018). Relative biomass (copy number) of the sensitive isolate Bc-7 (WT) and iprodione resistant isolate Bc-179 (I365S) as calculated by cdPCR. 'ns' indicates no significant difference at P > 0.05 using Student's t-test.

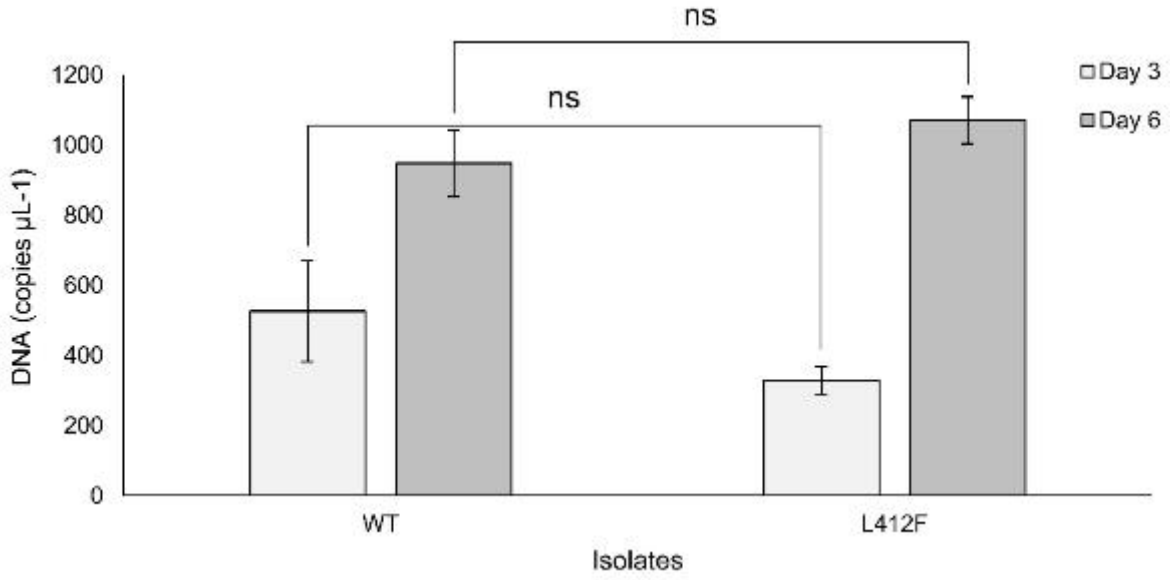


Figure 6.16. DBA experiment 2 (2019). Relative biomass (copy number) of WT sensitive isolates Bc-7, Bc-410 and Bc-486 (light grey) and L412F pyrimethanil resistant isolates Bc-132, Bc-396 and Bc-618 (dark grey) as calculated by cdPCR. 'ns' indicates no significant difference at *P* > 0.05 using Student's t-test.

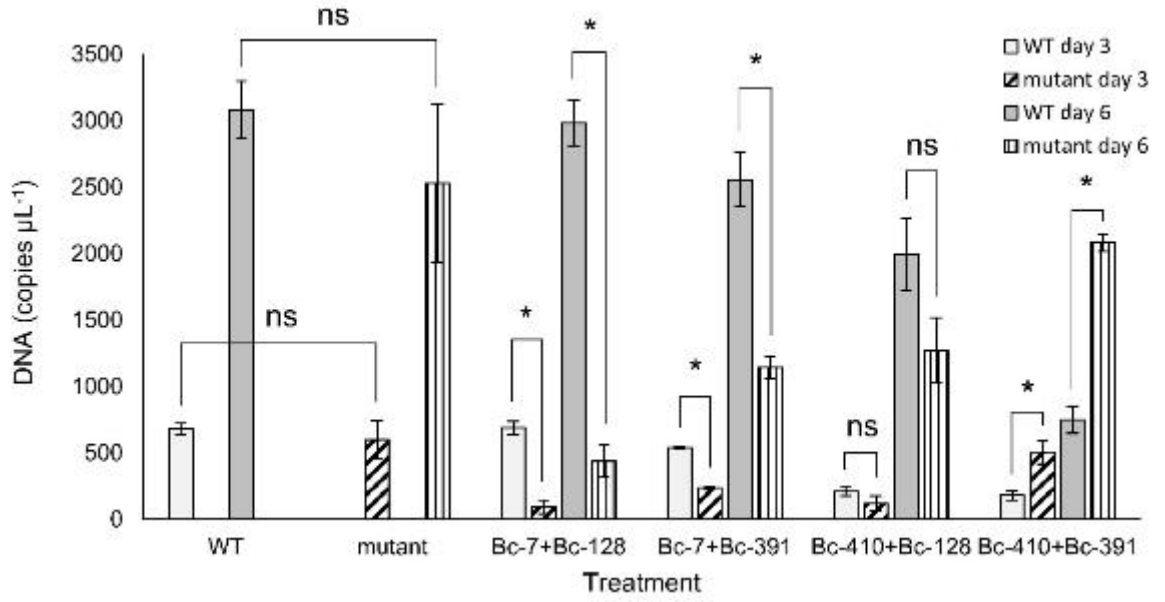


Figure 6.17. DBA Experiment 3 (2020). Relative biomass (copy number) of sensitive isolates Bc-7 and Bc-410 (WT) and mutant (D616G) fludioxinil resistant isolates Bc-128 and Bc-391 as calculated by cdPCR. WT and mutant columns indicate mean copy number for Bc-7 + Bc-410 and Bc-128 + Bc-391. * and 'ns' indicate significant (*P* < 0.05) and no significant difference (*P* > 0.05) using Student's t-test, respectively.

6.2.3.2. Vineyard experiments

In the five vineyard experiments (WA and SA, 2018–20), relative levels of WT or mutant biomass were established based on WT or mutant copy numbers of each resistance associated gene. In vineyard experiment 1 (2018, SA), the WT-only treatment (Bc-385) had significantly greater ($P < 0.05$) biomass in the absence of fungicide than the equivalent mutant treatment (Bc-401, I365S) (Figure 6.18). However, these differences could be due to the high levels of WT present in the mutant treatment, most likely from environmental cross-

contamination. There were no significant differences ($P > 0.05$) between the wild type and the mutant when twice the iprodione field rate was applied. Biomass of the WT was significantly greater ($P < 0.05$) with no fungicide treatments when the WT was co-inoculated with the mutant. The opposite occurred when twice the iprodione field rate was applied ($P < 0.05$) (Figure 6.18). In vineyard experiments 2 (2018, WA) and 3 (2019, SA), no significant differences ($P > 0.05$) between the wild type (Bc-7/Bc-385) and mutant (Bc-179/Bc-400) treatments were found in all single and co-inoculation treatments (Figure 6.19 and 6.20). In vineyard experiment 2, very low levels of mutant were detected in the WT Bc-7 fungicide exposed treatment (Figure 6.19). In vineyard experiment 3, WT and mutant biomass was detected in the uninoculated treatment (Figure 6.20). In vineyard experiment 4 (2019, WA), the WT-only treatment (Bc-7) had significantly greater ($P < 0.05$) biomass in the absence of fungicide than the equivalent mutant treatment (Bc-618) (Figure 6.21). Again, low mutant levels could be due to WT cross-contamination in mutant only treatment. WT biomass was also identified in the mutant only treatment exposed to fungicide, and in the 0.5 % gelatin control. The WT biomass levels were significantly greater ($P < 0.05$) than those from the mutant in the co-inoculations, including the treatment that had twice the field rate of Scala® (Figure 6.21). In vineyard experiment 5 (2020, WA), there were no significant differences ($P > 0.05$) when comparing the WT (Bc-7) and mutant (Bc-128) biomass levels in the single no fungicide treatments (Figure 6.22). The co-inoculation treatment in the absence of fungicide showed significantly greater ($P > 0.05$) levels of the WT. No significant differences ($P > 0.05$) were found when the co-inoculation treatment was sprayed with fungicide. WT biomass was detected at very low levels in treatments Bc-128, Bc-128 (1X) and 0.5 % gelatin. Very low levels of mutant biomass were detected in treatment Bc-7 (Figure 6.22).

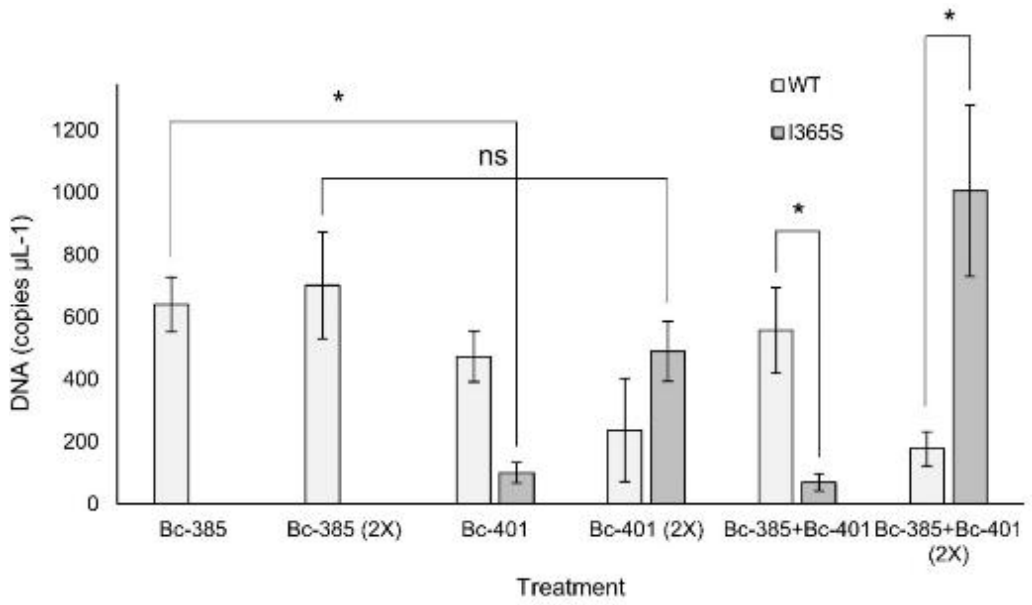


Figure 6.18. Vineyard experiment 1 (2018, SA). Relative biomass (copy number) of the WT (light grey) and mutant (dark grey) in treatments inoculated with Bc-385 (WT), Bc-401 (I365S) or the combination of both. (2X) indicates twice the field rate of Iprodione 500SC sprayed 6 weeks before harvest of berries for *Botrytis* expression (post-veraison). * and 'ns' indicate significant ($P < 0.05$) and no significant difference ($P > 0.05$) using Student's t-test, respectively.

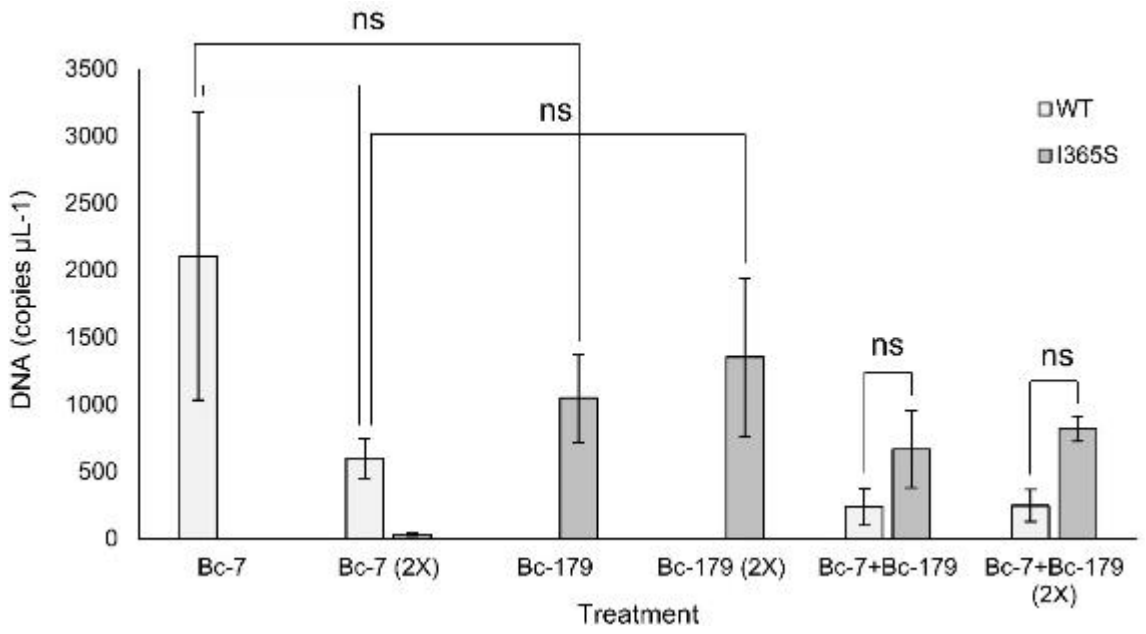


Figure 6.19. Vineyard experiment 2 (2018, WA). Relative biomass (copy number) of the WT (light grey columns) and mutant (dark grey columns) in treatments inoculated with Bc-7 (WT), Bc-179 (I365S) or the combination of both. (2X) indicates twice the field rate of Iprodione 500SC sprayed at day 4 post commencement of sprinkling. *Botrytis* was harvested 25 d post commencement of misting. 'ns'" indicates no significant difference at *P* > 0.05 using Student's t-test.

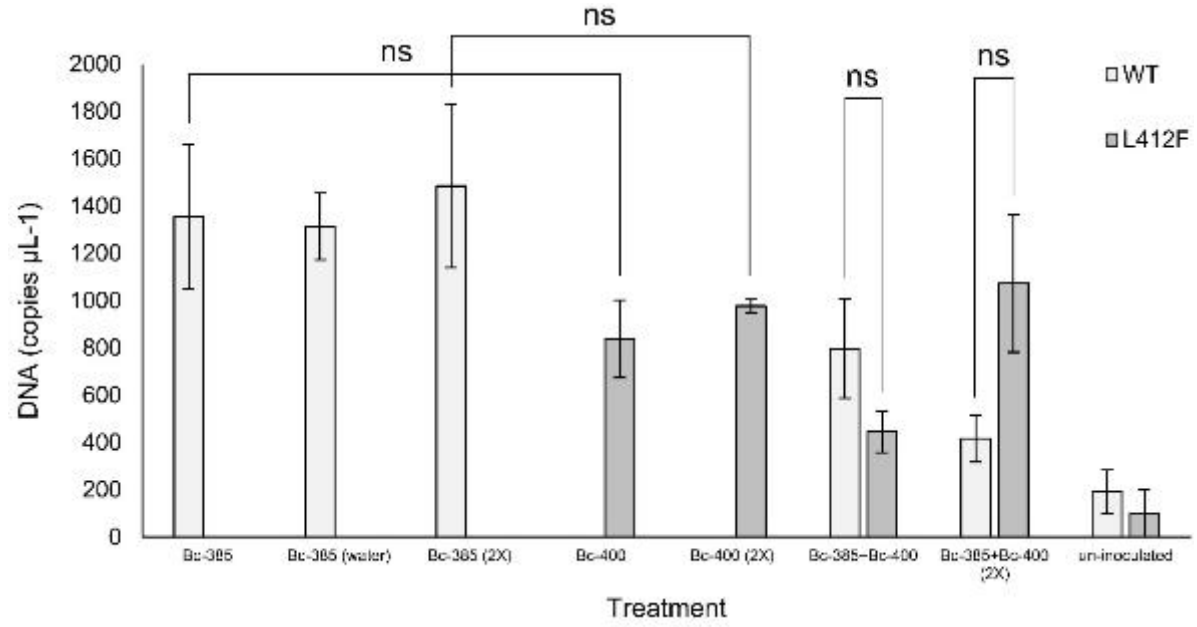


Figure 6.20. Vineyard experiment 3 (2019, SA). Relative biomass (copy number) of the WT (light grey) and mutant (dark grey) in treatments inoculated with Bc-385 (WT), Bc-400 (L412F) or the combination of both, as calculated by cdPCR. (2X) indicates twice the field rate of Scala® sprayed 6 h post inoculation. (water) indicates water applied 6 h post inoculation. Bunches with *Botrytis* were harvested post-veraison. 'ns' indicates no significant difference at *P* > 0.05 using Student's t-test.

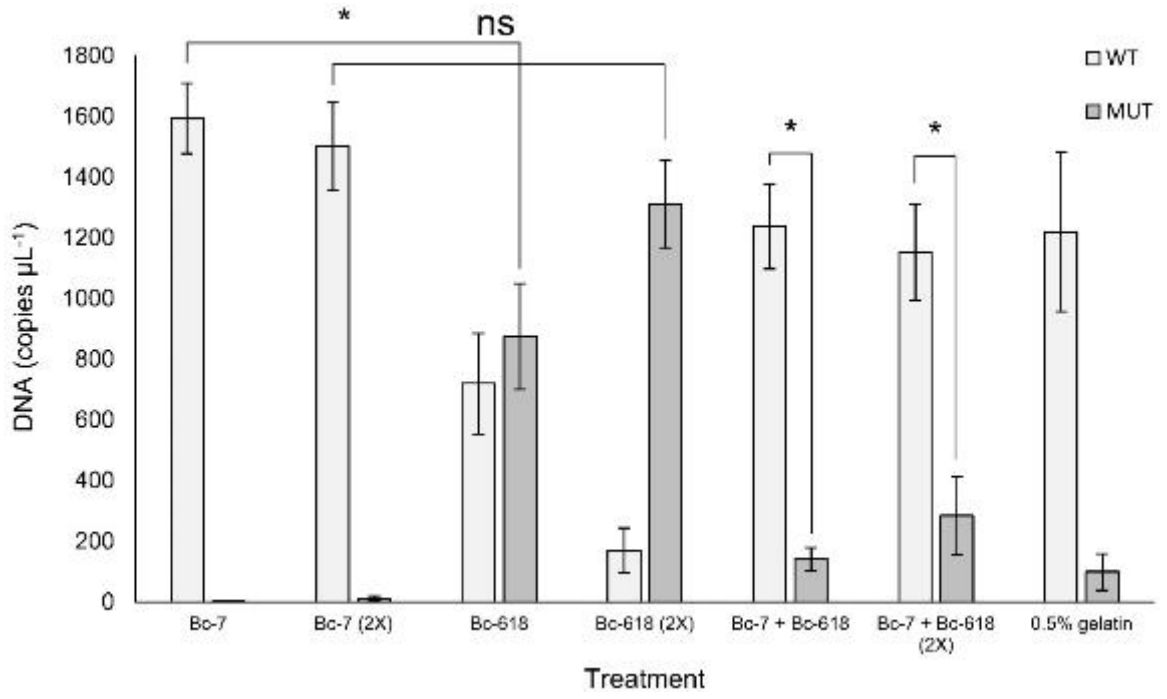


Figure 6.21. Vineyard experiment 4 (2019, WA). Relative biomass (copy number) of the WT (light grey) and mutant (dark grey) in treatments inoculated with Bc-7 (WT), Bc-618 (L412F) or the combination of both, as calculated by cdPCR. (2X) indicates twice the field rate of Scala® sprayed 24h before inoculations. *Botrytis* was harvested 14 d post commencement of misting. * and 'ns' indicate significant (*P* < 0.05) and no significant difference (*P* > 0.05) using Student's t-test, respectively.

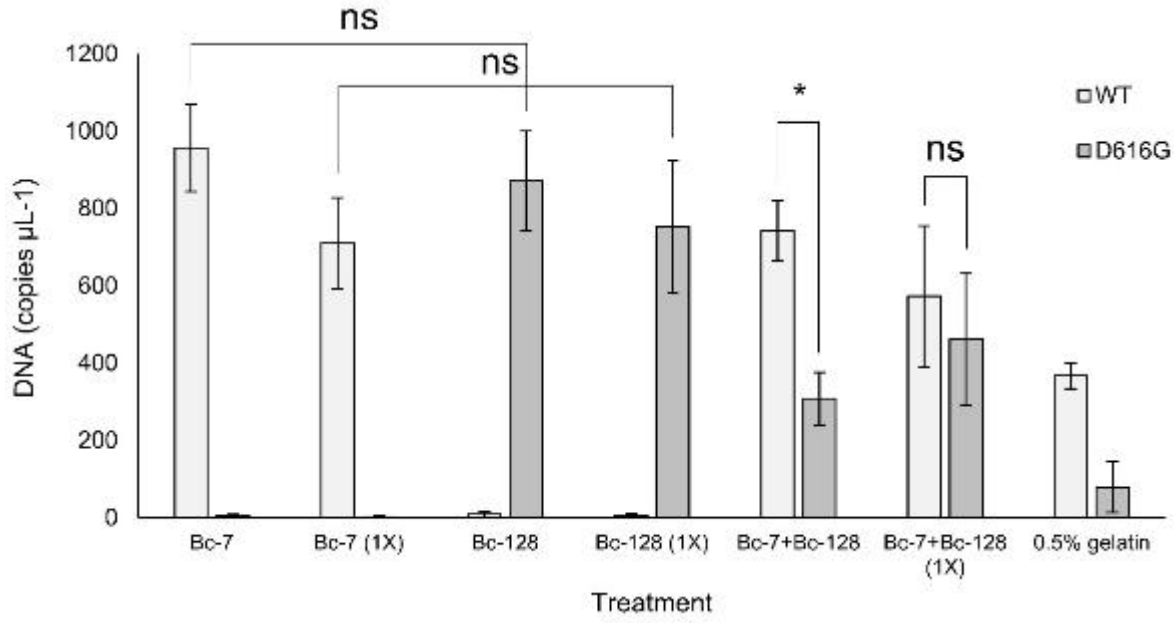


Figure 6.22. Vineyard experiment 5 (2020, WA). Relative biomass (copy number) of the WT (light grey bars) and D616G (dark grey bars) in treatments inoculated with Bc-7 (WT), Bc-128 (D616G) or the combination of both, as calculated by ddPCR. (1X) indicates field rate of Switch® sprayed at 24h before and 4 d after inoculation. *Botrytis* was harvested 18 d post commencement of misting. * and 'ns' indicate significant (*P* < 0.05) and no significant difference (*P* > 0.05) using Student's t-test, respectively.

6.2.4 Discussion

In this study, *in vitro* and *in planta* methods were used to compare relative biomass of sensitive and resistant isolates with the aim of characterising the presence of pathogenic fitness penalties in *B. cinerea* isolates resistant to group 2, 9 and 12 fungicides. In the *in vitro* DBA method, conidial germination and mycelial growth abilities are measured as part of the pathogenicity analysis. However, *B. cinerea* isolates did not sporulate on detached berries, so the sporulation component of any potential fitness cost could not be assessed. No evidence of differences in relative growth between WT and mutant isolates was identified when comparing single inoculum treatments in any of the DBA experiments. In two of the co-inoculation treatments in experiment 3, one of the WT isolates (Bc-7) exhibited greater relative growth than the mutant isolates. However, the other WT isolate (Bc-410) had similar or lower relative growth compared to the two mutant isolates. In all experiments, biomass levels appeared to be quite variable and isolate dependent, probably due to the highly polymorphic nature of *B. cinerea*. Additionally, isolates resistant to the four MOA (Bc-391 and Bc-396) did not appear to be any less pathogenic than isolate resistant to three or less MOA (Bc-128, Bc-132 and Bc-618). In this DBA study, there appears to be no considerable evidence for the existence of a pathogenic fitness penalty. Kim and Xiao (2011) found isolates resistant to azoxystrobin and boscalid to have a competitive disadvantage in co-inoculation with WT isolates on apple fruit. Chen et al. (2016) identified small reductions in *in vitro* fitness components in isolates resistant to five or six MOA compared to sensitive isolates. Grabke et al. (2014) could not correlate changes in growth rates on PDA or lesion sizes on strawberries when sensitive and dicarboximide resistant phenotypes were compared. Other studies with dicarboximide resistant isolates also failed to find fitness penalties (Beever and Brien, 1983, Cui et al. 2004, Oshima et al., 2006). Bardas et al. (2008) and (Fernández-Ortuño et al. 2013) could not characterise fitness penalties in AP resistant isolates after testing *in vitro* growth parameters. To our knowledge, reduced pathogenic fitness has only been described in two fludioxinil resistant *B. cinerea* isolates from apple and one isolate from strawberry (Li et al. 2014, Zhao et al. 2010). The results provided in this report emphasise the highly variable nature of *B. cinerea* isolates, which probably adds another layer of complexity to the adequate management of the disease in the field. Like the DBA method, measuring relative growth of WT and mutants on whole bunches from vineyard settings allowed for investigation into the presence of pathogenic fitness penalties. Unlike the DBA method, pathogenicity on bunches would involve sporulation, so this pathogenic fitness component would contribute to relative biomass levels of a particular isolate. In vineyard experiments 1 and 4, relative levels of WT biomass in the absence of fungicide were significantly greater in WT only inoculated treatments than those inoculated with the mutant only. These results could suggest the existence of pathogenic fitness penalties in the resistant isolates. However, primary, or secondary WT infections from other sources could have increased WT levels in WT only inoculated treatments and suppressed mutant biomass in mutant only inoculated treatments. In vineyard experiments 3 – 5, *B. cinerea* was found in non-inoculated treatments, which could indicate the presence of primary and/or secondary infections from natural sources. Fungicide application appeared not to have a clear or significant effect over fungal growth in the WT only inoculated treatments. This could be due to sub-optimal fungicide application timing/coverage or to late harvesting of latent/expressed disease as this could have concealed fungicide treatment effects. The application of fungicide to the co-inoculated treatments did not translate into a significant advantage for the mutant in the majority of cases. Kretschmer et al. (2009) could not identify any significant fitness cost in field trials inoculated with fludioxonil resistant isolates. However, Walker et al. (2017) found decreasing frequencies of fludioxonil resistant strains once fungicide was removed, indicating that a fitness cost may be present. In this study, DBA experiments provided very little evidence to support the presence of fitness penalties in *B. cinerea* isolates resistant to AP, dicarboximide and PP fungicides. Some evidence in co-inoculation treatments was found to support the presence of a competitive disadvantage in resistant isolates. However, the analysis of the vineyard experiment results suggested evidence of fitness penalty in some of the treatments. This is illustrated by the

significant biomass differences found when comparing WT only and mutant only inoculated treatments. However, these differences were only significant in two of the experiments. Furthermore, these differences may have been heightened by the presence of WT biomass in some of the mutant only treatments. Limitations in the DBA and vineyard experiments include a limited number of isolates and harvesting time points, which could have affected the reliability of the results obtained. Future work should consider this and possibly the need of incorporating additional *in vitro* growth parameters (e.g. mycelial growth rate, sporulation ability) to support findings in DBA and vineyards experiments.

7. Evaluation of spore trapping technologies

7.1. Introduction

Resistance can be monitored using either laboratory-based phenotypic bioassays and/or genetic tests to detect the associated mutations. Culture maintenance and bioassays with *Erysiphe. necator* require live plant tissue, and as a result these processes are time-consuming and laborious (Corio-Costet, 2015). These bioassays have several drawbacks associated to working with obligate pathogens including:

- Reliance on vineyard staff and viticulturalists to find, collect, appropriately package and send samples prompty to the research laboratory.
- *E. necator* quickly loses viability in storage.
- Culture collections for the disease samples must be continuously maintained, which is costly in terms of personnel time.
- A high risk of contamination from both other cultures of *E. necator* and fungal contaminants.

If the genetic mechanisms of resistance are known, molecular detection for resistance is faster, more sensitive, accurate and cost effective than bioassays (Falacy et al. 2007, Kunova et al. 2021).

Collecting samples from vineyards that are suitable for molecular resistance tests has been a focus of research in recent years, particularly using spore traps. Spore traps have been used for over 60 years to detect and/or quantify fungal pathogens in the field (West and Kimber 2015), including airborne powdery mildew spores (Mahaffee and Stoll, 2016). To date, detection of *E. necator* spores using spore traps has been primarily used for disease forecasting and decision making with respect to spray applications. There are broadly two types of traps, 1. passive traps that rely on airflow or wind to deposit spores onto the trap surface and 2. active or volumetric traps that require a power source and fan to actively draw the air containing spores into the trap.

Spores of *E. necator* move most readily within the vineyard in air currents between rows and vines (Gonzalez-Fernandez et al. 2019). Several designs of spore traps are commercially available, with spinning rod impaction style passive traps being the cheapest and most portable. A large research project led by Dr. Walt Mahaffee (Oregon State University/United States Department of Agriculture (USDA), Coravallis, Oregon, USA), is currently evaluating a network of these traps across viticultural regions of Oregon, Washington and California in the USA to detect and quantify fungicide resistance. *E. necator* has been successfully detected (Thiessen et al. 2016) as well as the G143A mutant (Miles et al. 2021).

Some fungicide groups, eg. QoIs, have a strong relationship between phenotyping and genotyping, which has been established in this project (section 5.1.3.6.2) and overseas (Miles et al. 2021). Therefore, establishing molecular marker (associated with the resistance) detection systems is going to replace the conventional phenotyping system, as has been done in USA for *E. necator* (Kunova et al, 2021, Miles et al. 2021). Therefore, finding a practical, reliable and cost-effective collection method is necessary to identify the fungicide resistance. However, the drawback of these methods is that they require a genetic marker, commonly a mutation, associated with resistance.

Several collection methods have been evaluated previously e.g. using cotton bud swabs or washing infected leaves and vine material with chasmothecia, to obtain cultures of *E. necator* (SAR 1204; (Hall et al. 2017). Material with chasmothecia was time consuming, with a low success rate and other methods were useful, hence they have been used in this project.

The aim of this research was to evaluate a number of spore traps compared with conventional methods of sample collection including a mini-vacuum spore collector, washing infected leaves and cotton bud swabs to obtain spore material suitable for molecular detection of *E. necator* and *Botrytis cinerea*, along with the fungicide resistance mutants G143A, Y136F and H242R/Y using Next Generation Sequencing (NGS).

7.2. Methods

7.2.1. Static volumetric Hirst-style spore trap

Prior to studies being carried out using the static Burkard volumetric Hirst-style spore traps (Figure 7.1), preliminary experiments were carried out to evaluate if the glue used on the tapes interfered with DNA extraction and/or sequencing reactions and the minimum threshold for number of spores detectable by NGS.



Figure 7.1. Burkard volumetric Hirst-style spore trap powered by a battery connected to a solar panel.

E. necator

Melinex spore tape used in the Hirst-style spore trap was cut in 5 x 5 mm squares and either coated lightly with a hexane-based glue (Tanglefoot, Tangle-Trap, Australian Entomological Supplies, Australia) as per the manufacturer's recommendation or left without glue as controls. Spore suspensions of *E. necator* were made in sterile Milli-Q (SMQ) water and serially diluted, with the aid of a haemocytometer, starting from 10^6 to 10 spores/mL. Using a micropipetter, spore suspensions were placed on to the pieces of spore tape (+/- glue) and air-dried in a laminar flow cabinet. Spore quantities of *E. necator* lower than 10 were added to the spore tapes by picking up individual spores with a single bristle of a sable hair paintbrush and depositing them on the spore tape. As the spores of *E. necator* are easily observed using a compound microscope, the number of spores deposited on each piece of tape was confirmed by visual observation, and tapes were kept at -20°C.

DNA was extracted from spiked tapes using the Powersoil kit (Qigen) (5.1.1.7) and PCR amplification of the *Cyp51* gene was performed (5.1.1.6).

Spore tapes were also obtained from the grapevine trunk disease (GTD) project (SAR 1701-1.1) from traps located in Hunter Valley (12 samples during Nov and Dec 2014) and Griffith (27 samples during Oct, Nov and Dec 2014). Tapes were originally split into two halves, with one half already processed as part of GTD research and the other half stored at -80°C. The spore tape was cut into small sections as described above, with a total of 39 pieces tested. DNA was extracted and subjected to ITS phenotyping using NGS (5.1.1.7).

B. cinerea

To determine the longevity and viability of spores from *B. cinerea* captured on spore tapes, the same method described above for *E. necator* was used for the preparation and application of spores of *B. cinerea.* However, the number of spores deposited was not counted. This is because the spores of *B. cinerea* are relatively small and tend to clump together, so it was difficult to pick up the desired number of individual spores. Tapes were spiked with *Botrytis* by holding a sporulating agar plate of *Botrytis* and tapping gently to dislodge the spores on to the tape with or without glue. Tapes were stored in 90 mm Petri dishes with lids at room temperature (RT) for up to two months. At 0, 3, 7, 14 28 and 56 days, pieces of spiked spore tape were removed from storage, cut in half, and one side placed glue side up and the other glue side down on to either potato dextrose agar (PDA) or V8 agar (V8A) media amended with streptomycin. Plates were placed in a plastic tray, sealed within a plastic bag and incubated at RT on the laboratory bench. Plates were checked after three days for growth of *Botrytis* from each half of the spore tape piece.

7.2.2. Mobile jet-sampler

Dr Mary Burrows (a visiting scientist from Montana State University, USA) undertook a one-day trial using the mobile jet sampler (Figure 7.2) developed as part of the iMapPESTs project (Davidson, 2018). The mobile jet sampler concept is to allow spore trapping over a wide-geographical area. The trap is compatible to downstream molecular diagnostics. On 19 March 2019, the jet sampler was driven around to collect samples from around vineyards located at the Waite campus, in the Adelaide Hills and at Langhorne Creek, South Australia. Fungicide resistance in *E. necator* has previously been detected in several vineyards located within these regions (section 5.1.1.1). DNA from the spore trap samples was extracted by the SARDI Molecular Diagnostics Centre and quantitative PCR carried out initially targeting *B. cinerea.* Analysis of *E. necator* was not performed, as there were insufficient spores to enable testing for resistant alleles.



Figure 7.2. Mobile jet sampler

7.2.3. Rotorod spore traps

Three rotorod spore traps were provided to SARDI by Dr Walt Mahafee. The rotorod spore trap units comprise a 6 V motor (12 VDC 5624 RPM, Nichibo Taiwan) with the motor shaft connected to a horizontal 4.5 mm sampling arm (Figure 7.3 A, B & C). The sampling rods are silicon bronze 1.6 mm welding rods cut to lengths of 98 mm (Figure 7.3 B). Three 2 mmdiameter holes were drilled in the sampling arm; the middle hole used to connect the sampling arm with motor shaft and the other two holes on the ends of the arms used to attach the sampling rods (Figure 7.3 B & C). Rubber O-rings are used to secure the sampling rods into the sampling arm (Figure 7.3 B). The spore trap was connected to a 12 V rechargeable battery, placed inside a weather-proof plastic box (Figure 7.3 D) The battery was connected to a 10 W solar panel (width 385 mm; height 253 mm and depth 30 mm, Powertech, Australia). The whole unit was installed on a metal pole using brackets, and was then attached to a vineyard post using screws (Figure 7-E). The solar panel was positioned 40 cm above the canopy, with the solar panel facing north. The sampling arm was positioned just above the canopy so that the rods could spin freely yet be close enough to the canopy to capture spores of *E. necator*. The sampling arm was moved upwards as required to allow for canopy growth. The sampling rods were coated lightly with a high vacuum grease (Molykote®, Dow Corning, USA) (Thiessen et al. 2016) prior to installing in the traps in the vineyard. The volume of air sampled for these units was reported in USA to be 48.3 $\pm$ 1.2 L air min^{-1} (Thiessen et al. 2016).

Prior to installation of the rotorod units in the field, a series of experiments were carried out to 1) evaluate the compatibility of the grease with the DNA extraction and sequencing protocols and 2) determine the minimum detection threshold of spores using NGS.

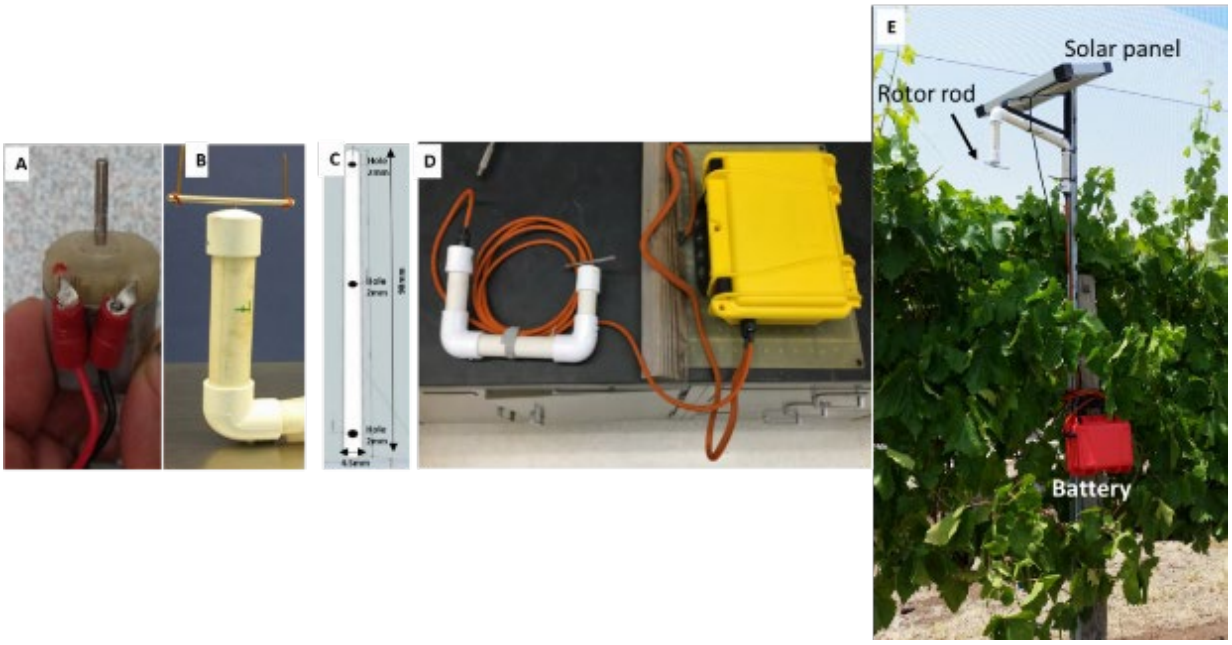


Figure 7.3. Diagram of the rotorod spore trap used in this study. (A) 6 V motor to operate the trap. (B) PVC tube used to enclose the motor and the shaft of the motor connected to the sampling arms, sampling rods attached to the sampling arms using rubber O-rings. (C) Sampling rod diagram showing the rod dimensions and specifications. (D) The spore trap connected to a battery. (E) Rotorod spore trap set up in the vineyard.

7.2.4. Other spore collection methods

To compare with spore trapping other conventional methods of collection were also evaluated.

Mini-vacuum

A mini electric cordless vacuum pump 4.2 V (unbranded, Kogan) was used to manually collect spores from infected leaves. The device was modified by gluing a plastic spout to enable connection of a plastic tube (Figure 7.4 A), which was connected to a standard cyclone separator. Rubber O-rings were used to connect the cyclone separator to 1.5 mL Eppendorf tubes, and the unit was assembled and fitted inside a 500 mL plastic container for easy transportation (Figure 7.4 B). Spores were collected by turning the vacuum on and moving the PVC tube in the cyclone separator along the surface of infected leaves (Figure 7.4 C). The Eppendorf tube with spores are removed and stored at -20°C until required for DNA analysis.

Leaf washing

Spores were collected by adding 10-15 mL of water to a zip-lock plastic bag containing infected leaves (Figure 7.4 D) and shaking vigorously for 30 seconds. The spore suspension was transferred to a tube, transported at 4°C to the laboratory and stored at -20°C.

Cotton bud swabs

Disinfested cotton buds were rubbed across the surface of infected leaves to collect spores (Figure 7.4 E). Swabs were placed in sterile tubes, transported at ambient temperature to the laboratory and stored at -20°C.

Gloves

Gloves were used in 2021-2022 season to collect powdery samples from the locations. Gloves and cotton swab used to collect fungal spores off the gloves of workers already moving through the vineyard. This method was adopted from Dr. Walt Mahaffee's group in Oregon State University in USA. After moving gloved hands through the infected canopy, swabs were taken using cotton buds and samples were kept at -20°C until required.

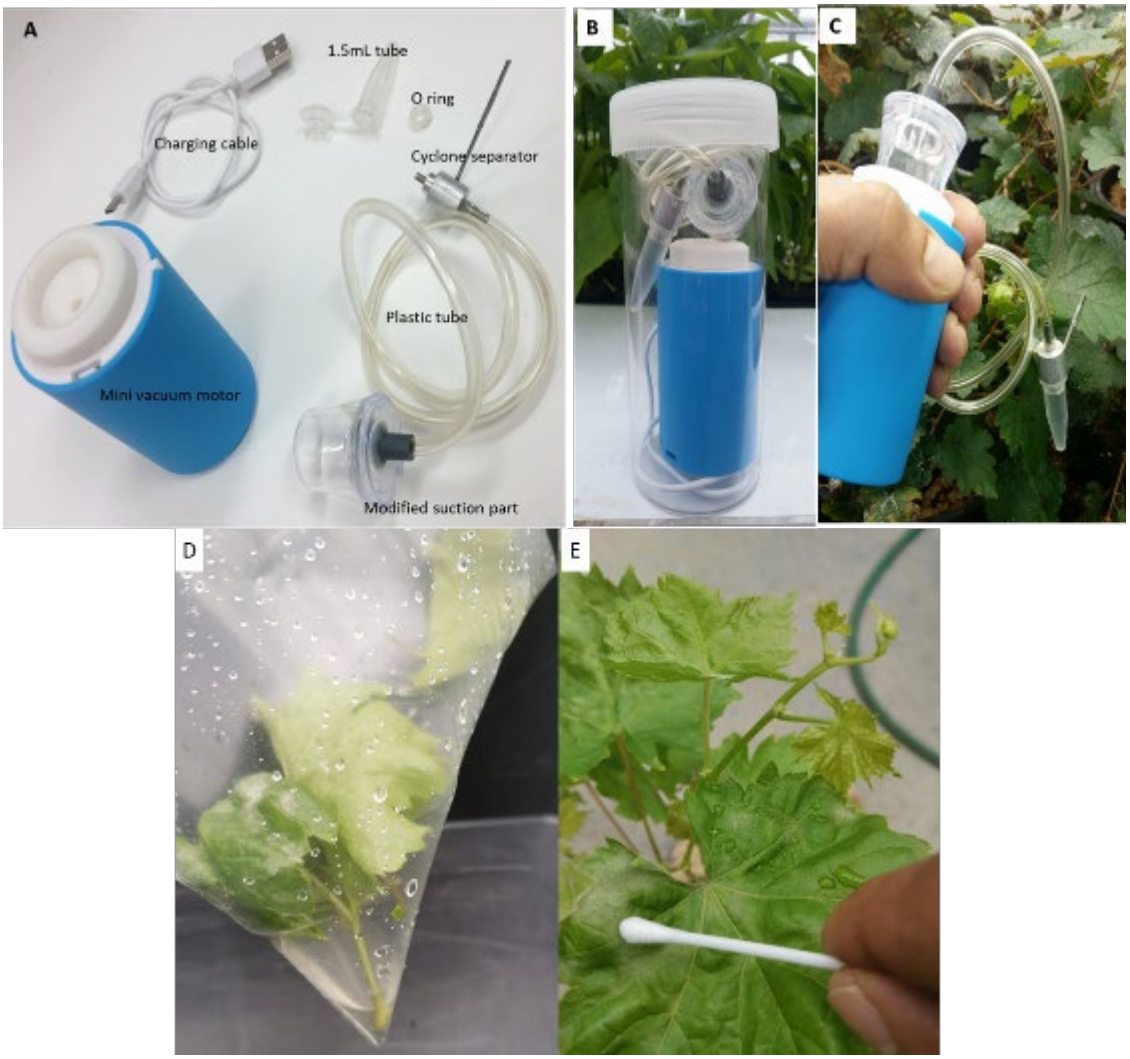


Figure 7.4. (A) Modified mini-vacuum for spore collection, (B) packaged in a container for easy transportation, (C) assembled ready to collect spores from leaves. (D) Leaves washed to collect spores and (E) cotton bud swab collecting spores.

7.2.5. Evaluation of spore traps and other collection methods

Laboratory evaluation

To determine the compatibility of the grease with DNA extraction, rods were disinfested using 80% ethanol and a thin film of vacuum grease was applied to each rod. The rods were then inserted into the motor arms of the trap. Detached grapevine leaves infected with *E. necator*, with different disease severities of powdery mildew, *viz*. low 10%, medium 50% and high 100% (Figure 7.5 A), were obtained from the culture collection. Spores were applied to the rods by

gently tapping an infected leaf above the rods whilst the motor was turned on and the rods were spinning (Figure 7.5 B). Airborne spores were trapped in the grease of the spinning rods. For the positive controls, grease-coated rods were wiped through spores on the 100% infected leaf and grease-coated rods with no spores were the negative controls. Rods were collected and stored in 1.5 mL sterile tubes at -20°C until required for DNA analysis.

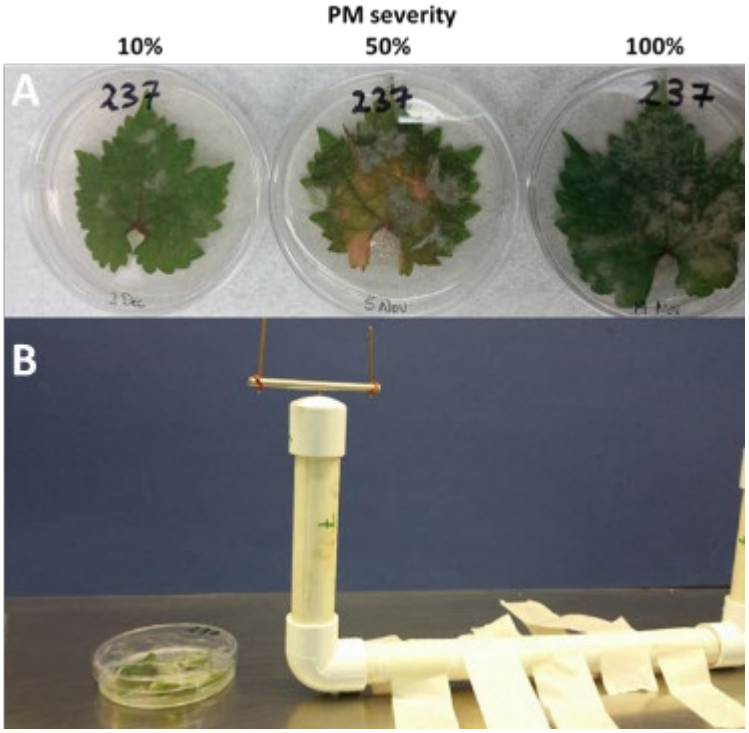


Figure 7.5. (A) Infected detached leaves with different infection severity. (B) spore trap set up inside a laminar flow cabinet to capture *E. necator* spores.

To determine the minimum threshold spore number required for detection, spores of *E. necator* were manually applied onto lightly greased rods under a dissecting microscope. Individual spores or chains of spores were transferred using an acupuncture needle to each sampling rod. The number of spores was 0, 1, 10, 50, 100, 250 and 500 based on methods described in (Falacy et al. 2007). For the positive controls, greased rods were rolled on a heavily infected leaf. There were three replicates for each spore number. Rods were then stored in 1.5 mL sterile tubes at -20°C until required for DNA analysis.

Field experiments

For the pilot experiment, in early January 2020, two spore traps were installed in vineyards, one at the Nuriootpa Research Station (cv. Chardonnay) and the other at the Waite Campus, Urrbrae (cv. Grenache). The traps were placed close to vines infected with powdery mildew as described above (Figure 7.3 E). On 13 January 2020, the units were turned on and ran as scheduled until 6 February 2020 (Table 7.1). Rod samples were collected after four days of running for a total of seven collection times at each site, making a total of 14 samples. Rods were then stored in 1.5 mL sterile tubes at -20°C until required for DNA analysis.

For comparison, samples were also collected at Nuriootpa on 6 February 2020 using the mini-vacuum and by leaf washing techniques (Table 7.1). Samples were stored at -20°C until required for DNA analysis.

Table 7.1. Spore trap collection times in two locations; Nuriootpa Research Centre and Waite Campus, Urrbrae in SA.

Date	Nuriootpa (sample #)	Urrbrae (sample #)
13/01/2020	ST1	ST8
16/01/2020	ST2	ST9
20/01/2020	ST3	ST10
24/01/2020	ST4	ST11
28/01/2020	ST5	-
31/01/2020	ST6	ST13
4/02/2020	ST7	ST14
6/02/2020	Vac*	-
6/02/2020	Wsh**	-

*sample was collected using mini vacuum
**sample was collected using leaf washing

In the 2020/21 and 2021/22 seasons, rotorod spore traps were installed in the same vineyards in Barossa Valley and Urrbrae, as well as an extra vineyard (cv. Chardonnay) each season near Renmark in the Riverland, SA.

In 2020/21, the traps were run continuously over four days for five separate time periods from December to February. For each sampling period, rods were collected from traps that had been running for 1, 2, 3 and 4 days, with a total of 20 samples collected each from the Barossa Valley and Urrbrae and 12 from the Riverland.

In 2021/22, traps were run continuously for two-day periods five times between December and February. Six samples for each site were collected in total of 18 samples.

In addition, the mini-vacuum, leaf washing and cotton bud swab methods were used to collect spores at the Barossa Valley site on 15 January 2021. No disease was observed at Urrbrae and Renmark in 2020/21 so samples were not collected. However, samples were collected from the three locations on 16 February 2022.

7.2.6. DNA extraction and sequence analyses

DNA was extracted from samples using the Mag-Bind Environmental DNA 96 Kit (Omega Biotek). To detect *E. necator*, a Next Generation Sequencing (NGS)-based fungal profiling methodology was applied (Sternes et al. 2017). This uses a DNA-barcoding strategy based on the fungal internal transcribed spacer (ITS) region to determine the proportions of species present in mixed environmental samples.

For resistance allele testing regions of the *CytB, Cyp51 and SdhB* genes that potentially contained resistance mutations G143A, Y136F and H242R/Y, respectively, were amplified by polymerase chain reaction (PCR). Proportions of resistant and sensitive alleles were determined from analysis of NGS data (Ramaciotti Centre for Genomics, UNSW Sydney, Australia) produced from the pooled the PCR products.

7.2.7. A multi-well agar plate spore trapping pipeline to detect L412F in *B. cinerea*

To overcome the limitations for directly testing captured spores, in this study, a combination of culturing, quick DNA extraction and qPCR techniques were used to detect the group 9 resistance associated mutation L412F (*Pos5*) in *B. cinerea*. Spores were captured on multi-

well plates containing agar that had been placed in a passive spore sampler within an experimental vineyard. Culturing prior to molecular analysis allowed *B. cinerea* to be first identified morphologically, and then it provided adequate amounts of biomass for molecular testing using a quick DNA extraction technique (section 5.2).

7.2.7.1. *In vitro* testing

The addition of a DC of fungicide to the spore capture and culturing steps was first investigated to see whether the media could be selective towards group 9 resistance. Sensitive (Bc-7, WT-*Pos5*, section 5) and pyrimethanil resistant (Bc-618, L412F-*Pos5*, section 5) were first tested on DCs. Isolates were sporulated, and spores were harvested and diluted as described in section 5.2. Spores of each isolate were added to 90 mm petri plates (50 spores/plate) containing yeast soluble starch agar (Fillinger et al. 2008) minus the yeast extract (YSSA-YA) and amended with pyrimethanil DCs (0. 4, 1, 2 and 4 µg/mL) or no fungicide. Plates were incubated for seven days and scored each day. The final DC was established at 4 µg/mL.

7.2.7.2. Field testing

Twelve-well plates (Corning®) containing YSSA-YA agar (2 mL/well) with the pyrimethanil DC were used to test the proposed pipeline. The multi-well plates would allow some separation of captured spores, so could assist in estimating resistance frequency. Field testing was undertaken by placing the plates vertically in a passive spore sampler in the Curtin University experimental vineyard (Figure 7.15). Testing under a *Botrytis* expression scenario was undertaken by inoculating the vineyard at flowering with Bc-7 and Bc-618 isolates and expressing *Botrytis* later in the season as described in section 6.2.2. Once *Botrytis* disease was well established, plates were placed in the spore sampler for 24h, with a total of six replicate plates tested. In each plate, the pyrimethanil DC was present in two of the four columns in each plate (Figure 7.16). To minimise the growth of other environmental fungal species, azoxystrobin, salicylhydroxamic acid (SHAM) and tebuconazole were also added at 0.1, 60 and 0.4 µg/mL, respectively. Plates were then exposed to near-UV for 5 - 10 days to enable sporulation of any germinated *Botrytis* spores. Any sporulated biomass was then harvested and used to produce quick DNA extractions (section 5.2). Following this, duplex qPCR was undertaken to detect the presence of WT and L412F genotypes in each sampled well as described in section 5.2.

7.3. Results

7.3.1. Static volumetric Hirst-style spore trap

E. necator

The experiments with spiked spore tapes both with and without glue revealed that the glue did not adversely affect DNA extraction or the PCR process. The *Cyp51* gene product was readily detectable using gel electrophoresis following 35 cycles of PCR in all samples and there was a clear relationship between the quantity of spores and the PCR intensity. Further PCR analysis of the spore tape samples determine that the lower limit of detection was 10^3 spores, to provide sufficient DNA quality suitable for NGS.

B. cinerea

B. cinerea grew from tapes irrespective of side placed on the agar, from both PDA and V8A (Figure 7.6). *Botrytis cinerea* grew from the tape pieces at 0, 3, 7, 14, 28 and 56 days after spiking, showing that it remains viable and culturable from spore tape stored at room temperature in the laboratory for up to two months.

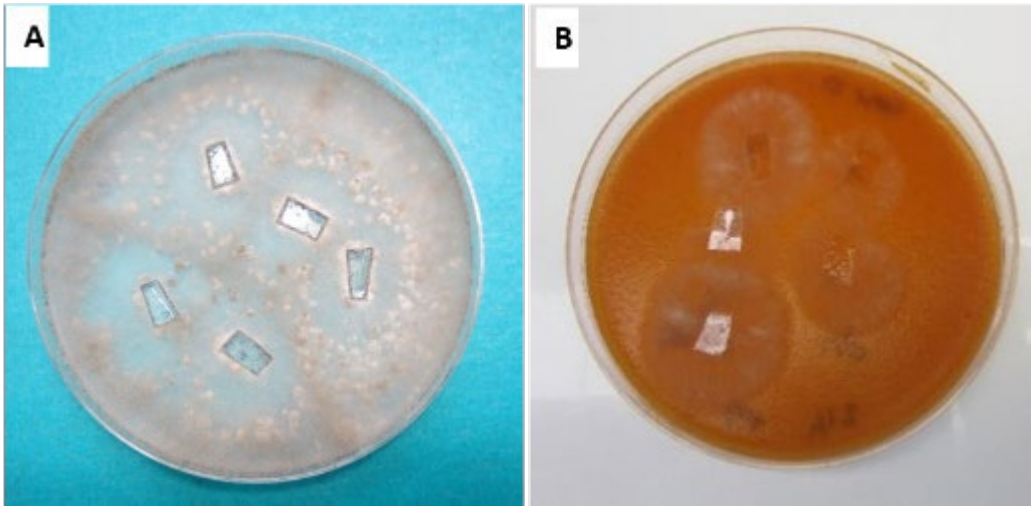


Figure 7.6. Spike spore tapes for *B. cinerea* growing on PDA (A) and V8 agar (B).

Next generation sequencing analyses of the ITS region of spores on the spore tapes obtained from the GTD project SAR 1701-1.1 (n=39) found over 35 different fungal genera were detected across the samples. *Alternaria* spp. and *Cladosporium* spp. showing the highest average abundance across the samples. Only two samples showed trace levels (<0.01%) of *E. necator* and at these levels it was not possible to specifically amplify any alleles associated with resistance.

7.3.2. Mobile jet sampler

Botrytis cinerea was detected and quantified in each of the five samples collected from the Waite campus, Adelaide Hills and Langhorne Creek regions (Table 7.2). The number of copies of DNA/g of sample material ranged from 0.2 to 2.9. However, *E. necator* was not detected.

Table 7.2. Quantity of DNA (kilo copies/g sample) of *Botrytis cinerea* detected using the mobile jet sampler in various locations in Urrbrae, the Adelaide Hills and Langhorne Creek regions in South Australia.

Sample	***B. cinerea*** **kDNA copies/g**	Location
BC67740	0.445	Deviation road, Adelaide Hills
BC67741	0.502	Gumeracha, Adelaide Hills
BC67742	1.717	Langhorne Creek
BC67743	0.221	Waite campus, Urrbrae
BC67744	2.900	Woodside, Adelaide Hills

* a kilo copy =1000 copies

7.3.3. Rotorod spore traps

Laboratory evaluation

The experiments confirmed that the grease was compatible with DNA extraction and PCR processes. DNA was successfully extracted from rods spiked with *E. necator* from both low and high powdery mildew severity categories, with no PCR product found in the negative controls (Figure 7.7). Also, both *Cyp51* and *CytB* genes were amplified. These results indicate that the greased rods were able to capture spores that are suitable for subsequent DNA extraction and PCR processing. Samples from the medium disease severity category were not required for processing due to the success of the PCR result for the low and high category samples.

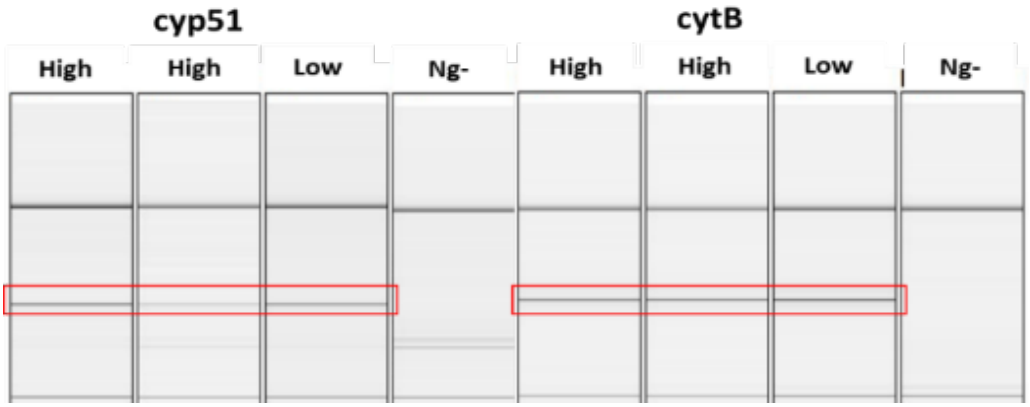


Figure 7.7. Results of PCR on DNA extractions from spore trap rods spiked in the laboratory with *Erysiphe necator*. PCR products from high and low inoculum spore rod samples are visible in lanes amplified with primers targeting *Cyp51* and *CytB* (red rectangles), but is absent in the negative control (Ng-) lanes.

The determination of the threshold for the minimum number of spores detectable with NGS revealed that as few as 10 spores provided sufficient DNA to detect the genus *Erysiphe* (Figure 7.8). In addition, 50, 100, 250, 500 spores and a positive control were also detected in high proportion, however a negative control and 1 spore were not detected.

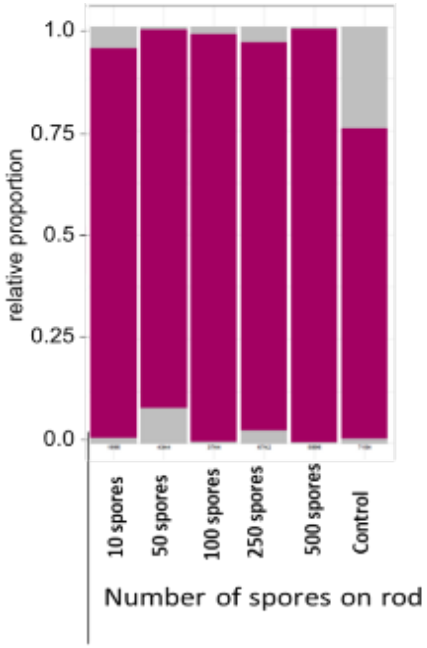


Figure 7.8. NGS results from DNA extractions from spiked rods of the spore trap presented as relative proportions within the population *E. necator* detection results. Only reliable data (above 100 reads) were presented in the graph, 0 and 1 spore treatment were below 1000 reads hence not presented.

Field experiments

Samples obtained from the spore traps installed in the field In January and February 2020 were processed by PCR using fungal ITS region primers. Fungal DNA was successfully extracted from all samples (Figure 7.9).

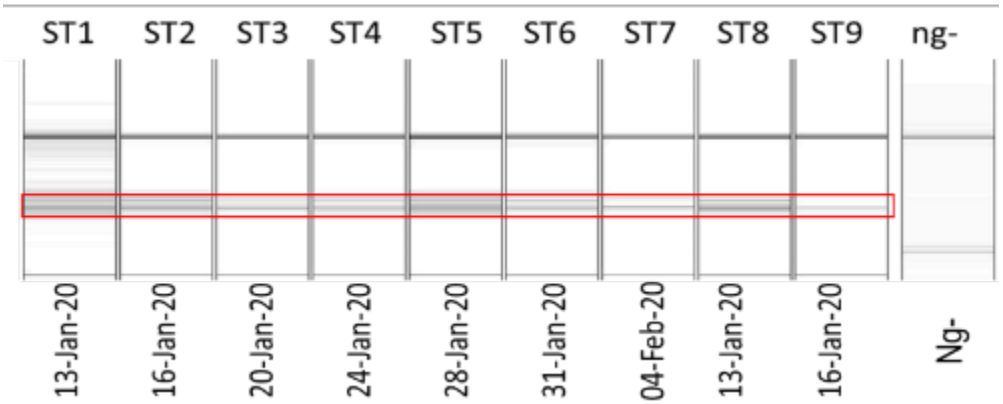


Figure 7.9. Results of PCR on DNA extractions from spore trap rods collected from vineyards in the Barossa Valley and Urrbrae, South Australia. PCR product from rod samples is visible in lanes amplified with primers targeting fungal ITS (red rectangles) but is absent in the negative control (Ng-) lanes.

NGS analysis showed that 17 fungal genera, including *Alternaria*, *Cladosporium*, *Epicoccum* and *Erysiphe* were detected at differing quantities from both the Nuriootpa and Urrbrae sites (Figure 7.10 A). In general, samples from Barossa Valley had more *Erysiphe* than those from

Urrbrae, and the highest detection rates occurred on 20 and 28 January 2020 at the Barossa Valley site.

Mutations were also detected from samples collected using the other methods; mini-vacuum and leaf washing in the Barossa vineyard (Figure 7.10 B).

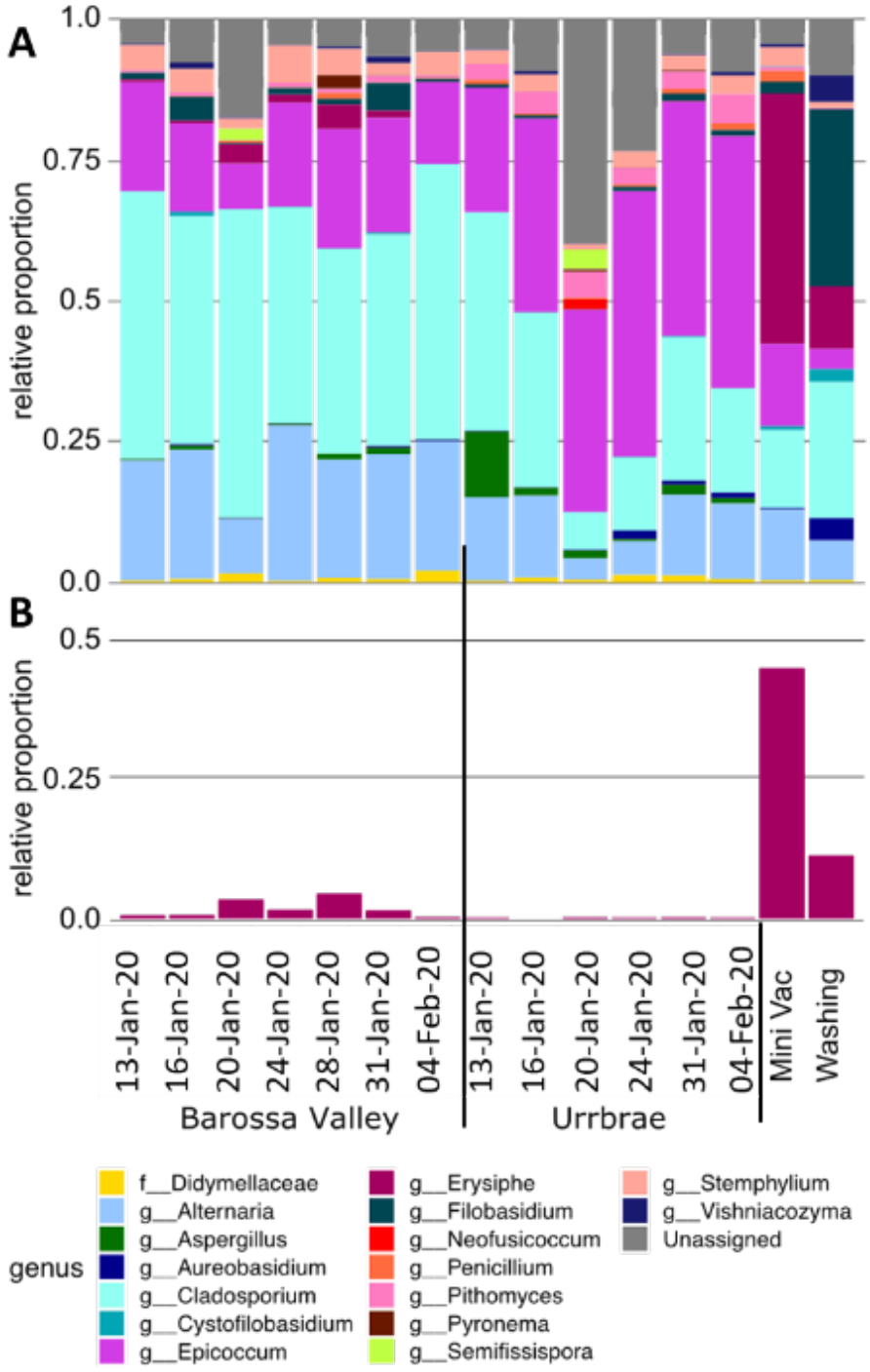


Figure 7.10. Fungal profiling of spore trap rods located in Barossa Valley and Urrbrae, mini-vacuum and leaf washing in 2020. (A) Population results, species were consolidated by genus and are presented as relative proportions within the population (B) *E. necator* detection results.

In the 2020/21 season, the G143A (associated with QoI resistance) and Y136F (associated with DMI resistance) mutations were detected in 18 of 20 samples collected at the Barossa Valley site (Figure 7.11). The frequency of G143A and Y136F mutants ranged from 54-77% and 53-100%, respectively. At Urrbrae, 6 of 20 samples were detected with G143A and frequency ranged from 8-42%, but no Y136F was detected. In the Riverland, seven samples were detected with G143A with frequency ranging from 2-32%, however only two samples were detected with Y136F, both with 100% frequency. The results also revealed that mutations were detected when the spore trap ran for 1-4 days.

In the 2021/22 season, samples from spore traps showed that G143A was detected four times at Nuriootpa over the sampling period and the frequency ranged between 22 to 63%, while Y136F mutation were 100% frequency at three time points (Figure 7.12). At the Urrbrae vineyards, a low range of G143A frequency was detected (18-22%) at three time points and Y136F has been detected only once in mid-December 2021. Samples from Renmark contained both mutations but with low range for G143A and higher range for Y136F (Figure 7.12).

7.3.4. Other spore collection methods

Mutations were also readily detected, in the 2020/21 season, from samples collected using the other methods; mini-vacuum, leaf washing and cotton bud swabs in the Barossa vineyard (Figure 7.13). Similarly in 2021/22 season, all these collection methods, beside the samples from gloves, were enabled detection for G143A and Y136F regardless of the location or the type of the collection (Figure 7.14). However, samples from gloves and the mini-vacuum from Nuriootpa did not yield enough fungal material to enable the detection of mutants (Figure 7.14).

The mutation H242R/Y, associated with resistance to the SDHIs, was not detected in any sample in all seasons.

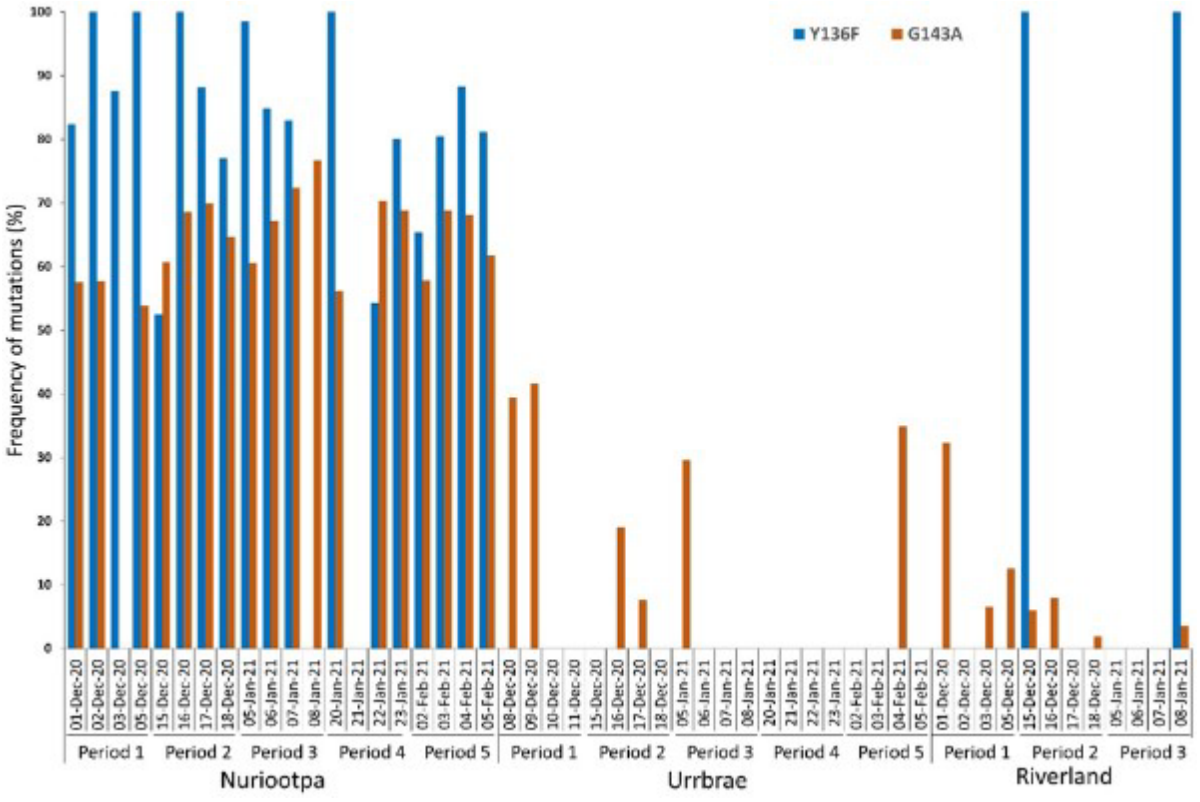


Figure 7.11. Detection of Y136F and G143A by next generation sequencing in *Erysiphe necator* samples collected over four-day sampling periods from Nuriootpa, Renmark and Urrbrae vineyards using the spinning rod spore traps in the 2020/21 season.

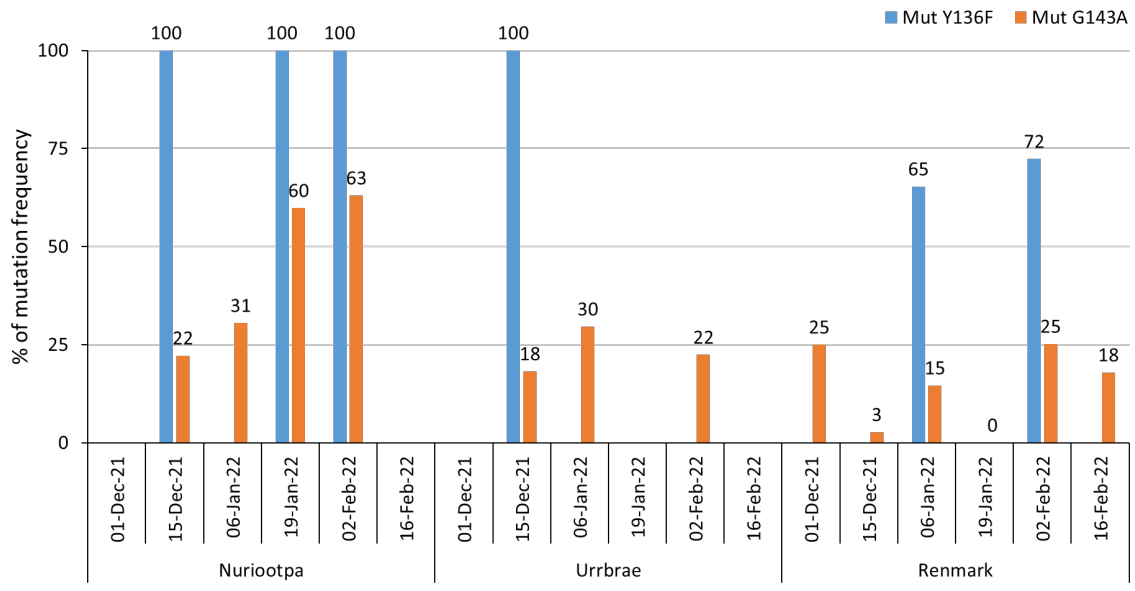


Figure 7.12. Detection of Y136F and G143A by next generation sequencing in *Erysiphe necator* samples collected over two-day sampling periods from Nuriootpa, Renmark and Urrbrae vineyards using the spinning rod spore traps in the 2021/22 season.

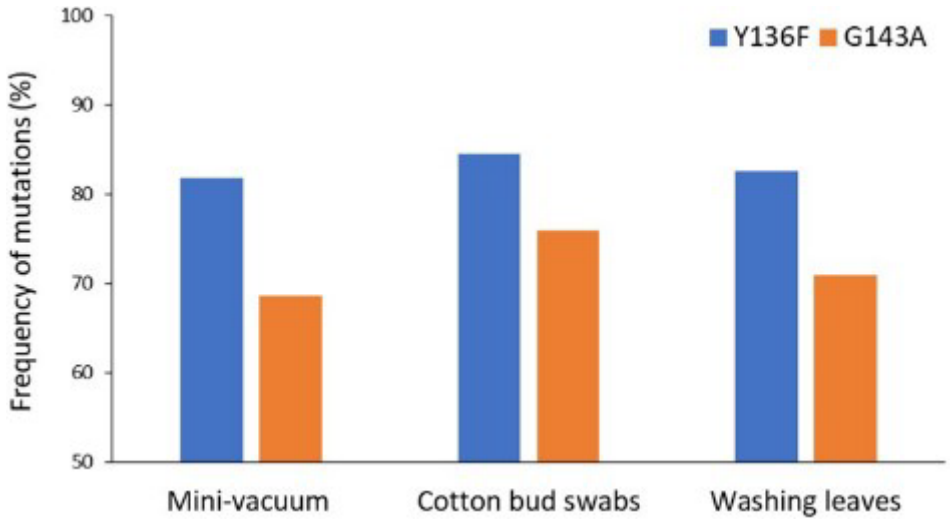


Figure 7.13. Detection of Y136F and G143A by Next Generation Sequencing in *Erysiphe necator* samples collected from the Barossa Valley vineyard using the mini-vacuum, cotton bud swabs and washing leaves in the 2020/21 season.

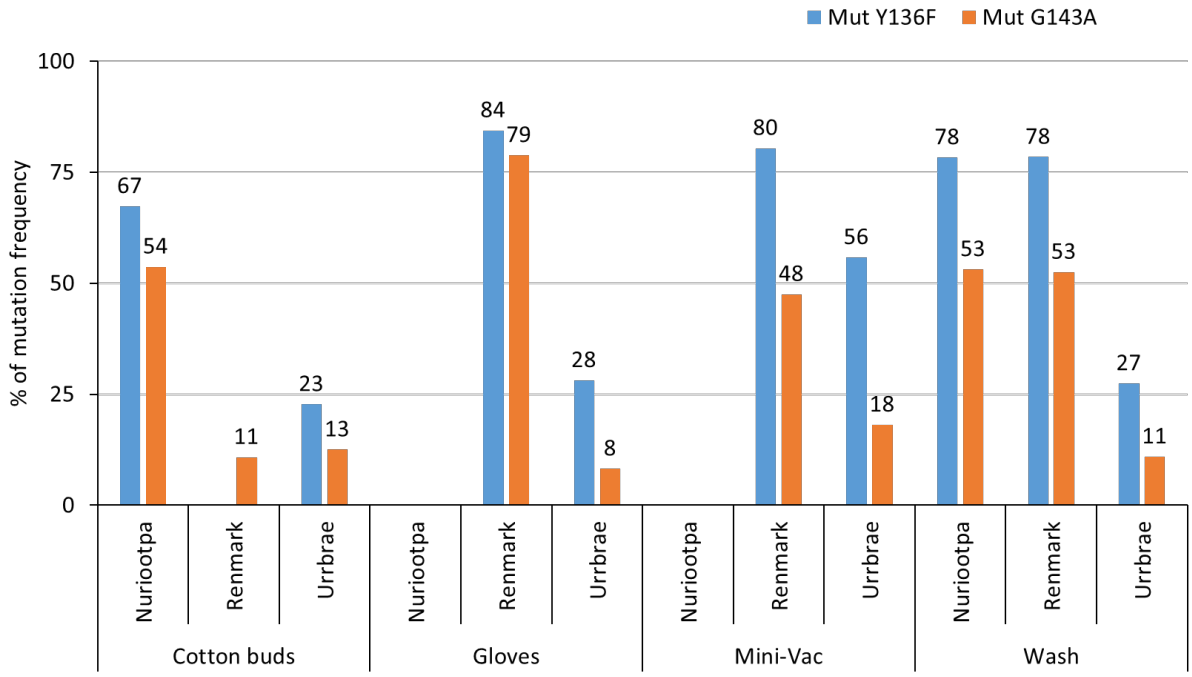


Figure 7.14. Detection of Y136F and G143A by Next Generation Sequencing in *Erysiphe necator* samples collected from Nuriootpa, Renmark and Urrbrae vineyards using the cotton bud swabs, gloves, mini-vacuum, and washing leaves in the 2021/22 season.

7.3.5. A multi-well agar plate spore trapping pipeline to detect L412F in *B. cinerea*

The pyrimethanil DC for testing within the field was established as 4 µg/mL. This concentration was chosen because it prevented the WT isolate Bc-7 from growing after incubation for seven days *in vitro* (data not shown). The YSSA-YA media desiccated significantly (approximately 20 – 50%) when placed in the spore sampler (Figure 7.15) for 24h, but subsequent growth and sporulation of *Botrytis* was still possible. The six field plates are shown in Figure 7.16. *Botrytis* was observed in plates 1, 4, 5 and 6, and all wells with visible *Botrytis* were tested for the presence of the L412F genotype. The WT genotype was present across all wells that were tested, with L412F only observed in one well (plate 1) in combination with the WT. The DC

prevented the growth of the WT biomass in all plates except for two wells in plate 1.



Figure 7.15. Passive spore trap used to test multi-well agar plates in the Curtin University experimental vineyard

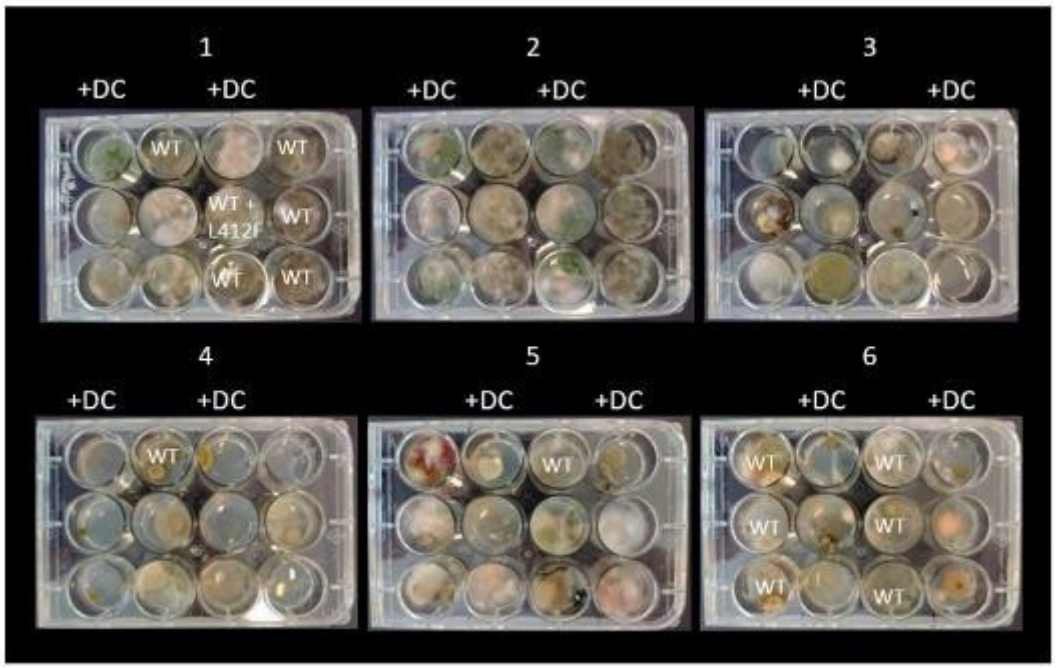


Figure 7.16. Multi-well plates after 24h exposure in the spore trap and induction of expression from near-UV exposure. numbers indicate each replicate plate. +DC indicates columns with agar amended with pyrimethanil DC (4 µg/mL). WT or L412F indicates the presence of that genotype using qPCR analysis.

7.4. Discussion

Tapes from spore traps showed that *B. cinerea* can survive and be re-isolated after two months storage at room temperature, and samples from a volumetric Burkard Hirst-type sampler were suitable for DNA extraction and ITS identification. Several previous studies used this type of spore trap to monitor *B. cinerea* and other pathogens in the field (Calderon et al. 2002, Gonzalez-Fernandez et al. 2021). Falacy et al. (2007) reported the collection of 684 and 1,228 conidia (0.05 and 0.09 conidia/L, respectively) using a Burkard sampler. However, the spore trap is large and heavy, making it difficult to place close to the canopy without adversely affecting normal vineyard operations. They are currently being used for the detection and quantification of spores from grapevine trunk disease (GTD) pathogens (SAR 1701-1.1; (Billones-Baaijens et al.2017), but traps are placed up to 20 m from vines and still readily detect pathogen spores. In the current study, *E. necator* was undetectable from the samples using NGS, most likely due to the location of the spore traps being below and far from the canopy. In addition, samples from the mobile jet sampler detected *B. cinerea* but not *E. necator*, so it may have potential for future regional monitoring of *B. cinerea*. Therefore, based on the results, it was decided that the existing spore trap tapes from the trunk diseases project were not suitable for purpose of fungicide resistance detection as they did not detect sufficient spores to enable testing for resistant alleles of *E. necator*. However, further trials need to be conducted to confirm the suitability of these traps.

Rotorod spore traps, cotton bud swabs, gloves, mini-vacuum and leaf washing all proved to be valid and practical methods for collecting spores of powdery mildew in the vineyard for subsequent molecular detection of the pathogen. The results showed that as few as 10 spores of *E. necator* on spore trap rods was sufficient to detect the fungus and the mutants that are associated with fungicide resistance. Falacy et al. (2007) found that as few as 10 conidia were occasionally detected on glass rods coated with silicon grease, however consistent PCR amplification required between 100 and 500 conidia. Thiessen et al. (2016) demonstrated that growers were able to effectively manage grape powdery mildew using spore trapping and molecular detection to initiate fungicide applications. In the USA, a spore trapping study conducted by Miles et al. (2021) reported that G143A had been detected in 58 of 132 samples from California and Oregon. In this study, two mutations (Y136F and G143A) were detected, indicating that multiple mutations can be detected from the same sample. As G143A is strongly associated with QoI resistance (Rallos et al. 2014) and Y136F is linked to DMI resistance (Kunova et al. 2021) the use of molecular methods makes detection of resistance faster, more targeted and accurate and can complement or replace the conventional bioassay phenotyping. Molecular detection for fungicide resistance has been adopted widely in the USA and a rapid DNA test for QoI resistance has been developed by the Fungicide Resistance Assessment, Mitigation and Extension Network (FRAME) and is being applied and reported across the major grape growing regions of California, Michigan, Washington, Oregon and Ohio (Oliver et al. 2021); https://framenetworks.wsu.edu/). In the current study, ITS profiling detected *E. necator* at low relative quantities from within a broad fungal population in samples collected in January-February 2020, indicating that the rotorod spore trap can capture spores from different fungal genera and consequently might help to manage other diseases and support resistance management.

The cheapest and most portable of the commercial spore traps is the rotorod impaction style traps Various brands of these are available commercially, however the cost can be prohibitive especially when multiple units are required. However, several research groups have developed low cost yet effective traps to detect and quantify spores suitable in in various pathosystems (Quesada et al, 2018). Dr. Walt Mahaffee (Oregon State University/USDA), is currently evaluating a network of low cost, effective rotorod traps across viticultural regions of Oregon, Washington and California in the USA to detect and quantify fungicide resistance in

E. necator (Thiessen et al. 2016). Dr Mahafee provided several spore traps for the current study, which were then replicated using material readily available at hardware stores and were subsequently used for experiments.

In the 2020/21 and 2021/22 seasons, G143A was detected using rotorod traps located in the Barossa Valley, Urrbrae and the Riverland and Y136F in the Barossa Valley and Riverland in 2020/21 but all locations in 2021/22. The high frequencies of G143A in Barossa Valley confirm the presence of genetic resistance. The low frequencies of G143A and Y136F detected in the Urrbrae and Riverland vineyards indicate that there may be potential for development of fungicide resistance in the future. The mutation H242R/Y, associated with resistance to the SDHIs, was not detected in any sample. The results also revealed that running the spore trap for one day was sufficient to detect mutations.

G143A and Y136F mutants were detected in samples collected from leaves with powdery mildew symptoms using the mini-vacuum, cotton bud swabs and direct leaf washing. Mini-vacuum devices can be modified from commercially available systems. The device could be used to collect individual samples of *E. necator* and may also be a useful tool to obtain samples for molecular sensitivity testing. The spore rods, mini-vacuum device and cotton swabs can be easily transported to a diagnostic laboratory at ambient temperature, whereas the spore suspension from leaves requires refrigeration to prevent DNA degradation. In addition, swabs from gloves were useful in detecting the mutants in the current study. Gloves have been tested for collecting powdery mildew to detect the G143A mutant in Oregon state and they found both glove swabs and spore traps detected the presence of mildew and the resistant mutation at similar rates (https://thegrapevinemagazine.net/2020/05/managing-powdery-mildew-in-the-vineyard/ .

Detection of mutants could be achieved using rotorod spore traps even when there were no visible powdery mildew symptoms, particularly early in the season. This could provide an early warning so that preventative management decisions can be made. However, visible powdery mildew infection was required for the other three collection methods; mini-vacuum, gloves, cotton bud swabbing and leaf washing. Therefore, if fungicide resistance is detected with rotorod spore traps, once symptoms appear, more comprehensive sampling can be undertaken using the other methods. Detection of fungicide resistance at low levels, before it can be detected in bioassays and/or manifests as field failure, may enable appropriate anti-resistance strategies to be applied in a timely manner, prolonging the effective life of fungicides. Thiessen et al. (2016) demonstrated that growers were able to effectively manage grape powdery mildew using molecular inoculum detection methods (e.g. G-LAMP assay) to initiate fungicide applications.

The rotorod spore trap system has many advantages. It is cost effective and easy to manufacture, portable, practical and casues minimal interference with vineyard maintenance. It is easy to operate by vineyard staff and does not require movement of plant material. Rotorod spore traps can detect low levels of *E. necator* and resistance mutations before symptoms appear and can facilitate the detection of other pathogens simultaneously. Rotorod spore trap sampling and molecular detection of mutants are powerful tools in powdery mildew management regimes (Falacy et al. 2007), hence it should be further developed in future research and include *P. viticola* and *B. cinerea*.

The multi-well agar plate spore trapping system allowed for separate spores to be captured and germinated, with subsequent *Botrytis* growth sporulated and analysed for the presence of the L412F genotype. The DC selected against the WT in all plates except for two wells in plate 1. The presence of WT biomass in these two wells containing the DC may indicate i) degradation of the fungicide in those wells, ii) the DC may be missing in these wells due to experimental error, iii) that the near-UV exposure was long enough for WT biomass to

eventually grow. It was not determined whether the *Botrytis* growth in wells that contain the WT only, are the result of single or multiple spore inoculum events. The theoretical detection limit for this system is extremely low, at one viable spore per well. However, one viable spore would still have to germinate and compete alongside other microbial species captured in the well. Across all plates, only one well (+DC) contained L412F biomass, and this well also included WT biomass. With only one well showing L412F biomass, an estimation of resistance frequency is difficult. WT biomass was frequently found across non-DC wells, indicating that WT spores were more prevalent than L412F spores during the sampling period. Additionally, this result could indicate that the L412F biomass was outcompeted by WT biomass in non-DC wells. Even though the agar volume was reduced by desiccation during exposure in the spore trap, *Botrytis* spores could still be captured and expressed. However, agar desiccation may reduce subsequent *Botrytis* growth, so this system would suit cooler and moister environmental conditions. Future work could include testing new types of media to reduce the effects of desiccation and the growth of other microbial species. A 24-well plate could also be tested, which would allow further spore separation. This pipeline was tested late in the season under high disease levels. Alternatively, trialing this system early in the season e.g. before flowering, would investigate its ability to capture *Botrytis* under low spore levels and before chemistry is applied. The early season estimation of resistance frequencies could give the grower valuable information for their resistance management strategies in-season and before botryticides are applied.

8. Efficacy of DMI fungicides when exposed to resistant populations

8.1. Introduction

To determine whether the efficacy of demethylation inhibitor (DMI) fungicides varied between resistant populations of the powdery mildew pathogen *Erysiphe necator*, sensitive (wild type, WT) isolates and those containing the Y136F mutation, associated with decreased sensitivity, were evaluated.

The use of a heterologous yeast expression system (HYES) provides a simple, reliable and high-throughput phenotypic methodology for the analysis of the relationship between mutations in *Cyp51* and demethylation inhibitor (DMI) sensitivity in organisms for which a transformation system is not available (Groeneveld et al. 2002, Revankar et al. 2004). The HYES can also provide insights into the presence of negative cross-resistance. Negative cross-resistance is whereby mutations can produce reduced sensitivity to one fungicide, but the same mutation increases sensitivity to another fungicide within the same chemical group. This phenomenon has been described in the wheat pathogen *Zymoseptoria tritici* (Fraaije et al. 2007, Leroux et al., 2007) and barley pathogen *Blumeria graminis* f.sp. *hordei* (Tucker et al. 2020) in relation to DMI sensitivities. Negative cross-resistance could be harnessed to suppress particular genotypes linked with reduced sensitivity to fungicides.

Previous research in SAR 1204 utilised the HYES to test *E. necator* WT *Cyp51* against five *E. necator* registered fungicides; myclobutanil, penconazole, tebuconazole, tetraconazole and triadimenol. The Y136F mutation in *Cyp51* has been previously linked with reduced sensitivity to DMIs in *E. necator* (Délye et al. 1997, Dufour et al. 2011, Frenkel et al. 2014, Miazzi and Hajjeh, 2011). The Y136F mutation has also been previously identified in Australian *E. necator* isolates (Hall et al. 2017). In this report, the *E. necator Cyp51* gene containing the Y136F mutation was synthesised and tested in the HYES against the five DMIs described above, and the additional DMIs; difenoconazole and fluquinconazole. Although fluquinconazole is not registered for use in grapes, it is of interest as negative cross-resistance was characterised for this DMI in the barley pathogen *Blumeria graminis* f.sp. *hordei* (Tucker et al. 2020). The aim of this work was to provide insights into the effect of the Y136F mutation on DMI sensitivity, and also identify any potential negative cross-resistance within the DMI fungicide group.

The next step is to transform the yeast cells with the mutation and carry out phenotype sensitivity assays on the yeast to determine which fungicides are theoretically more effective in controlling powdery mildew containing the Y136F mutation. As previously discussed, other yet to be well defined molecular mechanisms are also thought to be involved in decreased sensitivity of *E. necator* to the DMIs (Kunova et al. 2021). Once these mechanisms, and mutations, are known, yeast cells could be transformed with them to test their effects in DMI efficacy on *E. necator*. The HYES is a simple, reliable and high-throughput *in vitro* method, but results need to be confirmed *in planta* by infecting grapevine plant material with isolates of *E. necator*, with and without Y136F, and subjecting them to DMI fungicides.

In this study, the HYES was used to phenotype WT and mutant isolates of *E. necator* to a range of DMIs registered in Australia. Subsequently, a series of bioassays using both detached leaves and potted plants, was carried out to determine the efficacy of various DMIs exposed to WT and mutant isolates of *E. necator*, and compared with results of the HYES.

The aim of this study was to determine whether DMI fungicides vary in efficacy when exposed to ‘resistant’ populations (i.e. contain high levels of the Y136F mutation). This information will inform growers about which DMI is best to use if the Y136F mutation is present.

8.2. Methods

8.2.1. Heterologous yeast expression system (HYES)

Synthesis of the WT *Cyp51* gene (UNU72658) from *E. necator* was carried out by Bioneer pacific (Vic, Australia). *Hind* III and *Eco* RI restriction sites were added to the 5' and 3' ends prior to synthesis for cloning purposes. Using these restriction sites, the synthesised gene was ligated into the pYES3/CT (Invitrogen, Carlsbad, CA, USA) and chemically transformed into *E. coli* (DHα). The resulting construct, pYES3CT-Encyp51WT was validated via Sanger sequencing. The Y136F mutation was introduced into the pYES3CT-Encyp51WT vector (Hall et al. 2017) through a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The pYES3CT-Encyp51Y136 construct was then transformed into *E. coli* (DH5α) and validated via Sanger sequencing. The validated construct was transformed into the *S. cerevisiae* strain YUG::erg11 with its native *Cyp51* gene under the control of a tetO-CYC1 promoter, repressed in the presence of doxycycline (Parker et al. 2008). Complementation and fungicide sensitivity assays were completed as described by (Cools and Fraaije, 2008). The pYES3CT-Encyp51YWT and pYES3CT-Encyp51Y136 yeast transformants were tested against six grape registered DMI fungicides; difenoconazole, myclobutanil, penconazole, triadimenol, tebuconazole and tetraconazole. The pYES3CT-Encyp51WT yeast transformant was also tested against non-grape registered fluquinconazole. All statistical analyses were conducted in SPSS software (IBM). To compare mean EC_{50} values between the WT and Y136F transformants for each fungicide, values were analysed using a Student's t-test ($P < 0.05$).

8.2.2. Bioassays

Bioassays were conducted to compare the efficacy of DMIs when exposed to sensitive (S; wild type) and resistant (R; mutant Y136F) isolates of *E. necator* and to validate results of the HYES. A series of experiments were carried out using mixtures of S and R isolates on detached leaves and potted plants, with different combinations of fungicide rates and inoculation times (Table 8.1).

Table 8.1. Summary of experiments conducted using either detached leaves or potted plants to evaluate the efficacy of DMIs using a mixture of either sensitive or resistant isolates of *E. necator* with different times and concentrations.

Fungicides	Isolates	Bioassay	Experiment & Figure numbers	Fungicide rate (proportion of label rate)*	Fungicide application time
mefentrifluconazole difenoconazole myclobutanil penconazole tetraconazole triadimenol tebuconazole	Sensitive mixture (156, 159, 199 and 201) Resistant mixture (150, 154, 191 and 210) (Table 8.2)	Detached leaf	Expt. 1, Figure 8.2	1:10	1 day post-inoculation
					6 days post-inoculation
			Expt. 2, Figure 8.4	1:10	2 days post-inoculation
				1:100	
			Expt. 3, Figure 8.5	1:10	1 day pre-inoculation
				1:100	
		Potted plant	Expt. 4, Figure 8.6	1:10	1 day post-inoculation
					6 days post-inoculation
					1 day pre-inoculation
	Individual isolates, 215, 216 and 222 (Table 8.3)	Potted plant	Expt. 5, Figure 8.8	1:10	1 day pre-inoculation

*under label rate dilutions were used to evaluate the sensitivity of different genetic profiles of *E. necator* to lower concentration of fungicides.

8.2.2.1. Preparation of detached leaves and potted plants

Detached leaves for each experiment were prepared as previously described (5.1.1.3). They were all freshly prepared on the day of each experiment.

Rooted cuttings were planted in 20 cm pots as previously described (5.1.1.2) and grown in a greenhouse for up to 12 weeks. Grapevines kept free from unwanted infection of powdery mildew (5.1.1.2). To prevent cross contamination, each potted plant was placed inside a clear plastic cylinder, covered with two layers of fine curtain material (Figure 8.1). Two sentinel plants were also grown in cylinders to monitor for powdery mildew. Potted plants inside clear plastic cylinders, covered with fine curtain material to prevent cross contamination.



Figure 8.1.Potted plants inside clear plastic cylinders, covered with fine curtain material to prevent cross contamination.

8.2.2.2. *Erysiphe necator isolates*

For experiments 1 to 4, plant material was inoculated with either a mix of S isolates (156, 159, 199 and 201) or a mix of R isolates (150, 154, 191 and 210;Table **8.2**). Isolates were chosen based on fungicide exposure history and available genotype data from Sanger and Next Generation Sequencing (NGS). By the completion of experiments, genotype data were available for all isolates (**Table 8.2**). Although, Sanger genotype data found isolates 156, 159, 199 and 201 were Y136 (wild type), NGS revealed that all of these contained between 35 and 51% of the Y136F allele, therefore no true wild type isolates were used in experiments 1 to 4.

Table 8.2. Isolates used to determine the efficacy of DMIs on detached leaves and potted plant (experiments 1-4). Genotype status of Y136/Y136F alleles using Sanger and frequency percentage of Y136F using Next Generation Sequencing (NGS) and the history of exposure to DMI fungicides.

Sensitive/ resistant	Isolate	Genotype status		History of exposure
		Sanger	NGS (% Y136F)	
Sensitive mix (S)	156	Y136	41	unlikely
	159	Y136	44	unlikely
	199	Y136	35	nil
	201	Y136	51	nil
Resistant mix (R)	150	Y136F	100	yes
	154	Y136F	85	yes
	191	Y136F	100	yes
	210	Y136F	100	yes

Following a review of the results of experiments 1-4 and availability of new isolates of *E. nector,* an additional potted plant experiment was established (experiment 5). Three individual isolates were chosen, based on NGS genotype data, to represent three frequency levels of the Y136 allele (Table 8.3).

Table 8.3. Isolates used in potted plant experiment 5, using 1:10 dilution of seven different DMI fungicides, applied one day before inoculation; Y136F allele frequency, *Cyp51* copy number and history of exposure to fungicides.

Isolate	Y136F %	*Cyp51* copy number	Exposure
215	100	3.8	likely
216	38	5.29	yes
222	4	2.2	nil

8.2.2.3. *Cyp51* copy number

Ideally, isolates with the same Y136F frequency and high or low copy numbers would be used, however, none were available. Therefore, isolates were initially chosen based on frequency of Y136F allele, with copy number information provided once available (Table 8.3). CdPCR was used to determine *Cyp51* copy number in isolates 215, 216 and 222 (Table 8.3). Primers and probes were designed from *Cyp51* and *Actin* sequences; AF042067.1 and KM217171.1, respectively. *Actin* was used to normalise *Cyp51* copy number. DNA was extracted from leaf material (12 cm^2) infected with each isolate using a CTAB method as described in 6.2.2.3. The primers and probes were ordered from Macrogen Inc. and Sigma-Aldrich, respectively (Table 8.4). CdPCR was undertaken as described in 6.2.2.3 with an annealing temperature of 54°C. DNA was diluted accordingly to ensure 200 – 2000 copies/µL. *Actin* and *Cyp51* primer/probe reactions were carried out on separate chips. To calculate *Cyp51* copy number, mean *Cyp51* cdPCR copies/µL was divided by mean *Actin* cdPCR copies/µL for each isolate.

Table 8.4. Primer and probe details for determining *Cyp51* copy number in isolates 215, 216 and 222.

Gene	Primer name	Primer sequence (5′-3′)	Probe name	Probe sequence (5′-3′)
Cyp51	En-cyp-cdPCR-F3	ACTGGGCACCTCTACCT	En-cyp-560	CalFluor560/TCGTGATCATGCCCAAAGAACTGTT/BHQ1
	En-cyp-cdPCR-R3	AGTGGAACGCATCAACTC		
Actin	En-act-cdPCR-F	AGAGCGTGGCTATACATTC	En-act-FAM	6FAM/CCACCGCTGAGCGAGAA/BHQ1
	En-act-cdPCR-F	CACGTAACACAACTTCTC		

8.2.2.4. Inoculum preparation

Selected isolates of *E. necator* were grown and bulked up on detached leaves for up to four weeks prior to each experiment. Inoculum for experiments 1 to 4 was prepared as follows: spores from each isolate were harvested separately into sterile Milli-Q (SMQ) water using a sterile paintbrush. Spore suspensions from each isolate were diluted with SMQ water to ~1 x 10^5 spores/mL with the aid of a haemocytometer. The spore suspension from each isolate was then mixed to make 10 mL of either an S or R mix. Three aliquots of 500 µL were removed from each individual isolate suspension and from the S and R suspension mixes They were processed by AWRI using NGS to determine the starting frequency percentage of Y136F (5.1.1.7). NGS was carried out several months prior to commencement of experiments, to determine whether any changes due to cross-contamination had occurred. Inoculum for experiment 5 was prepared in a similar manner but spores were collected using the mini-vacuum cyclone separator (7.2.4), and individual spore suspensions of each isolate were made. Sub-samples of each suspension were processed by AWRI for NGS to determine Y136F %.

Inoculation of potted plants was carried out as follows: old senescing leaves and small, unexpanded leaves were removed from each plant. The remaining leaves, aiming at between 8 and 12 leaves, were marked with a black marker pen at the base of the leaf near the petiole (Figure 8.2) in order to identify the inoculated and fungicide treated leaves at the completion of the experiment. Marked leaves were then sprayed using a small hand-held sprayer to just before run off with either the S or R spore suspension. Leaves were allowed to air-dry and plants were returned to their cylinders.

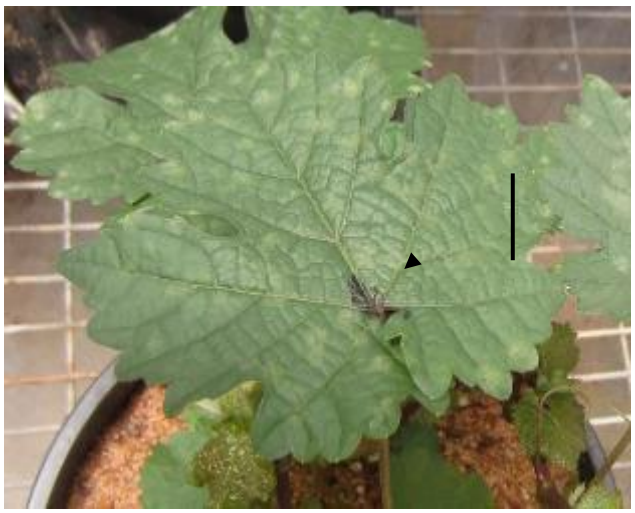


Figure 8.2. Grapevine leaf treated with the fungicide and inoculated with E. necator. Black arrow shows where the leaf was marked for easy identification

8.2.2.5. Fungicide preparation

Suspensions of seven fungicides were made in SMQ water at the recommended label rate (Table 8.5). They were then diluted to 1:10 (Rallos and Baudoin, 2016) and/or 1:100 depending on the experiment (Table 8.1). As the label rate might kill *E. necator* and prevent the infection, under label rate dilutions were used to evaluate the sensitivity of different genetic profiles of *E. necator* to lower concentration of fungicides. A 100 mL aliquot of each was decanted into a small hand-held spray bottle and was sprayed until just before run-off to each detached leaf or each marked leaf on the potted plants. SMQ water was used as the control.

Table 8.5. DMI fungicides used in experiments including label rate recommended for powdery mildew control.

Active ingredient	Trade name	Company	Label rate mL/100L
difenoconazole	Digger® (250 g/L a.i.)	Nufarm	25
mefentrifluconazole	Belanty® (75 g/L a. i.)	BASF	80
myclobutanil	Mycloss® Xtra (200 g/L a.i.)	Corteva™ agriscience	16
penconazole	Topas® (100 g/L a.i.)	Syngenta	12.5
tebuconazole	Orius® (430 g/L a. i.)	Adama	30
tetraconazole	Domark® (40 g/L a.i.)	Sipcam Pacific Australia	30
triademenol	Bayfidan® (250 g/L a.i.)	Bayer	10

8.2.2.6. Experimental design and analysis

Each detached leaf experiment was designed as a randomised block with up to four replicate plates for each combination of isolate mix and fungicide application. The potted plant experiments were a randomised block design, with two replicate pots per treatment.

Two weeks after inoculation, disease severity was rated as the percentage of the leaf area infected (LAI) with sporulating powdery mildew (Table 8.2) and the mean LAI +/- sd was calculated. A two-way analysis of variance (ANOVA) was used where possible, to determine if there were significant differences (p<0.05) among the fungicides. If there was a significant difference, a Tukey's test at 5% level was used to discriminate the differences. Mean and standard deviations were calculated using MS Excel and ANOVA using Genstat. Controls were included to confirm that inoculum was viable and disease could be established, so that any effects observed were due to the fungicides and not due to non-viable inoculum or poor infection. The focus of our investigations was to compare the efficacy of fungicides with one another and not with the controls, therefore controls were excluded from analyses, but shown in the graphs for comparison.

8.3. Results

8.3.1. Heterologous yeast expression system (HYES)

The mean EC_{50} values (µg/mL) of the pYES3CT-Encyp51WT and pYES3CT-Encyp51Y136F yeast transformants against difenoconazole, fluquinconazole, myclobutanil, penconazole, triadimenol, tebuconazole, and tetraconazole are shown in Figure 8.3. Resistance factors are indicated above each WT and Y136F EC_{50} combination for each fungicide and are calculated by dividing the Y136F EC_{50} value by the WT EC_{50} value. No shift in sensitivity was observed for difenoconazole (RF = 1) and fluquinconazole (RF = 1.1). A moderate significant shift in sensitivity was observed for myclobutanil (RF = 4.2), and a small significant shift in sensitivity was observed for tebuconazole (RF = 1.7). Small non-significant shifts in sensitivity were observed for penconazole (RF = 2.3), and tetraconazole (RF = 1.8). A large significant sensitivity shift was observed for triadimenol (RF = 7.5).

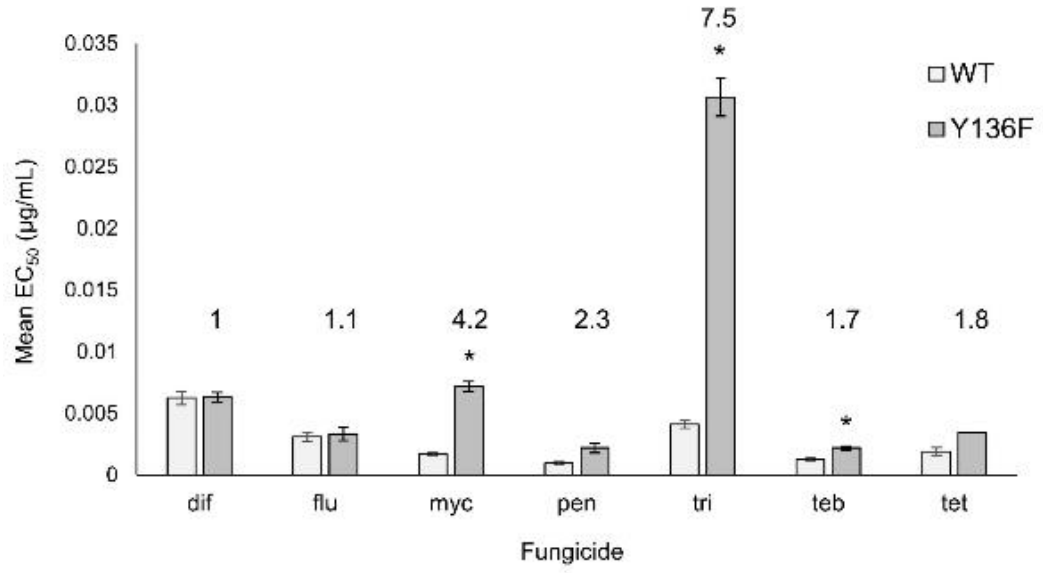


Figure 8.3. Mean EC50 values (µg/mL) for Encyp51WT (light grey columns) and Encyp51Y136F (dark grey columns) yeast transformants for seven DMI fungicides. dif = difenoconazole; flu = fluquinconazole; myc = myclobutanil; pen = penconazole; tri = triadimenol; teb = tebuconazole; tet = tetraconazole. Resistance factors are indicated above each WT and Y136F EC50 combination for each fungicide, and are calculated by dividing the Y136F EC50 value by the WT EC50 value * indicates significant difference at P < 0.05 using Student's t-test. 'ns' indicates no significant difference at P < 0.05 using Student's t-test

8.3.2. Efficacy of DMI fungicides on detached leaves

8.3.2.1. Experiment 1: effect of fungicide application time

Fungicides were applied at 1:10 dilution of label rate, two different times (1 and 6 days) post-inoculation. Disease severity on controls was 100% (Figure 8.4). Delayed application of fungicides to six days after *E. necator* inoculation resulted in greater disease severity compared to application one day post-inoculation, regardless of the sensitivity/resistance status of pathogen populations. When fungicides were applied at six days post-inoculation, disease severity was greater for the resistant population (95-100%) compared to the sensitive population (22-60%). When fungicides were applied one day post-inoculation, no disease developed with either the S or R populations, except for triadimenol with 52% disease severity with the R population (Figure 8.4).

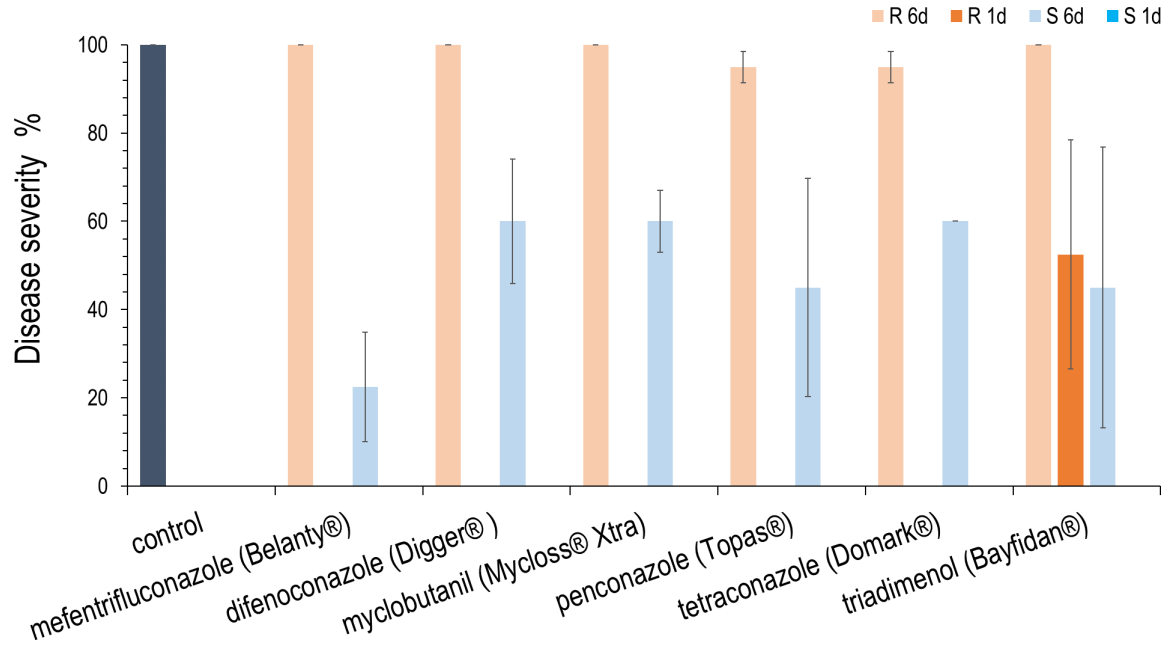


Figure 8.4. The effect of DMI fungicide application times, 1 day (1d) and 6 days (6d) after inoculation with mixtures of resistant (R) and sensitive (S) *Erysiphe necator* isolates on mean disease severity of powdery mildew on detached leaves. Fungicides were applied at 1:10 label rate. Bars represent standard error of the mean.

8.3.2.2. Experiment 2: effect of fungicide dilution when applied post-inoculation

Fungicides were applied at 1:10 and 1:100 dilutions of label rate onto detached leaves, two days post-inoculation with *E. necator*. In general disease severity was low, ranging between 0 and 50% (Figure 8.5). The 1:10 dilution was more effective at controlling powdery mildew than the 1:100 dilution on R populations compared with the 1:100 dilution, except with triadimenol and penconazole, which had similar disease severity for both dilutions. Even when diluted to 1:100, mefentrifluconazole and tebuconazole completely controlled powdery mildew for both R and S populations. Minimal disease developed with any fungicides on the S population, with only 2.5% severity for myclobutanil and triadimenol.

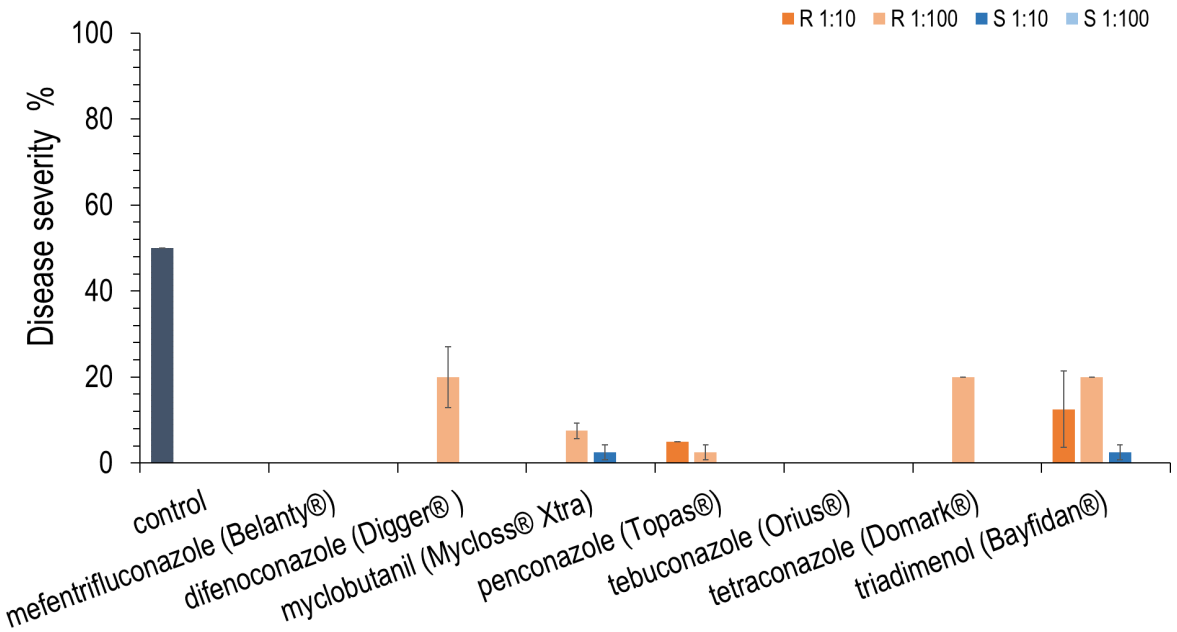


Figure 8.5. The effect of DMI fungicide concentration (1:10 and 1:100 dilutions of label rate) on powdery mildew disease severity on detached leaves, when applied two days post-inoculation with mixtures of resistant (R) and sensitive (S) isolates of *Erysiphe necator*. Bars represent standard error of the mean.

8.3.2.3. Experiment 3: effect of fungicide dilution when applied pre-inoculation
Fungicides were applied at 1:10 and 1:100 dilutions of label rate onto detached leaves, one day pre-inoculation with *E. necator*. Disease severity on controls was 100% (Figure 8.6). The 1:10 dilution was more effective at controlling disease than the 1:100 dilution for both R and S populations. Triadimenol was the least effective fungicide, and disease severity was 100% in both populations when diluted to 1:100. Overall mefentrifluconazole was the most effective fungicide on both R and S populations.

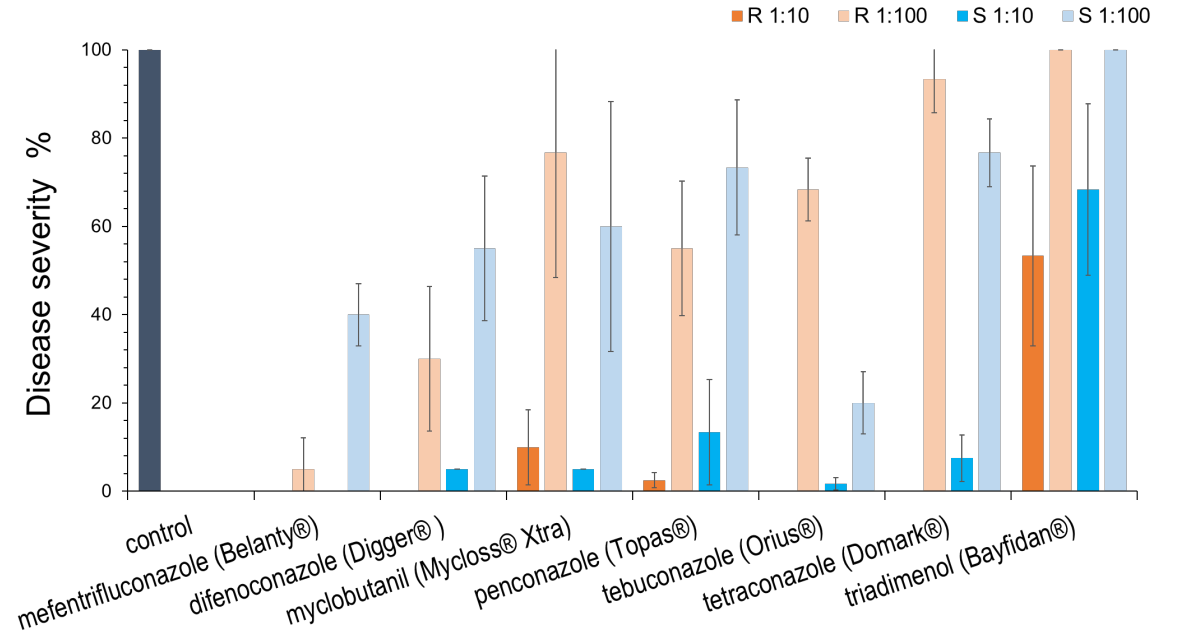


Figure 8.6. The effect of DMI fungicide concentrations (1:10 and 1:100 dilutions of label rate) on powdery mildew disease severity on detached leaves when applied one day pre-inoculation with mixtures of resistant (R) sensitive (S) isolates of *Erysiphe necator*. Bars represent standard error of the mean.

8.3.3. Efficacy of DMIs on potted plants

8.3.3.1. Experiment 4: effect of fungicide application time

Fungicides were applied, at 1:10 dilution of label rate, three different times: one day pre-inoculation, and then one and six days post-inoculation. Disease severity on controls was 89% (Figure 8.7). Results showed that the fungicides differ in efficacy, mefentrifluconazole was most effective with up to 19% disease severity, and difenconazole the next most effective with up to 44% disease severity. Triadimenol was least effective, especially on the R population when applied both pre- and post-inoculation. Also, none of the DMIs were effective at controlling the R population of *E. necator* when applied six days post-inocualtion.

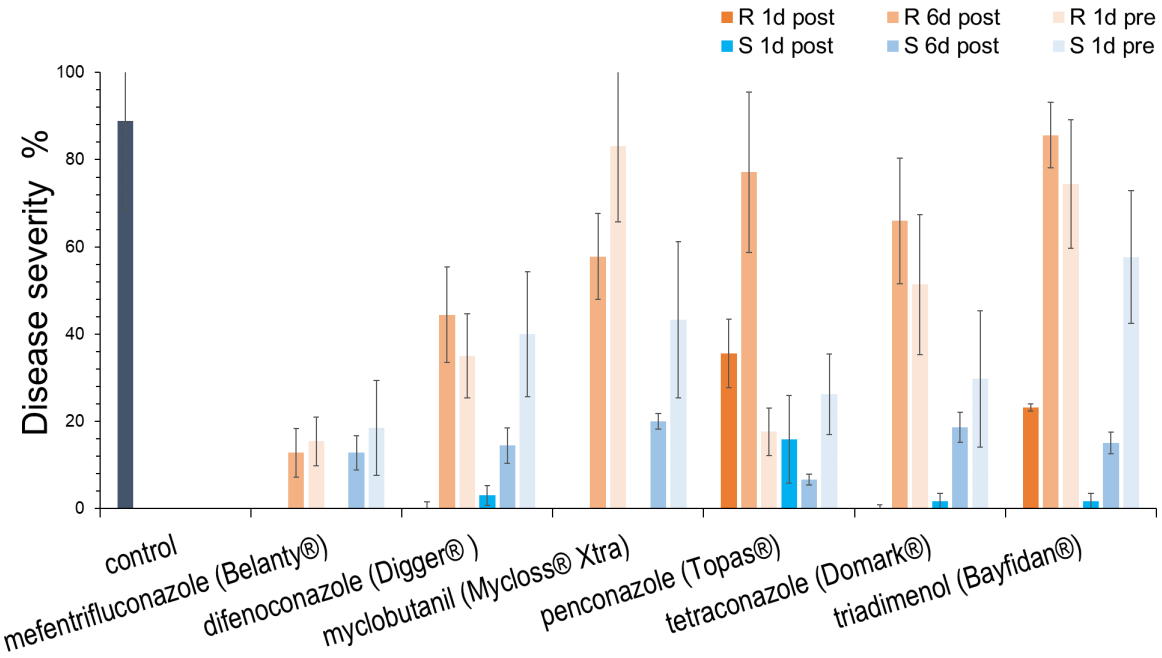


Figure 8.7. The effect of DMI fungicide application times, one day pre-inoculation (1d pre) or one and six days post-inoculation (1d post and 6d post, respectively) with resistant (R) and sensitive (S) isolate mixes of *Erysiphe necator*, on powdery mildew disease severity in potted plants. Fungicides were applied at 1:10 label rate. Bars represent standard error of the mean.

8.3.3.2. Experiment 5: effect of mutant Y136F allele and *Cyp51* copy number

Figure 8.8 shows the mean disease severity of powdery mildew for pooled data from the three isolates with different genetic profile. Mefentrifluconazole and difenconazole were generally significantly effective fungicides with less than 5% disease severity, while myclobutanil and triadimenol were the least effective fungicides with more than 30% disease severity.

Fungicides were applied, at 1:10 dilution of label rate, to three individual isolates that were chosen based on NGS genotype data, to represent three frequency levels of the Y136 allele, with associated *Cyp51* copy numbers also determined (Figure 8.3). Based on this, isolates 215 and 216 were considered resistant and isolate 222 was considered sensitive. Fungicides differed in their efficacy regardless to the genetic profile of the isolates. Mefentrifluconazole was the most efficacious while triadimenol and myclobutanil were the least efficacious (Figure 8.9). Disease severity of 2% or less was recorded on plants inoculated with each isolate and treated with mefentrifluconazole, and plants inoculated with isolate 216 and treated with tebuconazole and tetraconazole. The greatest disease severity (59%) was recorded on vines inoculated with isolate 215 (100% Y136F) and treated with triadimenol, and it was significantly different from isolate 216 and 222 indicating that resistance to this fungicide is associated with

the presence of the mutant Y136F allele. However, vines inoculated with isolate 216 (38% Y136F) and treated with myclobutanil, were recorded with relatively high disease severity (47%) and significantly different from 222 and 215. Isolate 216 had the highest copy number (5.29) of *Cyp51* (which contains the Y136F mutant). Furthermore, vines inoculated with isolate 216 developed some disease, but not significantly more than the other isolates when treated with difenoconazole (21%) and penconazole (18%), but the same isolate was effectively controlled by mefentrifluconazole, tebuconazole and tetraconazole.

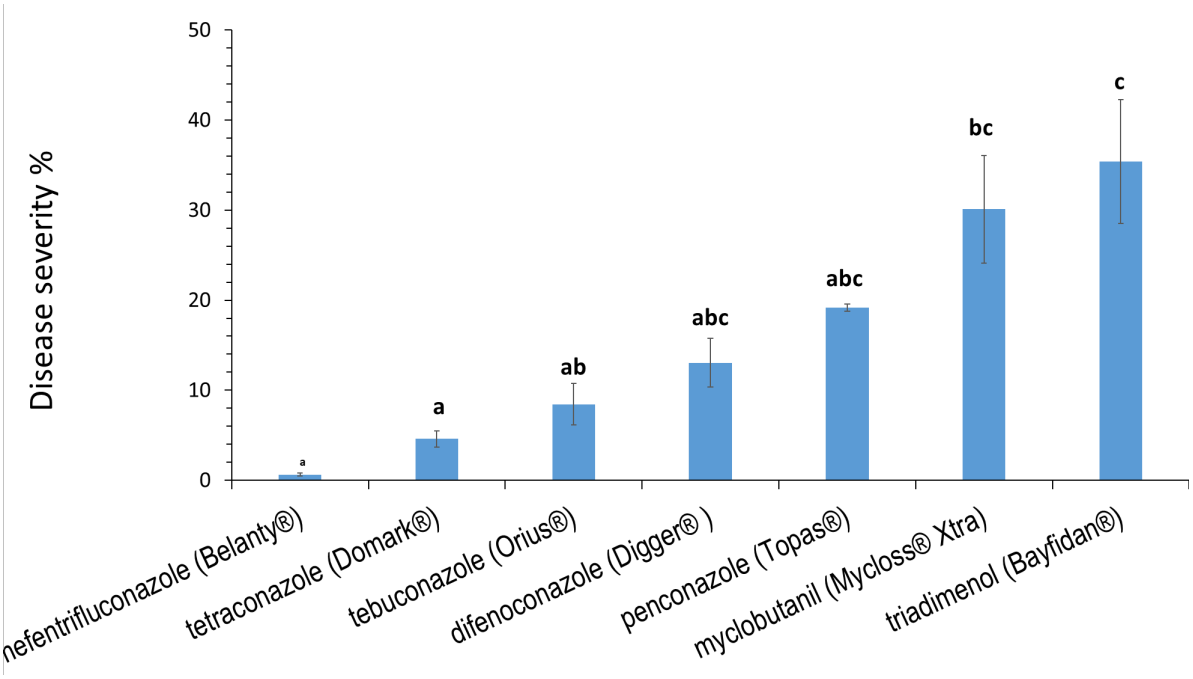


Figure 8.8. Mean disease severity of powdery mildew on potted plants treated with DMI fungicides. Data for three isolates with different genetic profile are pooled (Table 8.3). Bars represent standard error of the mean and LSD (P<0.05) was 7.85 between fungicides. Columns with no letters in common are significantly different from each other.

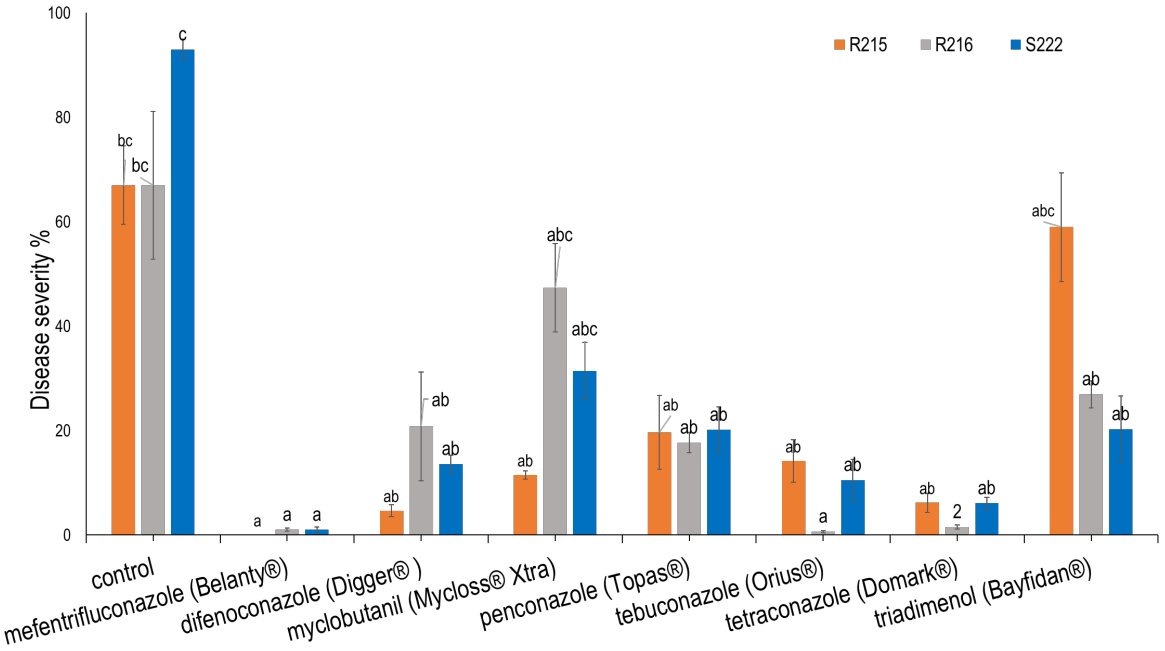


Figure 8.9. Efficacy of DMI fungicides on powdery mildew disease severity in potted plants using three isolates of *Erysiphe necator* with different genetic profiles of the mutant Y136 allele and associated *Cyp51* copy numbers (Table 8.3). Isolates 215 and 216 were considered resistant and isolate 222 was considered sensitive. Bars represent standard error of the mean and LSD was 7.85 and 13.59 between the interaction of fungicides and isolates respectively.

8.4. Discussion

In this report, the sensitivity of pYES3CT-Encyp51WT and pYES3CT-Encyp51Y136F yeast transformants against seven DMI fungicides was compared. Three DMI fungicides; penconazole, tebuconazole and tetraconazole, showed small shifts in sensitivity. Interestingly, myclobutanil and triadimenol exhibited a relatively moderate and high sensitivity shifts, respectively. In comparison to alternate studies using leaf disc bioassays as a phenotypic method, shifts in sensitivity to triadimenol and myclobutanil have been observed in Y136F *E. necator* isolates (Délye et al. 1997, Frenkel et al. 2014, Miazzi and Hajjeh, 2011). Délye et al. (1997) and Miazzi and Hajjeh (2011) identified the Y136F mutation in isolates with resistance factors of >5 and 2.6 – 10.7 when tested against triadimenol, respectively. The triadimenol resistance factors from these two studies are similar to those from this report (RF – 7.5). Frenkel et al. (2014) characterised a RF of at least 10 in Y136F isolates against myclobutanil, which is significantly greater than reported in this study (RF = 4.2). The pYES3CT-Encyp51Y136F yeast transformant showed no shift in sensitivity to fluquinconazole, whereby in a HYES study with the barley pathogen *Blumeria graminis f. sp. hordei*, Y136F (Bgh - Y137F) showed an RF of 9.7 (Tucker et al., 2020). Additionally, the RF for tebuconazole in this study (1.7) was greater than the tebuconazole RF (1.1) in Tucker et al. (2020). These differences in RFs between both studies may be due to the other CYP51 amino acid sequence changes between the two species. In this study, no negative cross-resistance was observed between the WT and Y136F haplotypes. This work has shown the feasibility of using the HYES to test mutations identified in the *E. necator Cyp51* sequence. Future work could involve testing any newly identified *Cyp51* mutations identified in Australian *E. necator* isolates or alternate mutations found in other mildew species.

The greater disease severity with the R population compared with that of the S population reflects the effect of the Y136F allele on efficacy of all fungicides, albeit on detached leaves, and at 1:10 label rate applied as a curative. Delye (1997) first associated Y136F with DMI resistance in grapevine powdery mildew to triadimenol. This mutation has been linked with azole resistance in *E. necator* from Virginia USA (Colcol et al. 2012), France (Dufour et al.

2011), Italy (Miazzi and Hajjeh, 2011) and eastern USA and Chile (Frenkel, 2014). In this study, we found an association between the presence of Y136F mutant and resistance to all the DMI fungicides tested. Recently, in addition to Y136F, Frenkel (2014) found another point mutation in *Cyp51*, A1119C, as well as an increased expression of *Cyp51* both linked to myclobutanil resistance in isolates of *E. necator* from eastern USA. Future research is required to develop assays to identify these mutants.

The results from these studies (Figure 8.5 and Figure 8.6) demonstrated that diluting the fungicides reduces the efficacy of DMIs significantly regardless of the genetic status of the *E. necator* population. However, the efficacy of DMIs at the lower 1:100 rate was improved when fungicides were applied after *E. necator* inoculation (section 8.3.2.2, Figure 8.5) compared to before inoculation (Figure 8.6). This was due to the lower disease pressure in experiment 2 compared to experiment 3. These results highlighted the importance of applying DMI fungicides at label rate, and not applied as a curative, especially when there is history of resistance in the vineyard.

In general, fungicide efficacy in potted plants was less compared to detached leaves, regardless of the genetic status of the *E. necator* population (resistant or sensitive), the application time (pre or post-inoculation) and DMI concentrations (1:10 or 1:100). Generally, powdery mildew on detached leaves is weaker than potted plants hence the ineffectiveness of DMI fungicides might be less. Applying fungicides at lower than the recommended label rate undermines the efficacy. These rates were for research purposes only and are not recommended for use in commercial vineyards.

Rallos and Baudoin (2016) demonstrated that gene duplication or increased gene copy number is possibly a requirement for at least one mutant copy of *Cyp51* for resistance. Results suggest an interaction between isolates and fungicides, most likely because there are multiple mutation alleles responsible for resistance, which may vary between fungicides. Recently, Frenkel (2014) found another point mutation A1119C, and together with an increased expression of *Cyp51*, was linked to myclobutanil resistance in isolates of *E. necator* from eastern USA. Future research is required to investigate this new mutant.

The label rate of DMIs fungicides might kill *E. necator* and prevent the infection, so under label rate dilutions were used to evaluate the sensitivity of different genetic profiles of *E. necator*. In general, applying DMIs below the label rate on the plants was not effective to control PM except for mefentrifluconazole. However, it showed that isolates with a high frequency of Y136F mutant and copy number were less sensitive to DMIs in general, with the exception of mefentrifluconazole. However, there is no clear relationship between copy number of *Cyp51*and DMI efficacy, and more research would be required to clarify this relationship.

Erysiphe necator is an obligate pathogen and sometimes does not interact with the host as anticipated, therefore inconsistent growth is sometimes observed, as was the case in experiment 2. Even though aseptic equipment was used in experiments, cross-contamination between populations can occur through airborne dispersion of pathogen spores. In addition, secondary infection may occur on leaves over the 14 day incubation period, where the life-cycle of the pathogen is only five days (Gadoury et al. 2012).

9. Effect of sulfur on the efficacy of DMI fungicides on powdery mildew

9.1. Introduction

Powdery mildew, caused by *Erysiphe necator,* is one of the most economically important diseases of grapevines in Australia. The demethylation inhibitors (DMIs) (FRAC code 3) and sulfur (FRAC code M02) are important fungicides for control of powdery mildew, and used in conjunction to manage fungicide resistance in grapevine (Emmett et al. 2003). There is growing concern about the possible antagonism between DMIs and sulfur when they are mixed in a spray tank. There are few reports that state sulfur might negatively impact the efficacy of DMIs. Steva (1994) reported that the uptake of the DMI Bayfidan® into grapevine foliage was reduced from 70 to 10% when it was tank-mixed with sulfur, decreasing the control of powdery mildew. Alternating sulfur with DMI fungicides in vineyards with fungicide resistance resulted in better disease control than tank mixing DMIs and sulfur (Ypema et al. 1997). However, agrochemical companies state that sulfur is compatible with DMI fungicides. Therefore, the aim of this research was to investigate the effect of tank mixing DMIs with sulfur on powdery mildew control.

9.2. Methods

Cabernet Sauvignon vines were grown from cuttings in pots for six weeks in Naturallife® mini-greenhouses maintained at 25-27°C (Figure 5.5). Mini-greenhouses have been routinely used to maintain *E. necator* isolates and the ventilation windows are covered with spore proof fabric to prevent cross-contamination (Section 5.1.1.3.3).

Plants were removed from the mini-greenhouses and placed in a humidity tent. Ten to 15 leaves on each plant were tagged for inoculation. Four wild type isolates (lacking the Y136F mutation associated with DMI resistance) and four isolates with high frequency of the Y136F mutant, previously confirmed by Next Generation Sequencing (NGS; 5.1.1.7), were used for the experiments. Inoculum was prepared by using 4-5 heavily infected leaves harvested from plants infected with a mix of either wild type or isolates with Y136F mutant and maintained in mini-greenhouses. These leaves were shaken and rubbed around the tagged leaves. Plants were returned to the mini-greenhouses and incubated for 48 h to establish the infection. Two separate experiments were conducted for each *E. necator* population (sensitive and resistant).

After 48 h of incubation in the mini-greenhouses three DMI fungicides (Table 9.1) were applied both individually and mixed with sulfur using hand-held spray bottles. Plants treated with sulfur only were separated by at least 7 m from the other treatments to prevent fungicide vapour activity interference (Parsons, 2021). The untreated control was sprayed with water.

Assessment of powdery mildew disease was conducted two weeks after fungicide treatment by measuring the percentage area of leaf infection. Each experiment was repeated using three biological replicates (plants), with treatments arranged randomly and data were combined for repeated experiments.



Figure 9.1. Mini-greenhouse system for culturing powdery mildew on grapevine.

Table 9.1. Fungicides and application rates used in experiments.

Trade name	Active ingredients	Application rate (/100 L)
Topas®	100 g/L penconazole	125 mL
Mycloss®	200 g/L myclobutanil	16 mL
Digger®	250 g/L difenoconazole	25 mL
Thiovit Jet®	800 g/kg sulfur	200 g

9.3. Results

Results showed that the untreated plants (no fungicide spray) had high disease severity (70 and 74% for sensitive and resistant populations, respectively) (Figure 9.2). The results showed that all DMI and sulfur combinations were very effective for both *E. necator* populations (wild type and with Y136F mutant), controlling the disease by between 98 and 100% compared with untreated controls under greenhouse conditions. There was no reduction in the efficacy of the three DMIs when mixed with sulfur to control powdery mildew. The results also showed that the individual fungicides were equally effective at controlling powdery mildew, regardless of the presence of the Y136F mutant in the *E. necator* population.

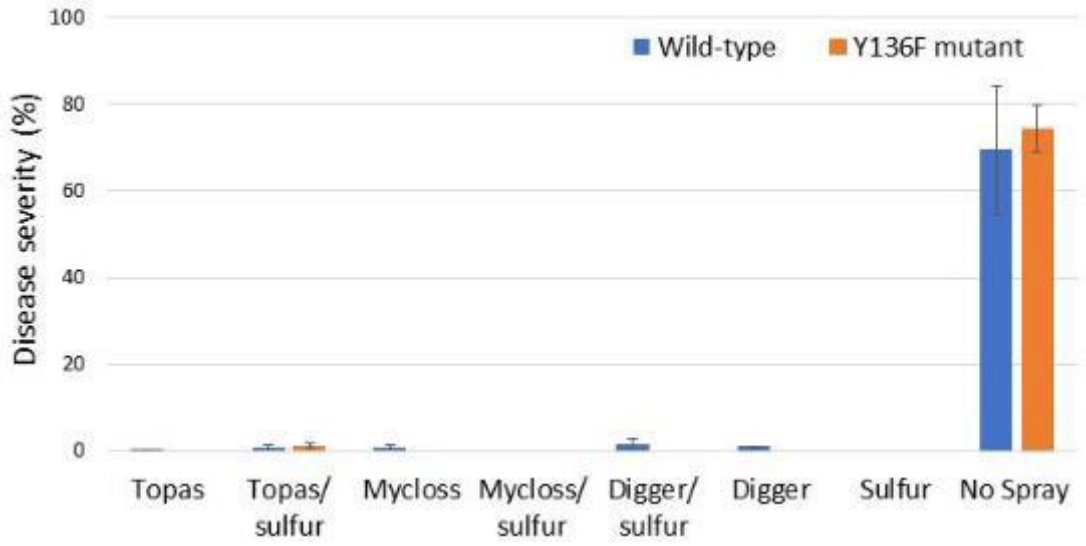


Figure 9.2. The effect of mixing sulfur with DMI fungicides on the severity of powdery mildew under greenhouse conditions using two *Erysiphe necator* populations; wild type (blue) and with Y136F mutant (orange). Each column represents the mean of two individual experiments with three biological replicates. Bars represent standard error of the mean.

9.4. Discussion

This study confirmed the efficacy of the DMIs Topas, Mycloss and Digger for controlling powdery mildew when tank mixed with sulfur. Tank mixing with sulfur is a common practice to manage fungicide resistance as it provides more than one mode of action to control powdery mildew and other pests and diseases. Chivers et al. (2007) reported no antagonism between myclobutanil (Mycloss) and sulfur and the mix of sulfur and myclobutanil is an important tool to control Erineum mite (*Colomerus vitis*) and powdery mildew on grape in New Zealand. Fungicide labels for Mycloss and Topas state that they are compatible with sulfur, and it has been recommended to mix sulfur with DMIs to manage resistance (Emmett et al. 2003). Tank mixing of DMIs and sulfur is an important strategy for the control powdery mildew disease and for managing fungicide resistance in the vineyards.

Although the resistant mix of *E. necator* has high frequency of Y136F mutant (linked to DMI resistance), Topas, Mycloss and Digger were still independently effective at controlling the disease. The resistance mechanism of DMIs are still not completely understood and Kunova et al. (2021) reported several mechanisms of resistance to DMIs, not only the Y136F mutant.

This study also showed that the mini-greenhouse protocol is very useful for evaluating efficacy of fungicides for powdery mildew, and could also be used for downy mildew. This would reduce the time and cost associated with evaluating efficacy compared with vineyard trials and will provide conducive conditions to guarantee disease development. It also facilitates the use of fungicide resistant populations for these evaluations.

10. In-field assays to detect resistant *B. cinerea* and *E. necator* populations

10.1. Introduction

Estimating fungicide resistance frequencies is critical to developing informed and effective resistance management strategies for economically important fungal pathogens. Current methodologies for determining resistance frequencies usually involves time-consuming laboratory based phenotypic techniques. As an alternative, *in vitro* genotypic techniques like the cleaved amplified polymorphic sequence (CAPS), allele specific-polymerase chain reaction (AS-PCR) and quantitative PCR (qPCR) methods could provide a rapid and cost-effective approach to estimating resistance frequencies (McCartney et al. 2003, Oshima et al. 2006). However, these techniques are still laboratory based and require specialist equipment and training. Therefore, adaption of genotypic techniques to a simple in-field pipeline could enable rapid *in situ* assessment of resistance frequencies. *In situ* qPCR has been previously shown to be a reliable, rapid and cost-effective method for the assessment of QoI resistance in the wheat pathogen *Blumeria graminis* f. sp. *triciti* (Dodhia et al. 2020). In this report, we have followed a similar approach to develop qPCR assays for the in-field detection of resistance-associated genotypes in *B. cinerea* and *E. necator*. Assays were developed to target the *B. cinerea* genotypes L412F/V (*Pos5*) and F412S (*Erg27*) previously associated with group 9 and group 17 resistance, respectively (Fillinger et al. 2008, Mosbach et al. 2017). An assay was also developed to target the G143A genotype (*CytB*) associated with group 11 resistance in *E. necator* (Baudoin et al. 2008). The pipeline was used to estimate resistance frequencies from samples in *Botrytis* infected blocks and in a *Botrytis* field trial where chemistry had been removed. The pipeline was also tested to see whether it could detect the *E. necator* G143A genotype in infected leaf and spore trap samples.

10.2. Methods

10.2.1. Design and optimisation of qPCR assays

Primer and probe sequences, and annealing temperatures for each assay are described in Table 5.40, Table 6.8 and Table 10.1. The BcL412F and BcF412S assays were used in the initial development (2019-20) and field testing to identify group 9 and group 17 resistance-associated genotypes L412F and F412S, respectively (Table 6.8 and Table 10.1). Due to difficulties in quantifying mixtures of genotypes within the same dual probe reaction (data not shown), the L412F and F412S assays were redesigned (Table 5.40). The probes were ordered as separate probes (Integrated DNA technologies, U.S.A.) and the new assays were termed BcL412Fv2 and BcF412Sv2. A BcL412F+V triplex assay designed which had an additional probe targeting L412V combined with the BcL412Fv2 assay. BcL412Fv2, BcL412F+V and BcF412Sv2 were designed as described in Section 5. The EnG143A assay was designed from *E. necator CytB* (NC_056146.1). All amplification primers were ordered from Macrogen Inc. (South Korea). For all *B. cinerea* assays, genomic DNA from isolates Bc- 7 (WT) Bc-396 (L412F, F412S) and Bc-403 (L412V) were used for initial testing and as subsequent control DNA (Harper et al. 2021). Control DNA was extracted from isolates as described in Section 5. The EnG143A assay was initially tested using synthesised *E. necator CytB* DNA in the form of gBlocks™ gene fragments (Integrated DNA technologies, U.S.A.). The sequences for the gBlocks™ were designed from the NC_056146.1 and span 152 bp, with a guanine (WT) or cytosine (G143A) at base pair number 77 (Table 10.2). After initial testing with the gBlock templates, DNA from grape leaves infected with WT (isolate 158) and G143A (isolate 216) *E. necator* isolates was extracted using a CTAB method (described in Section 6.2). The grape leaves were infected with *E. necator* spores as described in Section
5.1.1.4.1. For each *E. necator* isolate, approximately 12 cm^3 of infected leaf material was

ground once using a mortar and pestle using liquid N before CTAB extraction. To calculate *CytB* copy number in each extraction, cdPCR was used (Section 6). The cdPCR reaction mixture included 1.7 µL of each EnG143A assay probe (5 µM), 3.1 µL of EnG143A primer mix (5 µM each primer), 8.5 µL buffer (Bioline, U.K.), and 2 µL of diluted CTAB DNA (dilution factor of 20 – 160). The chip loading, PCR cycle conditions, chip reading and data analysis were carried out as described in section 6, with an annealing temperature of 62°C. The qPCR reaction mixtures for BcL412Fv2 and BcF412Sv2 are described in Section 5.2. The qPCR reaction mixtures for the pre-mixed probes sets (BcL412F, BcF412S) were as described in Section 5.2, except for differences in the probe and water components, 0.25 µL of each pre-mixed probe set (10 µM each probe, 36 µM each primer) and 5.75 µL of water. A BcL412F+V assay (triplex) was also tested as described in Section 5, except water at 5.25, and 0.25 µL of each L412 probe (Table 10.1). The qPCR reaction mixture for EnG143A was as described in Section 5. The qPCR protocol, optimisation and thermocyclers described in section 5 were used for optimising the L412F, F412S and EnG143A assays, with final annealing temperatures between 64 – 67°C (Tables 5.40 and 10.1). Standard curves were constructed by plotting DNA concentration (L412F and F412S) or DNA copy number (EnG143A) against Cq values (Figure 10.1 and Figure 10.2) for each assay and individual target DNA. Primer efficiencies and the Limit of Quantitation (LoQ) for each assay could be assessed using the standard curves. Mixed genotype reactions were also tested for the L412Fv2, F412Sv2 and EnG143A assays to investigate the ability of each assay to robustly quantify low abundance (e.g. 25%) target DNA.

Table 10.1. Primer details and annealing temperatures for *B. cinerea* and *E. necator* qPCR assays used for the development of the in-field detection pipeline. Assays BcL412F, BcL412Fv2 and F412Sv2 are described in Tables 5.1 and 6.3. *BcL412F+V assay combines the BcL412Fv2 assay (Table 5.1) and the BcL412V-TEX615 probe. + = a locked nucleic acid (LNA) on the following base.

Species	Assay name (Gene, FRAC group)	Primer name	Sequence (5-3)	Annealing temperature (°C)
B. cinerea	BcL412F+V (*Pos5*, 9) *	BcL412V-TEX615	5TEX615/AATGG+G+G+TG+CTGAA/3IAbRQSp/	64
	BcF412S (*Erg27*, 17)	BcF412-VIC	VIC/CTTACAAGGTAGAAGATCAA/MGB-NFQ	67
		BcF412-FAM1	FAM/CAAGGTAGGAGATCAA/MGB-NFQ	
		Bcerg27-412-F1	TCGGAGATCATGCCCTTGAATTT	
		Bcerg27-412-R1	GCCATTGGCTTCCCATCCAT	
E. necator	EnG143A (*CytB*, 11)	EnG143-HEX	5HEX/CCTATGG+G+G+TGCAACCGTT/3IABkFQ	64.5
		EnG143A-FAM	FAM/CCTATGG+G+C+TGCAACCGTT/3IABkFQ	
		EncytB-143-2F	TGGAACTTTGACAGCATTCTTGG	
		EncytB-143-1R	GTGATATTTTTCAACCGCTATTACCT	

Table 10.2. *E. necator CytB* gBlock™ sequences. The G to C nucleotide change (G143A) is bracketed.

Sequence name	Sequence (5′-3′)
EncytB.WT	ATTAGGTTATAGTAAGCTGGAACTTTGACAGCATTCTTGGGTTATGTTTTACCCTACGG GCAGATGAGCCTATGGG[G]TGCAACCGTTAAGTAGGTAATAGCGGTTGAAAAATATCA CTATATGCTGGAAACTTCTAAAGCTTTAAGTAGGTG
EncytB.G143A	ATTAGGTTATAGTAAGCTGGAACTTTGACAGCATTCTTGGGTTATGTTTTACCCTACGG GCAGATGAGCCTATGGG[C]TGCAACCGTTAAGTAGGTAATAGCGGTTGAAAAATATCA CTATATGCTGGAAACTTCTAAAGCTTTAAGTAGGTG

10.2.2. Using the in-field detection pipeline to test *Botrytis*-infected berries, *E. necator* infected leaves and spore trap samples.

Infected berries from the 2019 WA vineyard experiment (Table 6.7) and *E. necator* infected leaves acquired from the Perth metropolitan area (WA) were used to test the qPCR pipeline against these different forms of infected biomass (Dodhia et al. 2020). *Botrytis* infected berries were previously stored at -20°C prior to analysis. One infected berry was placed in a microcentrifuge tube, and the quick extraction method was undertaken as described in section 5. Multiple berries were taken as replicates. Using the quick extraction DNA, the L412F qPCR protocol was undertaken as described above. Leaf cores from *E. necator* infected leaves (fresh and dried) and spore trap rod samples (SARDI, SA) were tested for the presence of *E. necator* G143A using the EnG143A assay as described above. Eight *E. necator* infected leaf cores were taken from one leaf to form one replicate for testing, with a total of three replicates across three leaves taken. Spore trap rods were sampled at three SA locations (Table 10.4) using the trapping system described in Section 7. Sixteen spore trap samples were tested, with each sample consisted of a rod coated in grease and each rod was stored in plastic tube. To each tube, 200 µL of quick extraction buffer (described above) was added, and the tube was thoroughly inverted and mixed. The resulting mixture was then transferred to a 1.7 mL tube. Each of the leaf core and spore trap samples were hand ground with a micro pestle, diluted and used as template for testing the EnG143A assay with qPCR protocol as described in Section 5.

10.2.3. Using the in-field qPCR pipeline to estimate frequencies across field trial samples

The removal of a chemical group from a spray program can influence the persistence of resistance associated with that chemical group. In collaboration with Scott Paton (Nutrien), during the 2022 season a field trial at vineyard 3, Margaret River (WA), was established to investigate the effect of removal of group 9 and 17 chemistries on resistance frequencies. Attempts were made to identify additional potential trial sites in South Australia, but resistance frequencies at these sites were extremely low (data not shown). Vineyard 3 was an ideal trial site, as previous testing had detected the L412F and F412S genotypes at this site (data not shown). The different treatments (cv. Chardonnay) are shown in Figure 10.5, with Solaris® only (cyprodinil, group 9), Prolectus® only (fenpyrazamine, group 17) and un-sprayed treatments replicated across at least four panels (four vines/panel). Each fungicide was applied twice during flowering at EL23 (1 - 50% capfall) and EL25 - 27 (80 - 100% capfall) growth stages at a 100 mL/100 L rate. Once *Botrytis* expression was underway late in the season, 29 - 30 samples were collected per treatment for BcL412Fv2 and BcF412S qPCR for analysis using the in-field pipeline as described above. Swabs were also taken from treatments for single sporing and subsequent group 9 and 17 phenotypic analyses as described in section 5.2. QPCR, Sanger sequencing and the CAPS *Mbo* I test was undertaken on most swab isolates to determine genotypes as described in section 5. Sporulation of isolates and the quick extraction DNA method was undertaken prior to genotyping as described in section 5.

10.2.4. Running the qPCR assays on field samples at vineyard locations and in the laboratory

For the processing of all *Botrytis* field samples across the 2020/22 seasons, the in-field qPCR pipeline, which includes quick DNA extractions followed by qPCR, was carried out as described above. During the 2020 season, the L412F assay was used, while in 2021/22 the redesigned L412F assay (L412Fv2) was used. All field samples assessments used the F412S assay. All qPCR testing used either a duplex or triplex probe assay to allow for a reasonable sample throughput, especially for on-site testing using the 48-well MIC qPCR instrument (Bio

Molecular Systems, Australia). One berry sampled from one bunch was considered a single sample. Due to issues in quantifying targets in mixed samples across all assays, detection was the objective, with positives required to show a Cq value of ≤35. Pure genomic DNA was used as positive controls or standards in each qPCR run. All testing was completed at the vineyard (on-site), near the vineyard (near-site), or in the laboratory. Replication across the qPCR runs varied between 1 – 2 replicates per sample per assay. The laboratory testing involved using either the MIC instrument (Bio Molecular Systems, Australia) or 96-well CFX96 thermocycler (Bio-Rad laboratories, U.S.A.). During the 2020 season, a grower workshop to demonstrate the in-field pipeline was organised for a small group of grape growers from around the Margaret River wine region (WA). The growers each brought a collection of infected samples to a testing site just south of the Margaret River Township. At the workshop, 1 – 2 berries were taken per bunch. At the workshop, the BcL412F assay was run on site, while the BcF412S assay was run back in the laboratory. Secondly, a Sauvignon Blanc block (0.25 ha) was randomly sampled (n = 69) and tested on site with the Bc412F assay at a vineyard (vineyard 1) in Margaret River, WA (Figure 10.2). To enable a high throughput analysis of field samples on-site, only one technical replicate was tested per sample. The samples were tested against the BcF412S assay in the laboratory. At vineyard 1, the recorded sampling location was at the panel (between posts) level, not the specific vine in the panel. During the 2021 vintage, the BcL412F+V triplex and BcF412S assays were tested on berries randomly sampled from three blocks across two Margaret River vineyards (vineyards 1 and 2) to see if the L412V mutant could be detected (Figure 10.4). In each block, one berry from one bunch was sampled, with 39 – 40 samples taken across each block (Table 10.3). Testing was completed on-site, near-site and in the laboratory using the in-field pipeline.

10.3. Results

10.3.1. Optimisation of qPCR assays

The optimum annealing temperature for each assay was established and this temperature was used for subsequent qPCR testing (Table 5.22 and Table 10.1). For all *Botrytis* assays and the EnG143A assay, detection down to 0.01 pg/µL and 0.25 copies/µL (5 copies/reaction) was achievable, respectively (Figure 5.22, Figure 10.1 and Figure 10.2). The limit of detection (LoD) Cq value for all assays was between 34 – 37. For the L412Fv2, F412Sv2 and En143A assays, the testing of genotype mixtures showed that robust quantification of each target is difficult in a duplex assay reaction, with significant and highly variable distortion of DNA estimates compared to non-mixed controls (Table 10.3).

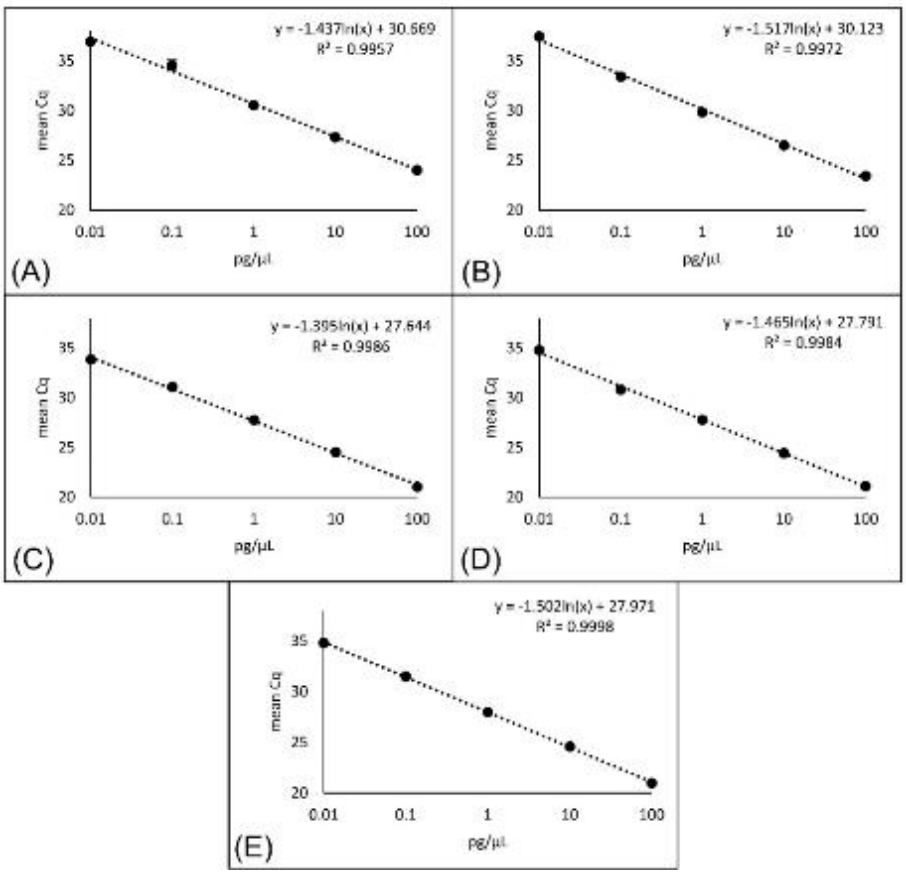


Figure 10.1. Standard curves of the B. cinerea Pos5 (group 9) duplex assays BcL412F and L412F+V against WT and mutant DNA. A = BcL412F with WT DNA, B = BcL412F with L412F DNA, C = BcL412F+V with WT DNA, D = BcL412F+V with L412F DNA, E = BcL412F+V with L412V DNA

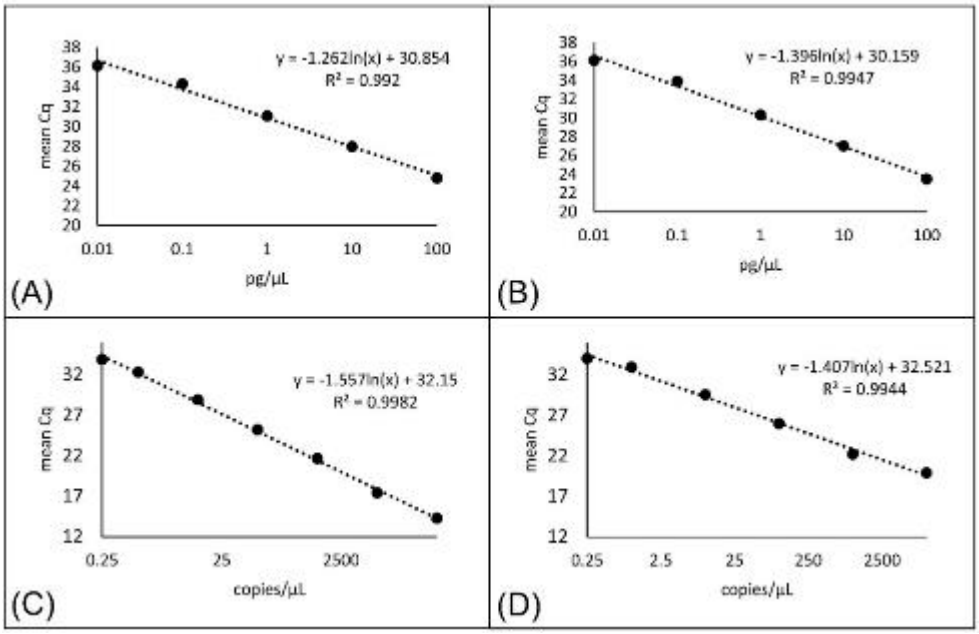


Figure 10.2. Standard curves for *B. cinerea Erg27* (group 17) and *E. necator CytB* (group 11) duplex assays against WT and mutant DNA. A = BcF412S with WT DNA, B = BcF412S with F412S DNA, C = EnG143A with WT DNA, D = EnG143A with G143A DNA.

Table 10.3. Difference (%) in estimated DNA concentration of mixed (25% target) reactions compared to non-mixed reactions. Percentage values for each replicate were ordered from low to high.

Assay	Target DNA (25%)	Total DNA concentration (ng/µL)	Difference (%) of mixed (25% target) reaction compared to non-mixed reaction			
			rep 1	rep 2	rep 2	rep 4
BcL412Fv2	WT	1.00E-03	-46.8	-3.5	+233.4	
		1.00E-04	-65.5	-0.6	+215.7	
	L412F	1.00E-03	-42.0	-37.6	+90.7	+242.2
		1.00E-04	-34.1	3.5	+146.1	
BcF412Sv2	WT	1.00E-03	-50.4	-3.3		
		1.00E-04	-39.3	+124.8		
	F412S	1.00E-03	-12.4	-9.7		
		1.00E-04	-5.1	+29.1		
EnG143A	WT	1.00E-06	+4.2	+23.9	+46.0	
	G143A	1.00E-06	-13.5	-8.5	-6.4	

10.3.2. Assessment of the in-field pipeline on infected berries, infected leaves, and spore trap samples

B. cinerea infected berries and *E. necator* infected leaves (fresh and dried) provided adequate DNA when these forms of biomass were processed using the quick extraction DNA method (data not shown). The results from the spore trap sample testing are shown in Table 10.4. The quick extraction DNA method is also suitable for processing spore trap rod samples, as five of the sixteen spore trap rods showed detection of the WT and mutant DNA simultaneously.

Table 10.4. Testing results for spore trap rods (n = 16) using the qPCR in-field pipeline.

Sample#	isolate/sample information	date of collection	qPCR result
ST3	Spore Trap Nurioopta 1st collection day 3	3/12/2020	no amplification
ST4	Spore Trap Nurioopta 1st collection day 4	5/12/2020	no amplification
ST6	Spore Trap Nurioopta 2nd collection day 2	16/12/2020	no amplification
ST8	Spore Trap Nurioopta 2nd collection day 4	18/12/2020	WT/G143A
ST11	Spore Trap Nurioopta 3rd collection day 3	7/01/2021	WT/G143A
ST13	Spore Trap Nurioopta 4th collection day 1	20/01/2021	WT/G143A
ST16	Spore Trap Nurioopta 4th collection day 4	23/01/2021	WT/G143A
ST20	Spore Trap Nurioopta 5th collection day 4	5/02/2021	WT/G143A
ST24	Spore Trap Waite 1st collection day 4	11/12/2020	no amplification
ST25	Spore Trap Waite 2nd collection day 1	15/12/2020	no amplification
ST29	Spore Trap Waite 3rd collection day 1	5/01/2021	no amplification
ST32	Spore Trap Waite 3rd collection day 4	8/01/2021	no amplification
ST36	Spore Trap Waite 4th collection day 4	23/01/2021	no amplification
ST47	Spore Trap Riverland 2nd collection day 3	17/12/2020	no amplification
ST49	Spore Trap Riverland 3rd collection day 1	5/01/2021	no amplification

ST50	Spore Trap Riverland 3rd collection day 2	6/01/2021	no amplification

10.3.3. Detection of L412F and F412S mutation at the grower workshop and at vineyard 1 and 2

The qPCR results for the grower workshop are shown in Table 10.5. The analysis of the samples brought by the growers detected the L412F and F412S in three out of the 24 berries tested. No WT DNA was detected in the three samples where mutant DNA was also detected. However, WT and mutant alleles were detected separately from different berries (sample K1 a – c) from the same bunch (Bunch 1, sample collection 7) (Table 10.1). QPCR results for the vineyard 1 (block a) testing are shown in Figure 10.3. Three samples (4.3 %) were positive for L412F, and one sample was positive for both L412F and F412S. L412F and WT genotypes were both detected in two samples. The qPCR results for sampling across vineyard blocks are shown in Table 10.6. In blocks 1b, 1c and 2a, F412S and L412F frequencies were 80 – 84% and 79%, 81 – 85% and 85%, and 4% and 9-10%, respectively.

Table 10.5. BcL412F and BcF412S mutation testing results from the grower workshop during the 2020 season. Detection of the mutant is indicated in red. ND = no detection.

Vineyard designation	Sample ID	Bunch number	BcL412F assay (on-site)	BcF412S assay (laboratory)
A	A1	1	WT	WT
	A SB	2	WT	WT
B	YTG 2	1	WT	WT
	YTG	2	WT	WT
	YSB	3	WT	WT
C	RB1	1	WT	WT
	RB2	2	WT	WT
	RB ST	3	WT	WT
D	X1	1	WT	WT
	X2	2	WT	WT
	X3	3	WT	WT
E	B10	1	WT	ND
	B10 b	1	WT	WT
	B12	2	WT	WT
	B13	3	WT	WT
	B14	4	WT	WT
F	Aug.2	1	WT	WT
	Aug2 b	2	WT	WT
	Aug2 c	2	WT	WT
G	K1 a	1	WT	WT
	K1 b	1	WT	WT
	K1 c	1	L412F	F412S
	K2 a	2	L412F	F412S
	K2 b	2	L412F	F412S



Figure 10.3. BcL412F (on-site) and F412S (laboratory) results from 69 samples at vineyard 1 (block a) in the 2020 season. The red dot indicates detection of both L412F and F412S in the same sample. Yellow dots indicate detection of L412F and WT genotypes in the same sample. White dots indicate WT only.



Figure 10.4. Approximate areas (white boxes) of block 1b/1c and 2a that were sampled during 2021 at vineyard 1 and 2, respectively

Table 10.6. qPCR results for the 2021 sampling at blocks 1b, 1c and 2a. F = L412F frequency

Vineyard/block number	Assay	Site of testing	Number of replicates	Number of samples tested	Number of samples with amplification	Mutant frequency (%) *
1b	BcF412S	on-site	1	39	30	24/30 (80%)
		laboratory			38	32/38 (84%)
	BcL412F+V	laboratory	2		24	F-19/24 (79%)
1c	BcF412S	on-site	1	40	31	25/31 (81%)
		laboratory			39	33/39 (85%)
	BcL412F+V	laboratory	2		34	F-29/34 (85%)
2a	BcF412S	near-site	2	40	28	1/28 (4%)
	BcL412F+V	near-site	1		23	F-2/23 (9%)
		laboratory	2		20	F-2/20 (10%)

10.3.4. Estimation of L412F and F412S frequencies in the vineyard 3 *Botrytis* field trial

The qPCR results for the vineyard 3 *Botrytis* field trial are shown in Figure 10.6. In the untreated, group 9 and group 17 treatments, F412S and L412F frequencies were 88 and 73%, 100 and 80%, and 71 and 65%, respectively. Phenotypic results from isolates originating from swabs taken from each treatment are shown in Figure 10.7. In the untreated, group 9 and group 17 treatments, group 17 and group 9 resistance frequencies were 76 and 71, 100 and 100, and 88 and 100%, respectively. Genotypic results from the swab isolates are shown in Table 10.7. The F412S and L412F genotypes were found in all treatments, but not in all isolates. The *Mdl1* changes E407K and S466R were identified in isolates from the untreated/group 9 plots and group 17 plots, respectively. Isolates that were WT in *Pos5* and *Mdl1* targeted regions simultaneously were identified in the untreated plots. The L412V (*Pos5*) and F412V (*Erg27*) changes were identified in isolates from the group 17 and untreated/group 17 plots, respectively.

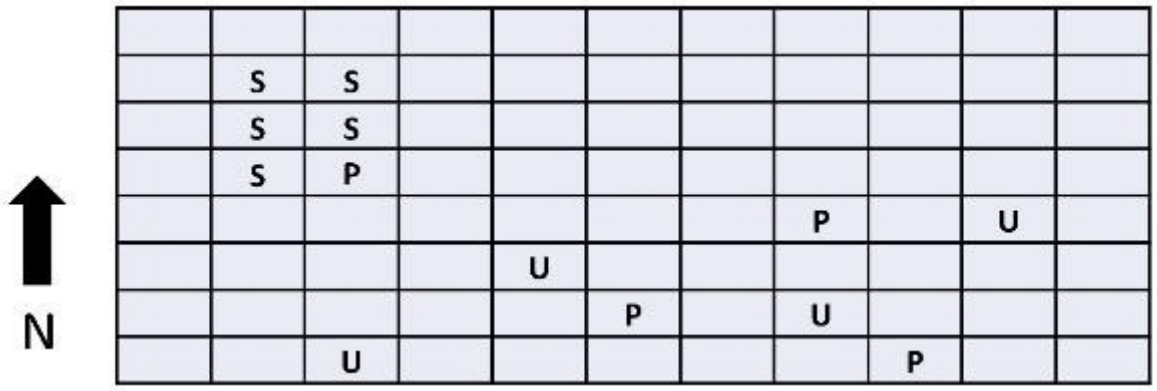


Figure 10.5. *Botrytis* field trial layout (vineyard 3, cv. Chardonnay). Each column represents a row. Each cell represents a panel of four vines. S indicates replicate panels in the Solaris® treatment, P indicates replicate panels in the Prolectus® treatment, U indicates replicate panels in the un-sprayed treatment. The trial was in small portion of a large block. Bottom row indicates the end of the rows.

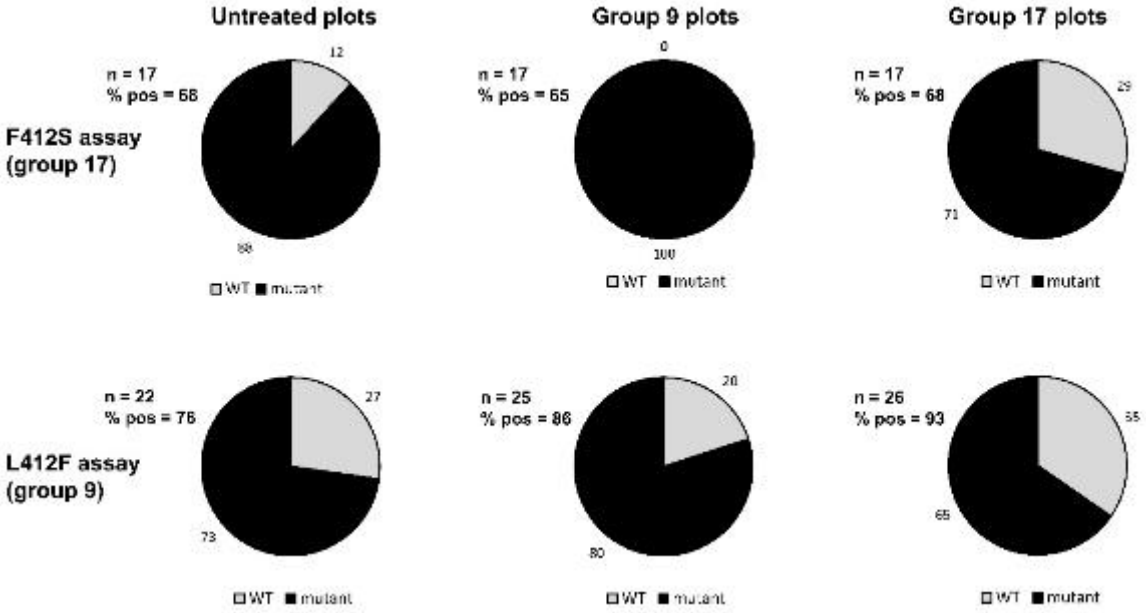


Figure 10.6. The frequency of the F412S and L412F alleles in untreated, group 9 and group 17 treatment in the 2022 *Botrytis* field trial. n = number of samples that detected *Botrytis*, % pos = the number of field samples that had detection of WT and/or mutant alleles.

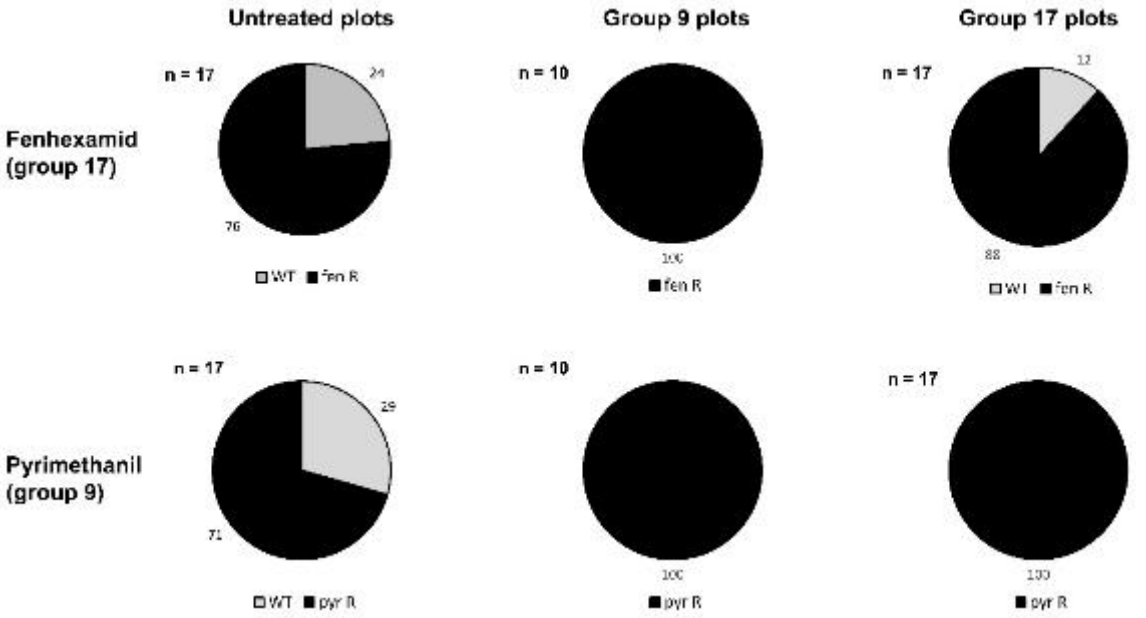


Figure 10.7. Phenotypic testing of swab isolates from the untreated, group 9 and group 17 treatments in the 2022 *Botrytis* field trial. n = number of isolates.

Table 10.7. Genotypic results from a subset of swab isolates. * = qPCR, ** = sanger sequencing, * = *Mbo* II CAPS. - = not present.**

	group 9						group 17			
	Pos5			*Mdl1*		*Pos5/Mdl1*	*Erg27*			
treatment	isolates tested	L412F*	L412V**	E407K**	S466R**	WT**	isolates tested	F412S*	F412V**	F412C/I/S/V***
untreated	9	5	-	2	-	2	10	4	3	3
group 9	9	7	-	2	-	-	9	4	-	5
group 17	15	13	1	0	1	-	17	13	2	2

10.4. Discussion

A rapid, cost-effective, and mobile in-field qPCR assay using a lightweight qPCR instrument has previously been established to characterise QoI resistance in *B. graminis* f. sp. *hordei* (Dodhia et al. 2020). In this study, this same pipeline was successfully adapted for the rapid field detection of group resistance-associated mutations in *B. cinerea* and *E. necator*. For both pathogens, the method was first optimised in the laboratory, followed by extensive sampling for *Botrytis* in block 1a (2020) and *E. necator* leaf and spore trap rod testing (Table 10.4 and Figure 10.3). During the 2021 season, a triplex assay was used to attempt to detect the *Botrytis* L412V genotype (Figure 10.4, Table 10.6). Previous studies have shown that qPCR is a robust method for quantifying *Botrytis* spp. from berries expressing the disease down to ~0.1 pg/µL (Cadle-Davidson, 2008, Saito et al. 2013). However, these qPCR studies were laboratory based and used time consuming and time consuming CTAB based DNA extractions. Furthermore, the method described here appears to be more sensitive than previous studies (Cadle- Davidson, 2008, Saito et al. 2013).

A *Botrytis* field trial was held in 2022, and the pipeline was successfully used to estimate L412F and F412S frequencies across different treatments (Figure 10.6). For both respective genotypes, the untreated plots showed a lower and higher allele frequency when compared to the group 9 and 17 plots, respectively. These results could indicate that application of group 17 had some control over F412S strains, while the group 9 application produced a relative increase of the L412F frequency compared to the untreated plots. The high background frequency of both resistant genotypes across vineyard 3, in combination with small plot sizes, could have prevented the WT to establish to a high frequency in the untreated plots. The sampling number was limited to 29 – 30 to ensure a short turnaround for results. Future use of this method in a trial may require more samples to reduce sampling bias and increase the statistical strength of any significant differences found between treatments. The sampling timepoint could have influenced allele frequency estimates. Sampling was undertaken once disease was well established, and secondary infections were present. An earlier sampling timepoint would reflect more on the underlying primary (latent) infection. Genotypic testing of isolates from each treatment identified alternate mutations at the L412 (*Pos5*) and F412 (*Erg27*) residues. These alternate genotypes could explain the low positive rates (65 – 68%) in all treatments when screened for F412S (Figure 10.5). Positive rates were not as low for the L412F testing (76 – 93%). Alternative genotypes could prevent the WT and mutant L412F/F412S probes to bind, resulting in no positive reactions. The presence of alternate genotypes must be considered when testing field samples with assays not targeting these genotypes. Testing for a limited number of resistance-associated mutations could therefore underestimate resistance frequency. Testing of blocks of 1b, 1c and 2a (Table 10.6) did not show the mutant allele in 100% of the samples, indicating that at these sites non L412F/V genotypes could also be present. Future work could involve developing additional assays to cover these alternate genotypes. Additionally, there were resistant isolates that were WT in both *Pos5* and *Mdl1* target regions, which at this stage makes them undetectable using qPCR.

Further work is required to determine the resistance mechanism and associated molecular changes in these group 9 resistant isolates lacking a mutation in *Pos5* or *Mdl1*.

In all three assays, quantification of low abundance target under a mixed target scenario is yet to be robustly achieved. In a mixed target sample, quantification is more desirable than detection, so that the ratio of mutant/WT can be established. Furthermore, quantification of each target in a mixed sample, not just one of the targets, must be robust to ensure the ratio estimate is not distorted. Across all *Botrytis* testing, only sampling of block 1a in 2020 showed samples (n = 2) with a mixture of WT and mutant genotypes. This seems to indicate that mixtures in a sample (one berry) are uncommon. Alternatively, mixtures could be more common than they seem, but low abundance targets in the mixed samples go undetected due to the dual probe assay dynamics. A mixed sample could occur from any number of scenarios; i) WT and mutant strains growing on the same berry, ii) heterokaryosis; whereby a particular strain can have multiple genotypes due to the multi-nucleic nature of *B. cinerea* (Korolev and Elad, 2016*),* iii) cross-contamination of samples. No mixtures of the mutant and WT were found on berries at the grower's field day. However, two berries from the same bunch each had a different genotype.

At block 1a, the frequency of the L412F was low at 4.3 %, and the need for speed in processing a high volume of samples resulted in the decision to carry out an un-replicated analysis. The lack of technical replication in this case makes it impossible to identify potential cross-contamination among other issues. However, all three positives from block 1a were confirmed after re-testing (in triplicate) in the laboratory 24 h after the vineyard test (data not shown). Furthermore, two samples still showed the presence of both the WT and mutant alleles after this *in vitro* testing (data not shown). The 24 h post-trial testing did show a general reduction in DNA levels compared to the initial field run, indicating degradation of DNA in diluted templates (data not shown). Degradation of the DNA is highly probable due to (i) the crude nature of the extraction, (ii) the freeze/thaw process when diluted samples are stored at -20°C and then retested. The non-replicated approach allowed for a high number of samples to be tested and allowed the research team to gauge the technical limitations imposed by the collection of the samples themselves. In future experiments, replication of qPCR tests will have to be balanced with adequate number of samples required to allow for resistance frequencies to be determined.

The confirmed viability of testing frozen infected material in this system is also of interest, as visibly infected berries could be conveniently harvested and stored at -20°C throughout the latter part of the season before analysis of all berry samples simultaneously. The re-testing of samples in the laboratory allowed additional assays to be tested after the on-site or near-site analysis. A portion of the laboratory testing used the 96-well CFX thermocycler, which allowed a higher throughput of samples than the 48-well MIC instrument. Laboratory throughput could further be increased if a bead beating machine was used to homogenise field samples.

Preliminary testing of the EnG143A assay on *E. necator* infected leaves showed that fresh or dried infected leaf material can provide adequate DNA to detect the pathogen. Dried infected material would have an advantage over fresh material, as prior to analysis, fresh material would need to be stored at 4°C where microbial contamination or decomposition could occur. Additionally, fresh samples that were packaged and transported interstate could degrade beyond their use. Subsequent EnG143A testing of 16 spore trap rods showed adequate WT/G143A detection, demonstrating that the in-field pipeline can detect WT or G143A *CytB* in this type of sample.

Future application of this technology would require establishing detailed sampling designs and number to provide adequate information to infer resistance frequencies in *B. cinerea* and *E. necator*. A fixed number of samples tested per hectare could be one component towards standardisation of the in-field assay. Due to the simplicity of the assay, and once standardisation is complete, local advisors could be trained to use the assay. New probes for existing or novel mutations linked with resistance could be easily adapted to this assay for use

in the field. Adaption of this method to assess other grapevine fungal pathogens of interest (e.g. *Plasmopara viticola*) is now underway. Furthermore, this method is yet to be tested for its ability to detect and quantify latent ('non-expressing') *Botrytis* spp. infections in grape bunches or conidia in plant detritus early in the season. Further work using this pipeline to estimate the resistance frequency from spore trap samples could enable frequency estimates to be made across any part of the growing season. The in-field qPCR method would suit being part of a preliminary step in gauging the resistance frequency at a particular site. This 'first look' would be especially important within a block with known control issues, to see whether more extensive phenotypic and genotypic lab-based testing is required.

11. DArTseq investigation of population structure in *B. cinerea* isolates

11.1. Introduction

Analysis of pathogen population structure within a vineyard where fungicide resistant isolates are known to exist could provide valuable information to adjust resistance management strategies. The necrotrophic fungal pathogen *B. cinerea* is a highly polymorphic species that can infect over 1400 plant hosts (Elad et al. 2015). Previous population studies in *B. cinerea* have sequenced and analysed a range of molecular markers including transposons, microsatellites, and various genes (Giraud et al. 1997, Isenegger et al. 2008, Ma and Michailides, 2005, Staats et al. 2004). An alternative to these methods is DArT (Diversity Arrays Technology) sequencing, which types several thousand loci in a single assay. In this report, a subset of *B. cinerea* isolates (*n* = 86) was selected for DArT sequencing analysis. The subset included sensitive and resistant isolates from Western Australia and Victoria (Harper et al 2021). The objective was to see whether DArTseq analysis could identify any underlying population structure that could be associated with sensitive and resistant phenotypes.

11.2. Methods

11.2.1. Isolate details and DNA extraction

Eighty-six *B. cinerea* isolates were selected for DArT sequencing analysis, all originated from WA and Vic, except for one that originated from Qld (Table 11.1). The subset of isolates was a mixture of sensitive and resistance isolates. Each isolate was sporulated, conidia were harvested, and DNA extracted as described in Section 5. DNA samples were concentrated by freeze drying. The DNA pellets were resuspended in 28 µL of distilled water, and DNA quality was tested by visualising 2 µL on an agarose gel. DNA quantity was measured using a Qubit flurometer (Thermo Fisher Scientific, USA) with a minimum final quantity of > 10 ng/µL required for DArTseq analysis. DNA samples were sent for DArT sequencing to Diversity arrays technology (Canberra, Australia). Each DNA sample was subjected to SNP and in-silico sequencing.

11.2.2. DArTseq analysis

Analysis of the DArT sequencing data was undertaken using the dartR package in RStudio. The SNP data set was filtered to remove loci that exhibited heterozygosity. Filtering also removed loci that did not pass the following criteria: reproducibility (=1), minimum allele frequency (=0.01), call rate of loci (=0.95), secondaries (best) and call rate of individuals (=0.8). Silico DArT data were filtered by the following criteria: reproducibility (=1), call rate of loci (=1), call rate of individuals (=0.8) and loci metrics (One Ratio 99.95 vs 0.05). A Principal Components Analysis (PCA; 2 axes selected) was performed on the filtered SNPs of the entire subset and viewed as both a graph and neighbour joining tree to observe the relationships between isolates. A Discriminant Analysis of Principal Components (DAPC) was performed on the filtered SNP and Silico DArTseq data to determine potential genetic clustering. This was performed with no population assignment and with population assignment based on state of origin.

Table 11.1. Eighty-six *B. cinerea* isolates selected for the DArT sequencing analysis. WA = Western Australia, Vic = Victoria, R = resistance, S = sensitive, Azo = azoxystrobin, Bos = boscalid, Fen = fenhexamid, Flu = fludioxonil, Ipr = iprodione, Pyr = pyrimethanil, Teb = tebuconazole. Grey boxes indicate resistance.

Isolate	State of origin	Sampling year	Azo	Bos	Fen	Flu	Ipr	Pyr	Teb
6	WA	2013	S	S	S	S	S	S	S
7	WA	2013	S	S	S	S	S	S	S
38	WA	2013	S	S	S	S	S	S	S
40	WA	2013	R	S	S	S	R	R	S
102	WA	2013	S	S	S	S	S	S	S
106	WA	2013	S	S	S	S	S	S	S
128	WA	2014	S	S	S	R	R	R	S
130	WA	2014	S	S	S	S	S	S	R
132	WA	2014	S	S	S	S	R	R	S
133	WA	2014	S	S	S	S	S	S	S
135	WA	2014	S	S	S	S	R	S	S
138	WA	2014	S	S	S	R	R	R	S
143	WA	2014	R	S	S	S	R	S	S
146	WA	2014	S	S	S	S	S	S	S
159	WA	2014	S	S	S	S	S	S	S
161	WA	2014	R	S	S	S	S	R	S
162	WA	2014	R	R	S	S	S	R	S
175	WA	2014	R	R	S	S	R	R	S
177	WA	2014	S	S	S	R	R	R	R
178	WA	2014	S	S	S	R	S	S	S
179	WA	2014	S	S	S	S	S	S	S
181	WA	2014	S	S	S	S	S	S	S
182	WA	2014	R	R	S	S	S	R	S
192	WA	2014	S	S	S	S	S	S	S
194	WA	2014	S	S	S	S	S	S	S
195	WA	2014	S	S	S	S	R	S	S
198	WA	2014	S	S	S	S	S	S	S
215	QLD	2014	S	S	S	S	S	S	S
243	VIC	2014	S	S	S	R	S	S	S
242	VIC	2014	S	S	S	S	R	S	S
240	VIC	2014	S	S	S	S	R	R	S
234	VIC	2014	S	S	S	R	S	S	S
244	VIC	2014	S	S	S	S	S	S	S
247	VIC	2014	R	R	S	S	R	R	S
259	VIC	2014	S	S	S	S	S	S	S
260	VIC	2014	S	S	S	R	S	S	S
270	VIC	2014	S	S	S	S	S	S	S
266	VIC	2014	S	S	S	S	S	S	S
263	VIC	2014	S	S	S	S	S	S	S
262	VIC	2014	S	S	S	R	S	S	S
272	VIC	2014	S	S	S	S	S	S	S
279	VIC	2014	S	S	S	R	S	R	S

280	VIC	2014	S	S	S	S	S	S	S
282	VIC	2014	S	S	S	S	S	S	S
284	VIC	2014	S	S	S	S	S	S	S
287	VIC	2014	R	S	R	S	R	R	S
290	VIC	2014	S	S	S	R	R	R	S
292	VIC	2014	S	S	S	R	S	S	S
297	VIC	2014	S	S	S	R	S	S	S
407	VIC	2015	S	S	S	S	S	R	S
408	VIC	2015	S	S	S	S	S	S	S
411	VIC	2015	S	S	S	S	S	S	S
413	VIC	2015	S	S	S	S	S	R	S
427	VIC	2015	S	S	S	S	S	S	S
434	VIC	2015	S	S	S	S	S	S	S
436	VIC	2015	S	S	S	S	S	S	S
437	VIC	2015	S	S	S	S	R	S	S
438	VIC	2015	S	S	S	S	S	S	S
440	VIC	2015	S	S	S	S	S	S	S
442	VIC	2015	S	S	S	S	S	R	S
446	VIC	2015	S	S	S	S	S	R	R
448	VIC	2015	S	S	S	S	S	S	S
451	VIC	2015	S	S	S	S	S	R	S
452	VIC	2015	S	S	S	S	R	S	S
456	VIC	2015	S	S	S	S	S	S	S
522	VIC	2015	S	S	R	S	R	R	S
523	VIC	2015	R	S	S	S	S	R	S
527	VIC	2015	S	S	R	R	R	R	S
545	WA	2015	R	S	S	S	S	S	S
551	WA	2015	S	S	S	S	S	S	S
552	WA	2015	S	S	S	S	S	S	S
570	WA	2015	S	S	S	S	S	S	S
591	WA	2015	S	S	S	S	S	S	S
613	WA	2015	R	S	S	S	S	R	S
614	WA	2015	S	R	S	S	S	S	S
618	WA	2015	S	S	S	S	S	R	S
631	WA	2015	S	S	S	S	S	S	S
639	WA	2015	S	S	S	S	S	S	S
657	WA	2015	S	S	S	S	S	S	S
683	WA	2016	R	R	S	S	S	R	S
711	WA	2016	S	S	S	S	R	S	S
725	WA	2016	S	R	S	S	S	S	S
735	WA	2016	S	S	S	S	S	S	S
727	WA	2016	S	S	S	S	S	S	S
757	WA	2016	S	S	S	S	S	S	S
760	WA	2016	R	R	S	S	R	R	S

11.3. Results

PCA produced several clades across the subset of WA and Vic isolates (Figure 11.1 and Figure 11.2). Within these clades there was a mixture of resistant and sensitive isolates (Table 11.1). With respect to the resistant isolates in each clade, there was no perceived prevalence of a particular resistant phenotype. Sampling year appeared to have no association with clustering in the PCA, shown by the WA clade with isolates 177, 727, 40, 545, 130, 38, 735 and 102 being sampled across 2013 – 2016 (Figure 11.2). DAPC with no populations assigned showed two major clusters across the SNP and silico data sets, with sub-clustering apparent (Table 11.2, Figure 11.3). This analysis also showed that in each of the four clusters across each state resistant and sensitive isolates are present (Table 11.2). DAPC with no populations assigned across both SNP and silico data sets showed WA isolates being divided into four clusters, while Vic isolates only had one. DAPC analysis of the SNP data set with populations assigned, showed a limited distinction between WA and Vic, with some overlap between the two states (Figure 11.4).

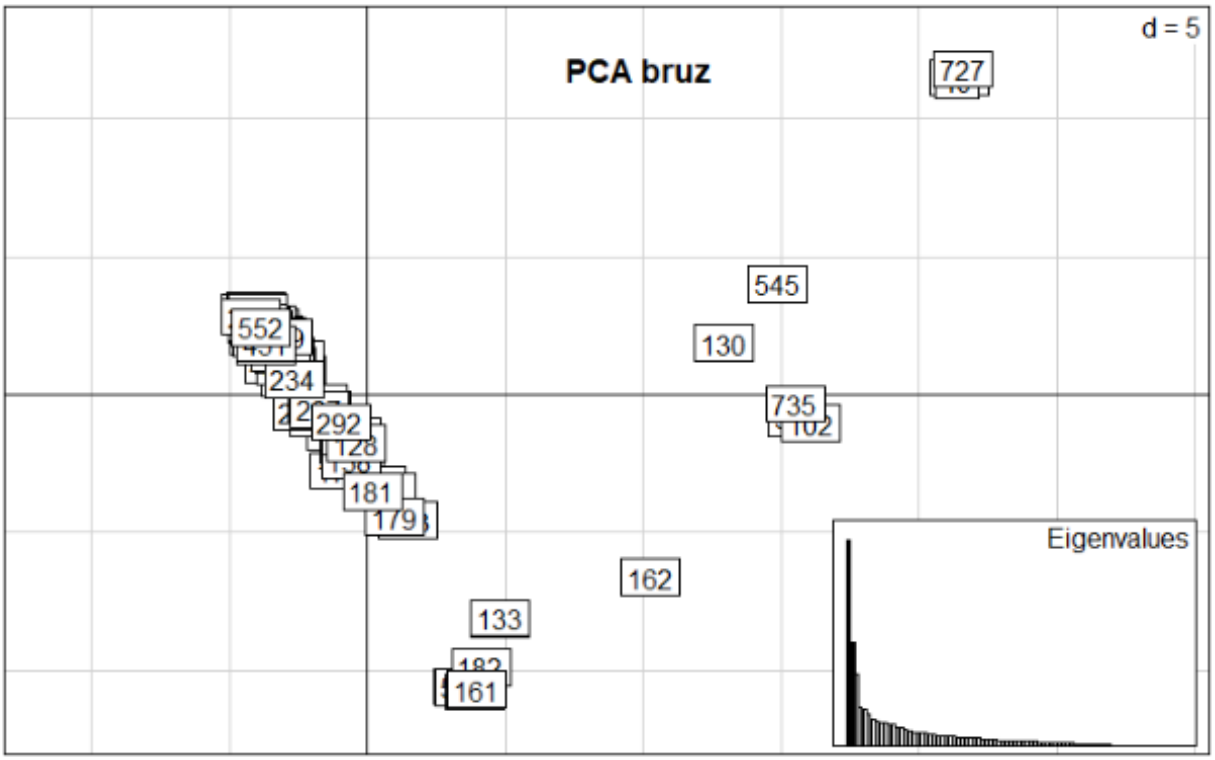


Figure 11.1. Graphical representation of the Principal Components Analysis (PCA) of the filtered SNP dataset

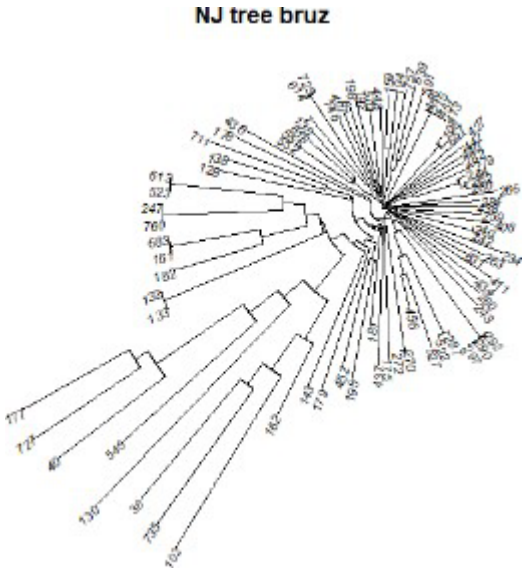


Figure 11.2. Neighbour joining tree of the Principal Components Analysis (PCA) analysis of the filtered SNP dataset. Numbers indicate isolate designations.

Table 11.2. Number of resistant and sensitive isolates from each state in each DAPC cluster for the SNP and silico datasets. No population assigned. R = resistant, S = sensitive

Data set	Cluster number	WA-R	WA-S	Vic-R	Vic-S
SNP	1	15	14	18	18
	2	2	3	0	0
	3	9	2	0	0
	4	3	1	0	0
Silico	1	10	2	0	0
	2	4	4	0	0
	3	7	1	0	0
	4	5	16	18	18

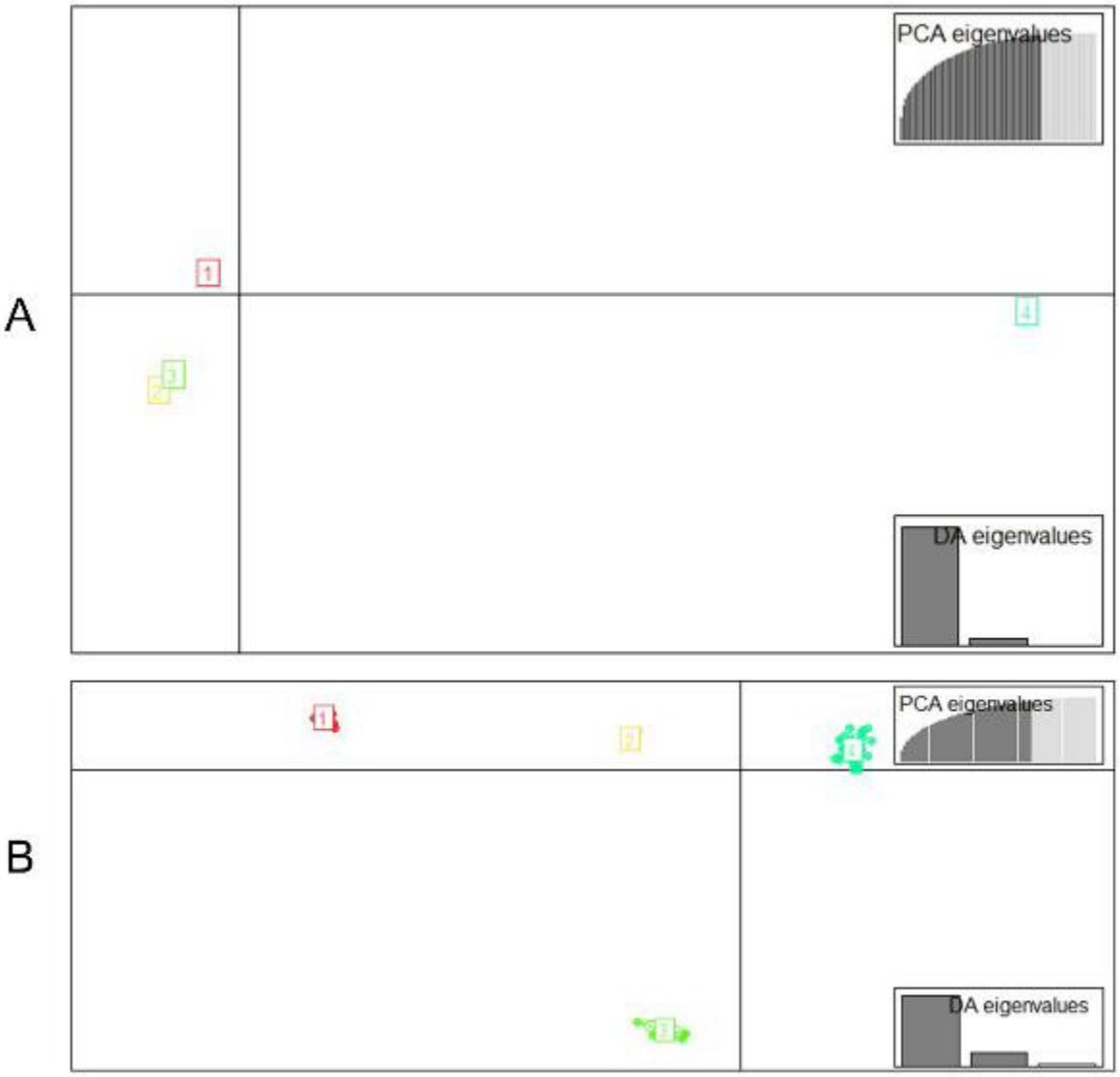


Figure 11.3. Discriminant Analysis of Principal Components (DAPC) using the SNP and silico data sets. A = SNP data set, B = silico data set.

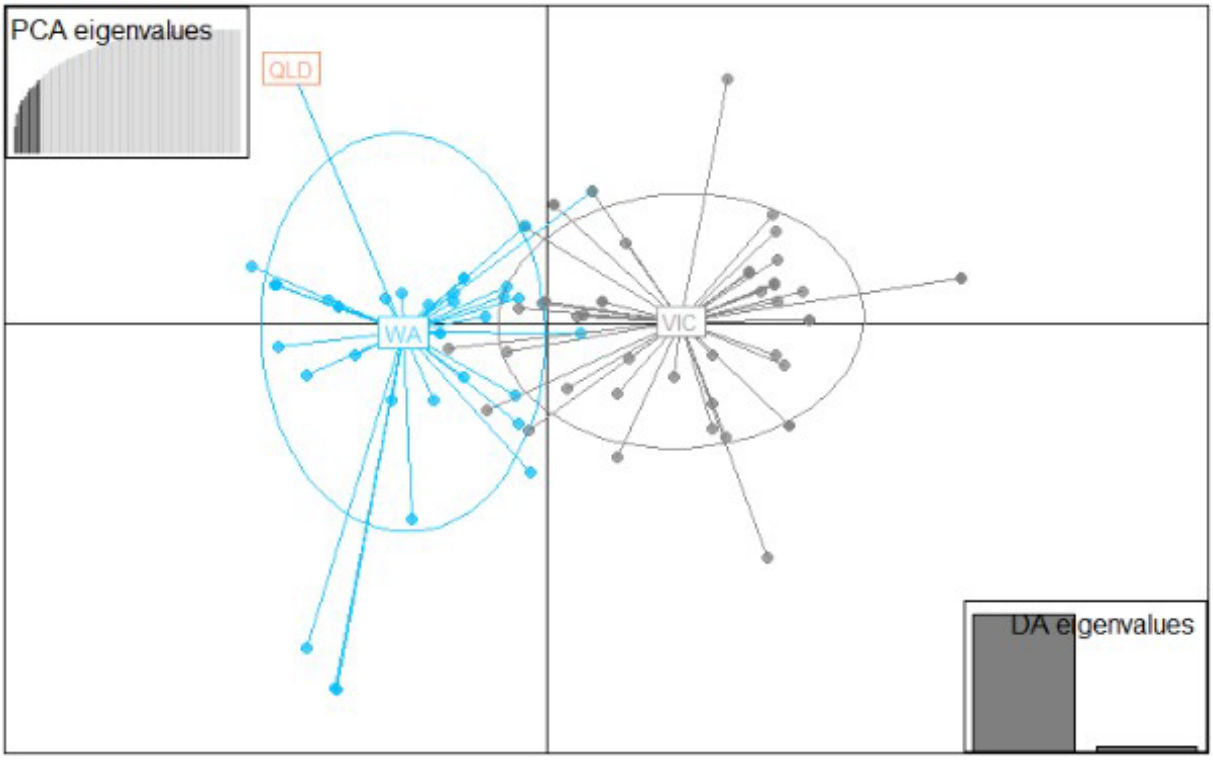


Figure 11.4. Discriminant Analysis of Principal Components (DAPC) using the SNP data set. Population assigned

11.4. Discussion

Analysis of DArT sequencing SNP and silico data from a subset of resistant and sensitive *B. cinerea* isolates showed no apparent clustering with respect to resistant or sensitive phenotypes. Major clustering and sub-clustering were present but appears to be driven by an association with the state of sampling origin, with some overlap between states.

A potential reason for the lack of association between fungicide resistance and the phylogenetic data generated by the DArT analysis can be found in the process by which resistance has emerged in the different regions surveyed in this study.

The resistant *B. cinerea* genotypes found are evenly distributed across the different grape growing regions and do not show evidence of clustering in any area. This, which is consistent with the DArT results shown above, is also indicative of an independent emergence of the resistance, or at least of the resistant genotypes used in the DArT analysis.

Resistance emerges independently in geographically distinct regions when selection pressure is similarly even in terms of strength and duration. The genotypic and phylogenetic evidence presented here is also in agreement with a chemical management approach that does not dramatically differ across regions. This is probably due to the limited number of actives available and, to a lesser extent, the management recommendations available to the industry.

A small pool of actives means that at the landscape level, the different combinations by which selection of resistance can occur are quite limited and, because of this, the number of potential resistant genotypes that can originate from this selection process is also small. Since fungicides have been in use for the control of Botrytis bunch rot in Australia for at least two decades already, the selection pressure has been in place long enough to allow for most relevant resistance mutations to accumulate in the background of the *B. cinerea* populations in the surveyed regions.

In addition to this, the airborne nature of this pathogen and the lack of distinct differences in the host and fungicides used, contributes to the homogenisation of population and the quick dilution of any genotypes that could be associated with fungicide resistance in specific grape growing regions.

Future work could involve a more in-depth look at the population structure in a larger subset of resistant and sensitive isolates. Furthermore, DArT sequencing data from additional isolates from other states in Australia (e.g. South Australia, New South Wales) could also be included in future analyses.

12. Outcomes and recommendations

12.1. Sensitivity of *E. necator*, *B. cinerea* and *P. viticola* to selected fungicides

1. Powdery mildew

Generally, *E. necator* isolates have shown reduced sensitivity to the selected fungicides, also resistance for group 13 has been detected. A new system was developed to maintain *E. necator* populations and effectively conduct experiments to obtain consistent results. In addition, new testing methodology was adopted, such as including field rate concentration in the testing regime and using fungal growth to evaluate the population sensitivity to fungicides. Consequently, the 'reduced sensitivity' category was introduced based on the fungal growth. These developments improved research outcomes and produced consistent results.

Group 11, Quinone outside Inhibitors (QoIs) *E. necator* isolates showed reduced sensitivity to the QoI fungicides pyraclostrobin, azoxystrobin and trifloxystrobin, however no phenotype resistance was recorded at field rate of these fungicides. Pyraclostrobin showed the most reduced sensitivity to *E. necator* populations.

Group 3, Demethylation Inhibitors (DMIs) *E. necator* isolates showed lower levels of reduced sensitivity to four DMI fungicides compared to the QoIs; difenoconazole was least effective and showed highest reduced sensitivity. Tetraconazole was the most effective fungicide. No phenotype resistance was recorded at field rate of DMI fungicides.

Group 5, Spiroxamine Most of the *E. necator* isolates were sensitive to spiroxamine and only 17% had reduced sensitivity. No resistance was recorded for this group.

Group 13, proquinazid and quinoxyfen Eight percent of *E. necator* isolates from NSW had high reduced sensitivity to proquinazid and grew at higher concentration of this fungicide and more than half of the tested isolates showed reduced sensitivity. However, 60% of the tested isolates were resistant to quinoxyfen, with the remainder having reduced sensitivity.

Group 7, boscalid and pydiflumetofen Reduced sensitivity was detected in *E. necator* isolates for boscalid and pydiflumetofen, 42 and 30% respectively. No resistance was detected at field rate of these fungicides.

Group 50 pyriofenone There was reduced sensitivity to pyriofenone by 41% of *E. necator* isolates, however no resistance was detected at the field rate of this fungicide.

Group U6 cyflufenamid All isolates tested were sensitive to cyflufenamid and no reduced sensitivity or resistance were detected.

Continued monitoring for *E. necator* resistance is required to detect any shift in resistance status especially where reduced sensitivity was detected. Regions detected with reduced sensitivity should consider rotating chemicals more frequently and avoid fungicides that have shown resistance or reduced sensitivity.

2. Botrytis bunch rot

Resistance frequency for groups 9, 12 and 17 was low at 10.1, 5.9 and 5.9%, respectively. The overall resistance frequency in this report is 16%. New genotypes associated with group 9 and 17 resistances were characterised, these genotypes have not been previously described in grape isolates. These include mutations in *Pos5* (P293S), *Mdl1* (E407K, G408R) and *Erg27* (F412I/V). These new mutations are ideal targets for future development of qPCR assays for *in vitro* or in-field testing. Further resistance monitoring of these three key chemical groups is still required in all wine regions, and especially in regions where frequencies are elevated. Of particular interest is group 12, where larger shifts in sensitivity are possible. The continual use of sound resistance management practices (e.g. a maximum of two sprays per MOA per season) is recommended to maintain low resistance frequencies.

3. Downy mildew

Resistance of *P. viticola* at field rate was detected for QoI fungicides (group 11; 8%) and PA (group 4; 19%), with the majority of resistant populations detected in NSW and Vic. In addition, reduced sensitivity was detected for group 40 (mandipropamid 2% and dimethomorph 23%), group 45 (ametoctradin 39%), group 11 (QoIs 47%) and group 4 (metalaxyl 46%). Therefore, PA and QoI fungicides should be withdrawn from the spray program where resistance to metalaxyl and pyraclostrobin has been detected. It is recommended that monitoring for resistance be continued, especially where reduced sensitivity has been detected.

12.2. Relationships between phenotype, genotype and field efficacy

Powdery and downy mildew

There were strong relationships between phenotyping and genotyping for group 11 (QoIs). Bioassay results matched with the presence of the mutant G143A (responsible for resistance to QoIs) with 92% and 90% agreement in powdery and downy mildew, respectively. Therefore, this mutant will be an ideal candidate for high-throughput and in-field testing.

The relationship between phenotyping and genotyping for group 3 (DMIs) in powdery mildew was weak and unclear as other factors could contribute to the resistance, such as gene copy number or other mutations. The Y136F mutant was detected in most of the isolates (87%) but the phenotyping results showed limited reduction in sensitivity. More research will be necessary before reliable genotyping can be performed for DMI resistance.

Data suggest that G143A and Y136F mutants were fit and stable for at least six months under laboratory and greenhouse conditions. This suggests that withdrawal of QoIs and DMIs from the spray program for six months may not be long enough to reduce mutant populations of *E. necator*.

The mutation linked to SDHI resistance was not detected in any *E. necator* populations, however reduced sensitivity was detected. Similarly, the G1105S mutant, linked to CAA (group 40) resistance, was not detected in any isolates of *P. viticola*, however reduced sensitivity was detected. Therefore, it will be important to continue monitoring for SDHI and CAA resistance in Australian vineyards.

Genotyping for other fungicide groups was not performed, as the mechanisms of resistance are unknown, hence only phenotyping was conducted. Future research should investigate the mechanisms of resistance in other fungicides to improve monitoring of fungicide resistance.

12.3. Fungicide efficacy when exposed to resistant populations

The heterologous yeast expression system (HYES) revealed that there were significant shifts in sensitivity associated with the Y136F mutation to the DMI fungicides myclobutanil and triadimenol These results support the findings that triadimenol is less effective at controlling Y136F isolates than WT isolates. These results have shown that the HYES is a suitable method to test additional *Cyp51* mutations that may be identified in the future.

DMI fungicides were different in their efficacy regardless of the genetic profile (mutant or wild type) of *E. necator.* Mefentrifluconazole and difenconazole were more effective than myclobutanil and triadimenol in controlling powdery mildew in glasshouse conditions. Triadimenol was less effective when the Y136F mutant was present.

The relationship between copy number of the *Cyp51* gene and phenotypic resistance is not clear, however there was a trend indicating that high copy number was associated with resistance to DMI fungicides. Further research is required to investigate this relationship and may lead to a marker for resistance to DMI fungicides.

In general, some of DMI fungicides were less effective when exposed to resistant populations. Therefore, efficacy testing (laboratory and or field) should be performed to evaluate the spray program.

12.4. Effect of sulfur on the efficacy of DMI fungicides on powdery mildew

Mixing sulfur with three demethylation inhibitor (DMI) fungicides (penconazole, myclobutanil and difenoconazole) had no effect on the efficacy of these fungicides in controlling powdery mildew in grapevine under greenhouse conditions. This supports label recommendations for the use of sulfur, and dispels any confusion raised by previous research on mixing sulfur with DMI fungicides.

12.5. Evaluation of spore trapping technologies

B. cinerea and *E. necator* were detected using various spore traps. The rotorod spore trap system has many advantages. It is cost effective and easy to manufacture, portable, practical and causes minimal interference with vineyard maintenance. It is easy to operate by vineyard staff and does not require movement of plant material. Rotorod spore traps can detect low levels of *E. necator* and resistance mutations (G143A and Y136F) before symptoms appear and can facilitate the detection of other pathogens simultaneously. Rotorod spore trap sampling and molecular detection of mutants will be used in future research for resistance monitoring via high-throughput and in-field testing and will include *P. viticola* and *B. cinerea*.

12.6. Development of in-field trapping and detection methodologies

An agar plate spore trapping method was successfully used to detect resistant *B. cinerea*. Future work on this method is needed to reduce agar desiccation and improve agar selectivity. Furthermore, a large-scale field trial could be undertaken to investigate whether this system could provide adequate data to estimate resistance frequencies.

A rapid in-field qPCR detection pipeline was successful in estimating *B. cinerea* group 9 and 17 resistance frequencies in commercial vineyards. This pipeline can now be used as a rapid and cost effective ‘first look’ step to estimate frequencies at vineyards with known control issues. The pipeline can also be used for the monitoring of frequencies where there are no known control issues, or to estimate frequencies within a field trial setting. Additional assays for alternate group 9 and group 17 genotypes could now be developed.

This pipeline used late season expressing *Botrytis* as a source of DNA. Future work could involve testing this system against spore trap samples to test for resistance at other stages of the growing season. The in-field pipeline was also successfully developed and optimised to target the group 11 associated mutation G143A in *E. necator*. Future work could also include testing the combination of this assay and spore trap samples.

12.7. Strategies to manage fungicide resistance

Reduced sensitivity of *E. necator* was recorded for all major groups of fungicides used to control grapevine powdery mildew in Australia and resistance was recorded for group 13. Therefore, resistance management strategies are required to minimise and delay the onset of resistance. These strategies include monitoring (using bioassays and/or genetic analysis), rotating fungicide chemistries, applying chemicals only when necessary, applying multi-site fungicides and other alternatives such as inorganic fungicides (sulfur) and avoiding chemicals that showed resistance in the pathogen populations.

Detecting fungicide resistance of powdery mildew in grapevine is a key element in managing resistance strategies, spore traps and mutant detections using molecular technology is a powerful tool to achieve this. Rotorod spore traps, mini-vacuum cyclone separators, washing infected leaves and cotton bud swabs, were equally successful tools to detect genetic resistance of *E. necator*. However, rotorod spore traps proved to be more sensitive and practical especially in the early stages of infection when there were no visible symptoms to be collected using the other methods.

Downy mildew pathogen populations showed resistance to metalaxyl (group 4) and QoIs (group 11), as well as reduced sensitivity to group 40. Therefore, it is important to avoid using metalaxyl and QoIs in the regions that showed resistance to these chemicals, in addition to continuing monitoring (using bioassays and/or genetic analysis), rotating chemistries and applying farm biosecurity practices, especially in the regions that exhibit resistance, by avoiding shared equipment if possible and practicing hygienic agriculture practices.

This project has indicated that the nation-wide resistance frequency of *B. cinerea* is low (16%), but some heavily sampled regions are showing elevated frequencies (25-35%). Therefore, sound resistance management practices are still required to maintain an overall low resistance frequency status and suppress any areas with elevated frequencies. Additionally, further monitoring of resistance is required to ensure these resistance management practices are still functioning.

The current in-field qPCR pipeline is a rapid and affordable means to estimate frequencies *in situ*. This pipeline can provide grape growers with a ‘snapshot’ of the current resistance frequency at the time of sampling. This pipeline will be a valuable tool for future monitoring of resistance frequency at vineyards, with and without control issues. *Botrytis* sampling for in-field qPCR is currently undertaken around the harvest period, where the results can then be used to adjust spray programs in the following season. Where frequencies are very high in a particular block, subsequent withdrawal of a chemistry may be necessary. This is especially the case for resistance to group 9 and 17, where resistance factors are very high. Combining in-field qPCR with spore trapping technologies could allow estimation of frequency throughout the growing season. Information on resistance frequencies early season prior to chemical applications would be invaluable to adjust chemical programs accordingly during the season. The pipeline could also be used in future field trials that investigate how changes to spray programs can affect development of resistance or changes in frequency once resistance has become established. This field trial information could help improve resistance management techniques and could include information on how long it takes for frequencies to decrease to undetectable levels once a chemistry is removed.

Future research will develop in-field and high-throughput techniques to achieve shorter turnaround and a high level of screening for known resistance-associated mutations.

13. Publications

13.1. Scientific publications

Ismail I, McKay S, Van Den Heuvel S, Borneman A and Sosnowski M (2023) Detection of fungicide resistance-associated mutations sampled by rotorod spore traps for powdery mildew on grapevine in Australia (in preparation)

Ismail I, Taylor AS, Van Den Heuvel S, Borneman A and Sosnowski MR (2023) Sensitivity of *Plasmopara viticola* to different groups of fungicides and the occurrence of G143A mutant in Australian isolates. (in preparation)

Harper LA, Paton S, Hall B, McKay S, Oliver RP and Lopez-Ruiz FJ (2022) Fungicide resistance characterised across seven modes of action in *Botrytis cinerea* isolated from Australian vineyards. *Pest Management Science* 78 (4): 1326-1340.

Harper LA, Derbyshire MC and Lopez-Ruiz FJ (2019) Identification and characterization of *Botrytis medusae*, a novel cryptic species causing grey mould on wine grapes in Australia. *Plant Pathology* 68, 939-953.

13.2. Conference papers

Ismail I, McKay S, Van Den Heuvel S, Borneman A, Hall B and Sosnowski M (2021) Sensitivity of *Plasmopara viticola* (downy mildew) to fungicides and the occurrence of associated mutants in isolates from Australian vineyards. 23rd Australasian Plant Pathology Society Online Conference, 23-26 November 2021, p 141.

Ismail I., McKay, Harper L, Lopez F, Borneman A, van den Heuvel S, Hall B & Mark Sosnowski (2021) The current status of fungicide resistance in powdery and downy mildew in Australian vineyards. Crush 2021, National Wine Centre, Adelaide, SA, 16 June 2021.

McKay SF., Harper L., Borneman A., Herderich M., Lopez-Ruiz F. and Hall B. (2019) Investigating the efficacy of Demethylation Inhibitor fungicides on combinations of *E. necator* isolates containing low or high ratios of wild type (Y136) and mutant (Y136F) *Cyp51*. The 19th International Reinhardsbrunn Symposium, Friedrichroda, Germany. 7-11 April.

Ismail I., McKay SF, Harper L., Borneman A., Herderich M., Lopez F. and Hall B. (2019) Powdery mildew and fungicide resistance: evaluation of in-vivo and in-planta bioassays. Australasian Plant Pathology Society conference, 26-28 November.

Harper, L., Hall, B., McKay SF., Paton, S., Oliver, R. and Lopez F (2019) Fungicide resistance characterised across six chemical classes in a *Botrytis cinerea* population from Australian vineyards. Australasian Plant Pathology Society conference, 26-28 November.

McKay SF., Harper L., Lopez F., Borneman A., Herderich M. and Hall B. (2017) Incidence and severity of QoI and DMI fungicide resistance of *Erysiphe necator* in Australia. The 8th International workshop on grapevine downy and powdery mildew. Corvallis, Oregon, USA. 17-20 July (~45 attendees).

13.3. Industry articles:

Ismail I, McKay S, Van Den Heuvel S, Borneman A and Sosnowski M (2022) Evaluation of spore collection methods for detection and quantification of fungicide resistance in powdery mildew. *Wine and Viticulture Journal* 37(4), 46-52.

Harper L, Lopez-Ruiz F, Paton S and Sosnowski M (2022) Development of an in-field molecular test to detect anilino-pyrimidine fungicide resistance in *Botrytis cinerea. Wine and Viticulture Journal* 37(3) 44-46.

Ismail I, McKay S and Sosnowski M (2022) The effect of sulfur on the efficacy of demethylation inhibitor (DMI) fungicides on powdery mildew. *Wine and Viticulture Journal* 37(3) 40-42.

Ismail I, McKay S, Riley L and Sosnowski M (2021) Using sulfur to protect vines, particularly under cool conditions. Agrochemical Updates, eBulletin, October 2021, The Australian Wine Research Institute [https://www.awri.com.au/information_services/ebulletin/2021/10/13/agrochemical-update-october-2021-using-sulfur-to-protect-vines-particularly-under-cool-conditions/]

Harper L, Lopez F, McKay S, Ismail I, Hall B and Sosnowski M (2021) Frequency and distribution of fungicide resistance in *Botrytis cinerea* collected from Australian vineyards. Wine and Viticulture Journal 36(4), 32-36.

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Ismail I and Mick H (2019) AI for early onset powdery mildew diagnosis coming to your smartphone. Australian and New Zealand Grapegrower and Winemaker 669, 34-35.

Hall B, McKay S, Lopez F and Harper L (2019) Regional differences in fungicide resistance. Australian and New Zealand Grapegrower and Winemaker 660, 18-19.

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McKay S and Taylor A (2018) Highlights from the 8th International Workshop on Grapevine Downy and Powdery Mildew. Wine and Viticulture Journal 33(2), 54-57.

Hall B and McKay S (2017) Fungicide resistance-where to now? Wine and Viticulture Journal, 32(5) 39-40.

13.4. Presentations, interviews

Ismail, I. 2022 Powdery mildew and fungicide resistance in Australian viticulture. Powdery Mildew Workshop (PMW), 14-18 Nov 2022 in Toowoomba, Qld (~20 atendees).

Ismail, I., Harper, L. and Sosnowski, M (2022) Fungicide resistance in Australian viticulture AWITC FR Workshop 26 June 2022 (23 attendees).

Ismail, I., Harper, L. and Sosnowski, M (2022) Fungicide resistance in Australian viticulture. Viticulture Meeting May 2022, Barossa (25 attendees).

Ismail, I. McKay S. and Harper L. (2022) Fungicide resistance in Australian viticulture. AWRI webinar, online. 20 January (102 attendees).

Ismail, I. (2021) Understanding fungicide resistance in Australian viticulture. SARDI Science Research Forum, Adelaide Showgrounds, 11 November (79 attendees).

Ismail, I. and Harper, L. (2021) Understanding fungicide resistance in Australian viticulture: Results update, NSW Spring Vine Health Day Online Workshop, 2 September (~100 attendees).

Ismail, I. (2021) The current status of fungicide resistance in powdery and downy mildew in Australian vineyards. Crush 2021, National Wine Centre, Adelaide, SA, 16 June (~150 attendees).

Ismail, I. (2021) Understanding fungicide resistance in Australian viticulture: Results update, Accolade Wines national viticulture conference, Sparkke at the Whitmore, Adelaide, 1 June (~25 attendees).

Ismail, I. and Harper, L. (2021) Understanding fungicide resistance in Australian viticulture: Results update, AgChem reference group online presentation, 19 May (~12 attendees).

McKay, S. and Ismail I (2021) Fungicide Resistance project overview and spore trap presentation. Syngenta: Disease field day for Viticulturalists, Agronomists and growers, Lenswood, 20 January (>90 attendees).

Ismail, I. (2020) Fungicide resistance in Australian viticulture; capability and potential collaboration. E.E Muir & Sons Hydroponic/Berry Conference: Microsoft Teams, 23 September.

McKay SF (2019) Fungicide resistance in Australian viticulture. AWRI roadshow, Barossa Valley, SA. 8 November (~ 15 attendees).

Hall, B. (2019) Fungicide resistance in Australian viticulture. Vitilink symposium, Clare. August (~20 attendees)

Ismail I (2019) Fungicide resistance in Australian viticulture. Vine to Wine, Agronomist and viticulturist symposium. National Wine Centre, Adelaide. 13 August (~30 attendees).

Hall, B. (2019) Fungicide resistance in Australian viticulture. Grower Supplies, Burton, SA. 18 June, (~ 15 attendees).

McKay SF and Hall B. (2018) Fungicide resistance: powdery mildew and downy mildew. BASF symposium, Adelaide. January, (~ 20 attendees).

Hall B. (2018) Fungicide resistance: powdery, downy and *Botrytis*. Nufarm symposium, Adelaide, May, (~20 attendees).

Hall, B. (2018) Fungicide resistance: powdery, downy and *Botrytis*. EE Muirs symposium, Yarra Valley, Victoria. August, (~30 attendees).

McKay SF. (2018) Fungicide resistance in Australian viticulture: focus on powdery mildew. Slides provided for a workshop on powdery mildews, University of Queensland, Toowoomba, Qld.

Hall B. and McKay SF. (2018) Slides provided for a Nufarm research update in Western Australia, conducted by Scott Paton, SAR 1701-1.2 Industry Partner from Nufarm. Western Australia. August.

Hall, B. (2018) What drives an epidemic or 'the one that got away'. Australian Society of Viticulture and Oenology seminar series. July.

Hall, B. (2018) Powdery mildew. AWRI roadshow. Orange, Murrumbateman, Tumbarumba, NSW. September.

Hall, B. (2018) Be alert: is fungicide resistance coming to your vineyard? AWRI road show. NSW. September.

Harper, L. (2018) Investigating fungicide resistance in grapevines. Riverina regional program workshops. NSW.

McKay, SF (2017) Be alert: Is fungicide resistance coming to your vineyard? AWRI Roadshow, Langhorne Creek. 17 May, (~20 attendees).

McKay SF. (2017) Fungicide resistance monitoring strategies. Fungicide Resistance Workshop, Arkaba Hotel, Adelaide, (~ 100 attendees).

McKay SF. (2017) What is the current status of fungicide resistance in viticulture? Crush-the grape and wine symposium, Plant Research Centre, Urrbrae. 13-14 November, (149 attendees).

McKay SF (2017) Fungicide resistance of grapevine powdery mildew: What is the current status? SARDI seminar series, Adelaide, 16 November, (~30 attendees).

McKay SF (2017) Powdery mildew 101: Biology, triggers, management. Orange, NSW (~ 25 attendees), Canberra, ACT (~ 20 attendees), Tumbarumba, NSW (~20 attendees) and Mudgee, NSW (~ 25 attendees).

Hall B. (2017) Fungicide Resistance in powdery and downy mildew. Tasmania, 3 workshops, 4-6 November.

Hall, B. (2017) Vititek symposium, Murray Valley Winegrowers Forum, Riverland. August (~20 attendees).

Hall, B. (2017) Three workshops: 1. Disease management 2. Grape Diseases 1014 and 3. Fungicide Resistance. Casella wines group. August.

McKay SF. (2017) Elders group. Fungicide resistance. Mildura, 22 August (~30 attendees).

14. STAFF

Project leader/supervisor: Mark Sosnowski (SARDI; formerly Barbara Hall)

Project supervisors: Suzanne McKay (SARDI), Fran Lopez (Curtin Uni), Markus Herderich (AWRI)

Principal investigators: Ismail Ismail (SARDI), Lincoln Harper (Curtin Uni), Anthony Borneman (AWRI)

SARDI technical assistance: Deborah Blackman and Anna Child

AWRI technical assistance: Steven van den Heuvel

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Appendix 1. Sampling protocol developed for fungicide resistance testing

Fungicide resistance sampling protocol. 2019/21

Before sending samples to the laboratory, please notify the contact person by phone or email. They will be able to answer any queries about sample handling for their particular disease.

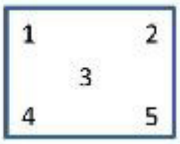
Contact laboratories can provide sample kits if required.

For all three diseases:

1. Select 5 separate sites within the vineyard block (area to be sampled).

Eg





2. Collect the samples as below (also see attached sampling procedure guide).
3. Keep samples cool and out of direct sunlight.
4. Place the 5 site bags from the block into a bag or box with the paperwork in a separate envelope or bag.
5. Send to the laboratory preferably by express post. Do not send samples on a Friday. Only use a courier if there is guaranteed service of less than 48 hours.

Downy Mildew

- At each of the 5 sites within a block, select 2-3 leaves with active downy mildew infection (does not need to be sporulating).
- Place the leaves from each sample site in a paper bag or wrap in dry paper towel, seal in a labelled press seal plastic bag.

Powdery Mildew

- At each of the 5 sites within the block, select 2-3 leaves or bunches with sporulating powdery mildew
- Place the leaves or bunches from each sample site in a paper bag or wrap in dry paper towel, seal in a labelled press seal plastic bag.

For Powdery and Downy

Contact: Suzanne McKay, 08 8429 2215, 0455 236 046, Suzanne.mckay@sa.gov.au
Ismail Ismail, 08 8429 3366, Ismail.ismail@sa.gov.au

Postal and courier address:
SARDI, Plant Research Centre, Gate 2B, Hartley Grove, Urrbrae, SA 5064

Botrytis

Bunches with sporulating *Botrytis*:

- At each of the 5 sites within the block, select 1 bunch with sporulating *Botrytis*.
- Rub the end of the sterile swab into the spores (***avoid getting juice on swab***), replace into the sterile container and label with the block, and site number. Use one swab per bunch.

Bunches with no sporulation:

- If the bunches have obvious *Botrytis* but no sporulation, they can be sent in a cool box to the laboratory for testing. Each bunch should be placed in a separate paper bag, and the 5 bags from each site placed in a labelled press seal plastic bag. ***Take care not to squash the berries.***
- Bunches can be collected at flowering in the same manner.

Leaves with *Botrytis*:

- At each of the 5 sites within a block, select 5-10 leaves with infection. Place the leaves from each sample site in a paper bag or wrap in dry paper towel, seal in a labelled press seal plastic bag.

Contact: Fran Lopez, 08 9266 3061/ 0468 766 584

Postal address: Curtin University, School of MLS, CCDM, Kent Street, Building 303, Room 189, Bentley, Western Australia 6102

Courier address: Curtin University, School of MLS, CCDM, Kent Street, Building 311, Bentley, Western Australia 6102

Some samples e.g. bunches near veraison, may require cooled transport which can be arranged using LabCabs.

Please contact Suzanne McKay, SARDI, 08 8429 2215, 0455 236 046 or Suzanne.mckay@sa.gov.au to arrange this service

LabCabs
Bookings Direct: 1300 522 222
F:1300 522 333 or +61 7 3890 7011
E: **data@labcabs.com.au**