Understanding fungicide resistance in powdery mildew, downy mildew and botrytis



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **SAR 1204**

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Research Organisation: **SARDI**

Date: **January 2017**

Project Number: SAR 1204

Project Title: **Understanding fungicide resistance in powdery mildew, downy mildew and botrytis**

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1. ABSTRACT

Investigations over the last four years into the incidence of fungicide resistance in powdery mildew, botrytis bunch rot and downy mildew have shown that with some fungicides resistance is widespread (e.g. QoI* in powdery mildew), whereas others are less common (e.g. DMI# in powdery mildew) or occur rarely (e.g. mandipropamid in downy mildew). Some populations of *Botrytis* are now resistant to more than one fungicide group. Further research is vital to relate the phenotypic and genotypic laboratory results to field performance. For effective resistance management it is critical that growers adhere to the recommended strategies and minimise exposure to at-risk fungicides.

* QoI – Quinone outside inhibitor
\# DMI – Demethylation inhibitor

2. EXECUTIVE SUMMARY

Botrytis bunch rot, powdery mildew and downy mildew caused by *Botrytis cinerea*, *Erysiphe necator* and *Plasmopara viticola* respectively, are the top three diseases in Australian vineyards, with high economic impact from management costs and reduced income. Fungicide resistant populations add to the cost due to reduced fungicide efficacy and the failure of spray programs to manage disease.

This project utilised a network of collaborators in research organisations, agrochemical companies and the viticulture industry to collect samples of all three diseases from vineyards in the main viticultural regions of Australia. The samples were tested against a range of commonly used fungicides to determine the incidence and severity of fungicide resistance in Australian vineyards. The results of the testing so far have confirmed the presence of resistant populations of these three pathogens to many fungicides throughout Australia.

Leaf disc assays were used to phenotypically test isolates of *E. necator* for sensitivity to pyraclostrobin, penconazole, myclobutanil and tetraconazole, and *P. viticola* to metalaxyl M, mandipropamid and pyraclostrobin. *Botrytis cinerea* was tested by mycelial growth assay for sensitivity to fenhexamid, iprodione, pyrimethanil and boscalid. Representative samples were genotyped for the presence of known mutations conferring resistance.

Results showed that QoI resistance in powdery mildew was found in most viticultural regions. While phenotypic resistance of *E. necator* to the DMIs was not widespread, the Y136F allele that confers resistance to DMIs was present in over 60% of the isolates. Populations of *P. viticola* resistant to metalaxyl have been confirmed in all states except South Australia, and the allele conferring resistance to QoIs has been detected. *B. cinerea* resistant populations to fenhexamid, iprodione, boscalid and pyrimethanil were detected. While one site had populations resistant to all four fungicides, 54% of sites had no resistance detected and the majority were resistant to only one fungicide. This suggests that a field management approach involving fungicides with different modes of action can contribute to the mitigation of resistance.

There have been significant advances in our understanding of the fungicide sensitivity and resistance mechanisms of these three pathogens to the key fungicide groups. The repertoire of well

described and novel mutations identified in this study can be used for the future development of in-field molecular high-throughput screening methods. In addition, the identification of novel mutations and a multi-drug resistance genotype will improve our ability to investigate resistance development under current fungicide management practices.

In conjunction with these studies, Dr Frank van den Bosch, Principal Investigator at Rothamsted Research, UK, developed two models to assist with fungicide choice in viticulture. The first analysed the risk resistance development in *B. cinerea*, *E. necator* and *P. viticola.* This gives an overview of what is known about the management of fungicide resistance, applies it to vineyards in Australia, and provides a decision tree (adapted from Walker et al. 2013) that can be used to determine the appropriate action. The second was a model for the economics of fungicide application programs when resistance is developing, to provide a mechanism to calculate the cost and benefit of fungicide treatment programs. Further development of the models in conjunction with appropriate field experimentation will make it possible to apply the analysis to actual fungicide application programs for multiple diseases threatening crop yield.

The results of this work have highlighted significant gaps in our knowledge, with the main area of uncertainly being the correlation between the laboratory testing results and field performance of a fungicide. Areas that required further research to improve understanding of the resistance mechanisms include the competitive ability of resistant isolates and fitness costs associated with mutations and how fungicide applications affect resistance development and the frequency of resistant isolates in the field. In addition, the importance of the role multi-drug resistance plays in the Australian pathogen populations needs to be elucidated; research into the management of multi-drug resistance is urgently needed because of its potential importance in the development of effective resistance management methods.

Techniques have been developed during this project such as *in planta* and *in vitro* assays that will enable more detailed research into these areas. These will be assisted by the utilisation of next generation sequencing techniques and the development of high throughput assays. With this further research we will be able to fill significant knowledge gaps that will be utilised not only in Australia but internationally.

This project was undertaken by a consortium of researchers from SARDI, Curtin University, AWRI, Charles Sturt University and the Western Australian Department of Agriculture. Funding from Wine Australia is gratefully acknowledged, as are the in-kind contributions from the research organisations and the industry partners. These have been acknowledged in any presentation with a collection of logos provided for all presenters to include.

The researchers sincerely thank all the growers, agronomists, company technical staff and viticultural consultants who have provided samples.

The assistance of industry partners had been invaluable in not only providing samples, but also technical advice and assisting with dissemination the information. Utilisation of these networks has resulted in over 24 presentations to growers and industry personnel, 11 industry journal articles and six conference presentations, three of these international. It is critical that this dissemination continues, to ensure that growers and viticulturists are aware of the issues and the work being undertaken to provide them with sustainable resistance management strategies for the at-risk fungicides.

Project team & Industry collaborators



Industry reference group





- Vitisolutions

3. BACKGROUND

Botrytis bunch rot, powdery mildew and downy mildew are the top three diseases in Australian vineyards, with high economic impact from management costs and reduced yield and therefore income. Fungicide resistant populations of the causal organisms of these three diseases add to operating costs due to reduced fungicide efficacy and failure of spray programs to manage the diseases. While the industry is advised to follow the recommended anti-resistance strategies, there is confusion over which compounds are best to use as mixtures or in alternation strategies. A considerable number of cases of fungicide resistance in all three diseases have been reported within the grape industry, with the most recent being resistance of *E. necator* populations to the QoI group of fungicides. As this group of fungicides is also used to control downy mildew, it is vital to determine if resistance also occurs in populations of *P. viticola*.

It is important to establish the geographical distribution and severity of fungicide resistance to enable adaptation of spray programs to maintain optimal efficacy. The viticulture industry needs access to tests for rapid and accurate detection of fungicide resistance; such tests are currently not available for routine testing at a population level due to time- and resource-demands of conventional genotyping and phenotyping. This is complicated by the fact that the genetic basis of resistance is not known for all fungicides.

This project aimed to: 1) determine the geographical distribution and the severity of resistance of populations of *E. necator*, *P. viticola* and *B. cinerea* to at-risk fungicides within Australia; 2) validate high throughput (HT) genotypic testing using established markers and next generation sequencing techniques to develop rapid and accurate tests to detect resistance in populations; 3) develop and evaluate effective and sustainable resistance management strategies for the at-risk fungicides and 4) disseminate the information to industry through existing networks. This project utilised a network of collaborators in research organisations, agrochemical companies and industry agronomists. It is vital for the future of the Australian wine sector that the extent of fungicide resistance is elucidated, so that grapegrowers can continue to utilise the at-risk fungicides to maintain crop health and grape quality. Development and evaluation of sustainable and environmentally safe disease management practices will assist in reducing the impact of disease from pathogens that have developed resistance to registered fungicides. Maintaining effective use of these fungicides into the future will contribute significantly to the objective of reducing the economic and environmental impact of disease in Australian viticulture.

4. PROJECT AIMS AND PERFORMANCE TARGETS

These aims and targets were specified in the SAR 1204 project contract and amendments.

Identify and quantify resistant populations in Australia

- Through established collaborators, sample vineyards with reported loss of control and organic vineyards for comparison and collect spray records
- Develop effective sampling techniques, pathogen maintenance and storage techniques
- Optimise standardised phenotypic testing methods for each fungicide/pathogen combination
- Validate genotype testing of known resistance alleles
- Workshop to share optimised phenotyping techniques and pathogen storage
- Test samples to determine levels of resistant populations
- Develop next generation sequencing rapid tests and validate to provide a quantitative measure of the proportion of each sample population that contain resistant alleles and optimise sampling strategies

Develop and validate spray programs

- Model existing and project developed data to determine potential fungicide strategies
- Develop field efficacy levels of single actives
- Evaluate variable efficacy of fungicides through phenotypic studies
- Evaluate spray programs

Industry reference group

- Convene an industry reference group from Viticulture and Agrochemical collaborators including CropLife and FRAC to oversee project outcomes

Extension

- Provide annual updates to industry on testing outcomes through industry journals and seminars, targeting areas with highly resistant populations
- Extend management strategies though existing networks, including industry and scientific journals, Dog book, seminars and roadshows

Project Objectives

1. Determine the geographical distribution and the severity of resistant populations of *E. necator*, *P. viticola* and *B. cinerea* to a range of key at-risk fungicides within Australia, utilising an industry network through established contacts to collect samples from vineyards in the main viticultural regions and assess for resistant populations.
2. Refine and develop robust phenotyping methods that can be used to accurately determine pathogenic fungicide resistance.
3. Develop genotyping methods based on known resistance alleles using established markers and techniques currently available and recognised by the Fungicide Resistance Action Committee (FRAC).

4. Develop rapid and accurate tests for detecting and quantifying resistant populations via high throughput next generation sequencing techniques. Evaluate the technique against established phenotyping and genotyping methods. Availability of rapid and accurate genotyping tests will ensure that growers are advised of the detection and distribution of resistance on their properties with sufficient time to amend spray programs and achieve effective disease control.
5. Develop and validate effective and sustainable resistance management strategies for the at-risk fungicides, through modelling and testing of new actives and the effect of dose, mixtures and alternations of active ingredients on resistance management. Existing data and data from this project will be incorporated into models to determine the most effective combinations of products to evaluate.
6. Disseminate the information to industry through existing networks such as AWRI roadshows, Dog book, industry and scientific journal articles.

Planned Project Outputs

Outputs and Activities 2012–13

Year 1	Output	Target Date	Activities
a	Industry reference group formed and project meeting schedule developed for the life of the project	28/02/2013	Canvass and secure members for a project industry reference group Hold an initial meeting with the industry reference group and project research team
		30/06/2013	Develop and execute co-funding arrangements with consortium research organisations
b	Field samples collected for PM, DM, BOT from commercial vineyards in SA, WA and NSW for phenotyping and genotyping testing (Y1)	30/06/2013	Identify and confirm field sampling collaborators. Develop and define consistent field sampling strategy. Collect at least 20 field samples for each pathogen (Powdery Mildew - PM, Botrytis - BOT)

Outputs and Activities 2013–14

Year 2	Output	Target Date	Activities
a	Field samples collected for PM, DM, BOT from commercial vineyards in SA, WA and NSW for phenotyping and genotyping testing (Y1)	30/9/2013	Identify reliable pathogen storage methods for Powdery mildew and Botrytis
b	Standard Operating Procedures (SOP) developed for phenotypic testing for PM, DM and BOT and shared with research partners.	30/09/2013	Test and refine existing phenotypic testing for BOT resistance and develop SOP Test and refine existing phenotypic testing for PM resistance and develop SOP Undertake research team meeting to share

			findings and SOP techniques
c	Genotypic testing of known alleles tested and validated as a method to identify fungicide resistant pathogens	30/09/2013	Extract DNA from PM, DM and BOT samples for genotyping Undertake PCR testing for key fungicide/pathogen combinations Test samples for PM and BOT fungicide resistance Cross-validate genotypic findings with phenotypic tests for Botrytis.
	Preliminary project findings disseminated to industry in at least one published industry journal article	30/09/2013	Hold an industry reference group meeting to review and discuss project findings and outcomes Compile and submit at least one article for industry on preliminary pathogen resistance findings
d	Field samples collected for PM, DM, BOT from commercial vineyards in SA, WA, Vic, Tas and NSW for phenotyping and genotyping testing (Y2) Resistant populations of PM, and BOT identified for specific winegrowing regions	31/05/2014	Collect at least 20 field samples for each pathogen (PM, DM and BOT).Undertake phenotypic and genotypic tests

Outputs and Activities 2014–15

Year 3	Output	Target Date	Activities
a	Progress report, including a review of progress and recommendations for change accepted by AGWA and changes executed	30/09/2014	Hold a project research team and Industry reference group meeting to review and discuss project findings and outcomes Undertake a project review meeting with AGWA to discuss future direction of project
b	Genotypic testing of known alleles tested and validated as a method to identify fungicide resistant pathogens New knowledge on pathogen fungicide resistance disseminated to industry through at least one industry article published	31/12/2014	Cross validate genotypic findings with phenotypic findings for PM Compile and submit at least one article for industry on updated pathogen resistance findings

c	High throughput (HT) technology tested and validated as a method to identify large samples of fungicide resistant pathogens. Resistant populations of PM quantified for specific winegrowing regions	31/12/2014	Cross-validate HT method for individual samples of BOT and PM against DNA of known phenotype and genotype to ensure consistency of results between labs. Extract DNA from PM samples for genotyping Perform high throughput (HT) testing to identify resistant pathogenic strains Establish HT genotyping of pooled samples Apply next generation sequencing to determine resistance in PM populations
d	Field samples collected for PM, DM, BOT from commercial vineyards in SA, WA, Vic, Tas and NSW for resistance testing (Y3) Resistant populations of PM, DM and BOT identified for specific winegrowing regions	31/05/2015	Collect field samples for each pathogen (PM, DM, and BOT) Undertake genotypic and phenotypic testing as required and cross validate
	New knowledge on pathogen fungicide resistance disseminated to industry through at least one industry article published	31/05/2015	Compile and submit at least one article for industry on updated pathogen resistance findings Hold an industry reference group meeting to review and discuss project findings and outcomes
e	Genotypic testing of known alleles tested and validated as a method to identify fungicide resistant pathogens	30/06/2015	Collect samples of DM Test and refine existing phenotypic testing for DM resistance and develop SOP Extract DNA from DM samples for genotyping. Undertake PCR testing for key fungicide/pathogen combinations Cross validate genotypic findings with phenotypic findings for DM
	Co-funding agreement with agrochemical companies for field trials	30/06/2015	Develop and execute co-funding arrangements with agrochemical companies and industry partners
f	Preliminary data available on best practice spray and resistant management strategy using a robust model	30/06/2015	Obtain real spray program data for at least 10 winegrowing regions Incorporate spray data and information from international literature into a resistant strategy model Undertake controlled glasshouse trials to test efficacy of single actives, applied at registered dose rates to understand how rates influence fungicide resistance development of PM, DM and BOT. Using the model data, identify potential fungicide mixtures and develop potential spray programs

Outputs and Activities 2015–16

Year 4	Output	Target Date	Activities
a	Resistant populations of PM, DM and BOT quantified for specific winegrowing regions using high throughput (HT) next generation sequencing approaches	31/12/2015	Extract DNA from PM, DM and BOT samples for genotyping Perform high throughput (HT) testing to identify resistant pathogenic strains Cross-validate HT method for individual samples against DNA of known phenotype and genotype Enhance HT method to include additional alleles and/or population markers
b	Field samples of DM collected from commercial vineyards for phenotyping and genotyping	30/6/2016	Collect new field samples and retest stored samples for phenotypic resistance. Genotype selected samples for cross validation
c	Knowledge on changes in resistant populations of *Botrytis* due to fungicide pressure	30/6/2016	Inoculate vines with botrytis genotypes and apply different fungicides. Evaluate population changes under fungicide pressure by testing the genotypes present
d	Sample strategy to detect resistant alleles (PM and BOT)	30/6/2016	Determine the minimum number of samples required to accurately detect resistant alleles in a field population (PM and BOT)

Outputs and Activities 2016–17

Year 4	Output	Target Date	Activities
a	Validated, robust phenotypic and genotypic tests for assessing pathogen resistance to fungicides available to industry for commercialisation	30/12/2016	Determine the proportion of resistant alleles related to phenotypic resistance tested on shootlets in greenhouse. Heterologous yeast expression system (HYES) developed for PM to explore how presence of mutant alleles affects sensitivity of PM to different DMIs
b	Updated resistance management strategies endorsed and submitted for the new edition of the AWRI Dog book	30/12/2016	Field trials of known resistance to test efficacy and validate at least three alternate spray fungicide resistant management programs developed by the model Develop and prepare updated industry resistant management guidelines and submit to the AWRI Dog book review group for endorsement and inclusion in new edition
c		30/6/2016	Hold a project research team and Industry reference group meeting to review and discuss project findings and outcomes Compile and submit at least one industry article and one peer-review paper to convey project findings Organise state workshops in at least three

			locations around Australia (SA, NSW and WA) to present findings.

5. METHOD

The project was a collaboration between five research organisations, each with specific areas of research activity:

SARDI:

- Management of sample collection in SA, Victoria and Tasmania
- Phenotypic testing of powdery mildew
- Phenotypic testing of downy mildew as required
- Cross validation of phenotypic and genotypic results for powdery mildew
- Preliminary investigations into whole block sampling strategies
- Preliminary investigations of proportion of resistant alleles related to resistance in powdery mildew

Curtin University:

- Phenotypic testing of *Botrytis*
- Genotypic testing of *Botrytis*, powdery mildew and downy mildew
- Cross validation of phenotypic and genotypic results for *Botrytis*
- In collaboration with Dr Frank van den Bosch (Rothamsted, UK) develop models for analysis of risk resistance development and economics of fungicide application programs when resistance is developing
- Greenhouse and laboratory trials for efficacy of single actives
- Development of heterologous yeast expression system for powdery mildew
- Preliminary investigation of proportion of resistant alleles related to resistance in *Botrytis*

CSU:

- Management of sample collection in NSW and QLD
- Phenotypic testing of downy mildew
- Cross validation of phenotypic and genotypic results for downy mildew

AWRI:

- High throughput and next generation sequencing of powdery, downy and *Botrytis* samples

DAFWA:

- Management of sample collection in WA

All members of the project team were responsible for providing scientific input into the research and disseminating information through articles, journal papers, presentations and workshops.

5.1. Identify and quantify resistant populations in Australia

5.1.1. Vineyard sampling

Samples were collected by the research team and through collaborating agencies, viticulturists, agrochemical companies and growers.

Initially sites were targeted with known loss of control and organic vineyards for putative sensitive samples. In the third season, viticultural regions with no or limited samples previously tested were targeted.

Spray records for up to five years were requested from growers.

5.1.2. Sampling methods

The sampling and collection methods were developed at the first project meeting in February 2013. They were refined following each season to ensure that the most effective method was used to achieve a sample in the laboratory suitable for testing.

5.1.3. Pathogen establishment, maintenance and storage techniques

Techniques to maintain and store the pathogens used in current literature, or provided by other scientists working in the area, were evaluated.

5.1.3.1. Powdery mildew (*Erysiphe necator*)

Isolate establishment and maintenance

To successfully grow and maintain *E. necator* on detached grapevine tissue, green leaf material that will remain healthy for at least two weeks is required. Leaves of three *Vitis vinifera* varieties, Cabernet sauvignon, Chardonnay and Sultana were tested to determine which would survive best when detached from the plant.

Leaves were surface sterilised in bleach for 3 mins, then washed in sterile distilled water. Excess moisture was removed by blotting with sterile paper towel then leaves were placed adaxial surface facing upwards onto tap water agar plates (Evans et al. 1996) amended with pimaricin.

Methods to transfer active powdery mildew spores from the infected field samples to the sterile leaf included (i) removing spores with a single hair of a sable paint brush or an acupuncture needle under a binocular microscope (ii) placing a sterile cotton bud into the mildew on the sample and then touching onto the fresh leaf and (iii) directly touching infected material to a fresh leaf.

Storage method #1 (from Stummer et al. 1999)

Conidia were harvested using a cyclone separator into 1.5 mL Eppendorf tubes. The collected conidia were dried by leaving tubes open inside a laminar flow cabinet overnight for approximately 18 hrs. The tubes were sealed and snap frozen in liquid nitrogen and stored at -80^{o}C.

Storage method #2 (adapted from method used by James Brown, John Innes Centre UK for storage of *Blumeria graminis*, barley powdery mildew)

Conidia were harvested using a cyclone separator into 500 µL tubes with no lid contained in 1.5 mL Eppendorf tubes. Two to three sterile silica beads were added to the 1.5 mL tubes, the lids closed and sealed with Parafilm. After approximately 10 hours at room temperature the tubes were stored at -80^{o}C.

Storage method # 3 (from Stummer et al. 1999)

Infected leaves were cut leaves into strips (~5 x 15 mm) and placed in sterile 1.5 mL Eppendorf tubes. The conidia on leaves were dried by leaving tubes open inside a laminar flow cabinet overnight for approximately 18 hrs. The tubes were sealed and snap frozen in liquid nitrogen and stored at -80^{o}C.

5.1.3.2. Downy mildew (*Plasmopara viticola*)

Isolate maintenance

A leaf disc culture method was adapted for establishment and maintenance of downy mildew isolates (Wong and Wilcox 2000). *Vitis vinifera* varieties Sultana and Cabernet sauvignon were evaluated for their ability to survive and become infected following inoculation. Leaves were surface sterilised in 0.5% sodium hypochlorite and 0.1% Tween20 for 90 secs and rinsed three times, for 2 min each time, in sterile distilled water. The leaves were blotted dry with sterile paper towel and 9 mm discs were cut from each leaf. The leaf discs were placed with the abaxial side facing upwards onto water agar plates.

A number of different techniques were trialled to transfer the *P. viticola* sporangia from the infected field samples to the sterile leaf discs including; (i) rubbing the diseased sample directly onto the leaf discs, (ii) using an artist brush to collect the sporangia and transfer them to the leaf discs and (iii) preparing a spore suspension to transfer to the leaf disc.

Storage method #1

Field leaf samples and pure subcultured samples on leaves were frozen at -20^{o}C. Leaves were thawed prior to use.

Storage method #2

Air-dried pure subcultured leaf samples were maintained at room temperature in paper bags prior to being wetted with water and the *P. viticola* spores collected for experimentation.

Storage method #3

P. viticola spores were collected from infected field leaf samples in sterile distilled water in 1.5 mL Eppendorf tubes, adjusted to 1 x 10^{6} spores/mL and stored at -20^{o}C prior to being used in phenotypic testing assays.

Storage method #4

Pure subcultured leaf samples were freeze-dried in 1.5 mL Eppendorf tubes and stored at room temperature prior to use for further experimentation such as DNA extraction for genotyping studies.

5.1.3.3. Botrytis (*Botrytis cinerea*)

Isolate maintenance

Isolates were stored as mycelial plugs at 4^{o}C and as spore suspensions at -80^{o}C and removed and utilised as required.

Storage method #1 Hyphae

Isolates were inoculated onto Botrytis Media Agar (BMA) and incubated in the dark at room temperature. Once sufficient growth was reached (3-4 days), agar plugs containing hyphae were removed and stored in distilled water at 4^{o}C.

Storage method #2 Sclerotia

Isolates were inoculated onto BMA and incubated in the dark at room temperature. Once sufficient growth was reached (3-4 days), fruiting bodies were removed and freeze dried for 45 min stored at 4^{o}C.

Storage method #3 Spores

Isolates were inoculated onto BMA and incubated at 22°C for 7-10 days under near-UV light to induce sporulation. Spores were harvested by flooding the plate with 20 mL sterile Milli-Q water and scraping with glass or plastic rod. The suspension was filtered through glass wool and centrifuged at 3220g for 10 min at 4^{o}C. Following removal of supernatant the pellet was washed in sterile Milli-Q water and again centrifuged at 3220g for 10 min at 4^{o}C. The pellet was resuspended in 20% glycerol and stored at -20 and -80^{o}C.

5.1.4. Phenotypic tests

5.1.4.1. Powdery mildew (*Erysiphe necator*)

The methods for performing the fungicide sensitivity bioassays were adapted from previous work carried out by Erickson and Wilcox (1997) and Savocchia et al. (2004) using a leaf disc assay. All aspects were tested to ensure the most effective method was chosen.

Three media were tested: 1. 1.5% water agar (TWA, Bacto™ agar, BD, USA) amended with pimaricin (2.5 µL/mL, Sigma Aldrich); 2. TWA with one piece of sterile filter paper moistened with 1 mL sterile water on the surface; and 3. Two layers of sterile paper towel covered with two layers of Miracloth (EMD Millipore USA) moistened with 2 mL sterile water. The 1.5% TWA amended with pimaricin (aTWA) provided the most consistent results so was used for all further experiments.

Young, glossy, disease-free leaves, cv. Cabernet sauvignon, were removed from the third and fourth node from the growing tip, surface sterilised in 1.0% sodium hypochlorite solution (White King® bleach) for 3 mins, washed 3-4 times in sterile distilled water (SDW), then blotted dry between two layers of sterile paper towel. Leaf discs were cut from each leaf using a sterile 10 mm diameter cork borer and placed abaxial surface upwards in a Petri dish lined with sterile filter paper containing 5 mL of fungicide at a given concentration. After 30-60 mins, discs were removed and blotted dry between two layers of sterile paper towel. Discs were placed adaxial surface upwards on aTWA in 30 mm petri dishes.

Fungicide sensitivity was tested using commercial formulations of the DMI fungicides, Topas® 100 EC (penconazole 100 g/L ai, Syngenta), Mycloss™ Xtra (myclobutanil 200 g/L ai, Dow AgroSciences) and Domark® (tetraconazole 40 g/L ai, Sipcam) and, the QoI fungicides, Cabrio® (250 g/L ai pyraclostrobin, Nufarm) and Amistar® (azoxystrobin SC 250 g/L ai, Syngenta). Fungicides were freshly prepared immediately prior to each assay and were diluted in sterile type 1 water (SMQ). The following concentrations were used for testing Topas®, Domark®; Cabrio® and

Amistar® 0.001, 0.01, 0.1, 1.0 and 10.0 µg/mL and 0.002, 0.02, 0.2, 1.0, 4.0 and 16.0 µg/mL for Mycloss™ Xtra. Cabrio® at 20 µg/mL was used in preliminary experiments but it caused extensive phytotoxicity so was not used in subsequent experiments. SMQ was used for the controls. For Cabrio® only, isolates established from samples collected in late 2014 and 2015/16 season were tested in a leaf disc assay using discriminatory doses of 0, 0.1 and 1.0 µg/mL, with 10 µg/mL included in some tests.

Four inoculation methods were evaluated: 1. Tapping an infected leaf at the top of a spore settling tower (plastic tube, 270 mm diam × 270 mm high) to dislodge conidia; 2. A single conidium, transferred using a single bristle attached to a sable hair paintbrush; 3. A 10 µL droplet of spore suspension (2×10^5 spores/mL) and 4. Transferring spores from a fresh leaf culture to the leaf disc with a sterile cotton bud. Controls were not inoculated. Method 1 was initially chosen as the most effective, however later testing showed that method 4 was equally effective and less time consuming, so was used in preference.

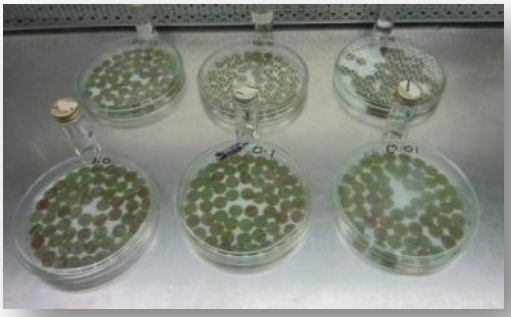
Dishes were incubated at 22 ^{o}C 12/12 hr day/night under fluorescent light. Fourteen days after inoculation, the percentage of each disc covered in sporulating powdery mildew was assessed under a dissection microscope at x 20 magnification. The mean of the six discs infected was calculated and the EC_{50} µg/mL was determined for each isolate by Probit analysis using Genstat (18th edn, VSN International Ltd).

EC_{50} is the concentration of fungicide inhibiting fungal growth by 50%. Resistance factor (RF) is another measure often used to define whether an isolate is resistant or not. It is calculated from the EC_{50} value for each isolate: RF=EC_{50} selected isolate/mean EC_{50} wild type isolates. However RF varies considerably between studies and is dependent on having unexposed isolates which are true wild type. As it is difficult to be sure that we have isolates with no exposure, EC_{50} has been used as the determination for resistance.

The testing methods are illustrated in Figure 1. Further details are in the SARDI report section 9.4.2.



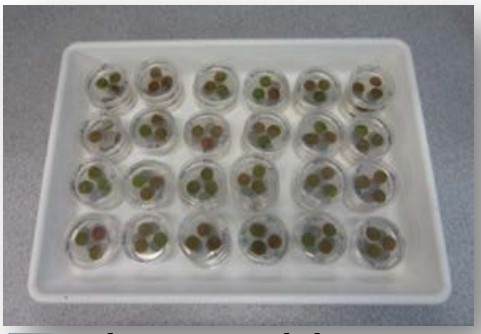
Cut leaf discs



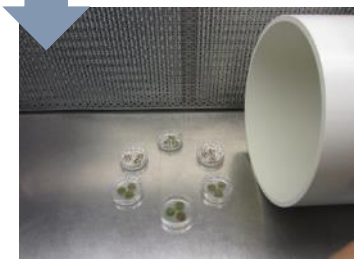
Soak in fungicide

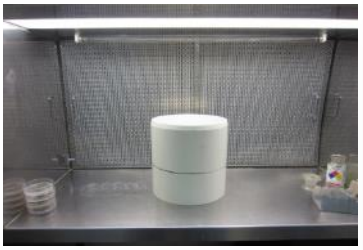


Blot dry



Place on amended agar







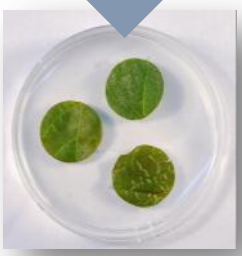
Either inoculate with spores using a settling tower

or:





Transfer spores with a sterile cotton bud





Assess infection and calculate EC_{50} µg/mL

Fig 1. Phenotypic testing method for *Erysiphe necator*

5.1.4.2. Downy mildew (*Plasmopara viticola*)

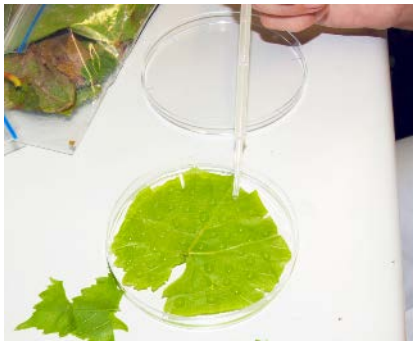
The leaf disc culture method was adapted for phenotypic testing of downy mildew isolates. Disease-free immature, bright, shiny leaves were harvested from the leaves taken from the fourth to sixth node from the distal shoot tip of glasshouse-grown *V. vinifera* cv. Sultana and surface sterilised as described above.

Technical grade pyraclostrobin, mandipropamid and metalaxyl were dissolved in 1 mL of acetone and diluted to the desired concentrations. Pyraclostrobin was diluted to concentrations of 0.1, 0.05, 0.01, 0.005, 0.001, metalaxyl to 10, 1.0, 0.5, 0.1, 0.05 and mandipropamid to 10, 1.0, 0.1, 0.01, 0.001 µg/mL in sterile distilled water immediately prior to each assay.

In the biohazard cabinet, discs were cut from each leaf using a sterile 9 mm diameter cork borer. Two methods were tested for the bioassay (i) floating the leaf discs in fungicide solution or (ii) imbibing the leaf discs in fungicide solution and then placing them onto water agar. The latter method produced the most consistent results so was the chosen method for the all bioassays. The leaf discs were placed into sterile beakers with a given fungicide concentration, agitated for 1 hr (Wong and Wilcox, 2000) and then dried in the biohazard cabinet. The discs were randomised and 10 were placed into each Petri plate containing 1.5% water agar amended with 25 µg/µL pimaricin (Sigma Chemical Co., St Louis) and 50 mg/L streptomycin (Sigma Chemical Co., St Louis) and with the abaxial surface facing upwards. Sterile distilled water was used for the control. Each disc was considered a pseudo-replicate and each plate was considered a replicate with a total of three replicates for each treatment and isolate combination.

Each of the 10 replicate discs was inoculated with a single isolate of *P. viticola* by placing a 10 µL droplet of 1 x 10^6 spore suspension onto each leaf disc. The discs were incubated for 24 hr in the dark and then dried in the biohazard hood until the inoculation droplet was no longer visible before transferring back to the incubator for a further seven days. Disease incidence was recorded by assessing for presence or absence of *P. viticola* relative to the control. The effective doses for 50% control (EC_{50}) were calculated by plotting the percentage of leaf discs that were infected with *P. viticola* against the fungicide concentration. Linear regression was used to determine the EC_{50} concentrations for each isolate (Microsoft Excel 2013).

The testing methods are illustrated in Fig 2. Further details are in the CSU report section 9.4.5.





DM subcultured onto fresh leaves, incubated for 1 wk prior to phenotyping.



Day 1: Leaf discs cut, soaked in fungicide solutions, dried, plated onto water agar. Inoculated with suspension.

Day 3: Discs dried in biohazard hood (2-3 h), transferred to incubator at 23^{o}C, 12 h light/dark.



Day 7: Assess % of infected leaf discs, determine % inhibition and EC_{50}.

Fig 2. Phenotypic testing method for *Plasmopara viticola*

5.1.4.3. Botrytis (*Botrytis cinerea*)

Phenotypic testing of *Botrytis* was undertaken using a 96 well microtitre assay system and a discriminatory dose agar assay.

Fifty-four *Botrytis* isolates were screened against four fungicides using a microtitre assay to establish EC_{50} and the minimum inhibitory concentration values (MIC, the concentration that inhibits growth by 100%) and to define a discriminatory dose for each fungicide. Technical grade fenhexamid, boscalid, pyrimethanil and iprodione were dissolved in absolute ethanol and seven dilutions between 0.01 and 10 µg/mL of each fungicide were evaluated. Re-testing of isolates that exhibited a significant reduction in sensitivity was carried out with at least one or more ranges of increased concentrations of fungicides, up to 150 µg/mL. In in each well 0.5 µL of fungicide stock and 0.5 µL of 10% Tween20 was added to 94 µL of liquid media. Five µL of *B. cinerea* spore suspension (10^5/mL) was added to 95 µL of the media mixture resulting in a final concentration of 5000 spores/mL. Each plate was wrapped in Parafilm to prevent evaporation and incubated. There were two biological replicates each with two technical replicates for each isolate. Immediately following the addition of the spore suspension the optical density (OD) was measured at 450 nm wavelength in a Synergy HT microplate reader (BioTek). After 72 h incubation at room temperature in darkness, or 96 h for pyrimethanil, the OD was again measured. Final OD values were adjusted by subtracting the readings taken immediately following the addition of the spore suspension. The EC_{50} was estimated by linear regression of percentage reduction in OD (compared to zero fungicide control) against the log concentration of the fungicide.

Results from the microtitre screen allowed discriminatory doses to be defined for the four fungicides listed above: 1 µg/mL fenhexamid, 3 µg/mL iprodione, 0.4 µg/mL pyrimethanil and 1 µg/mL boscalid.

The remaining isolates were tested in a mycelial growth assay using discriminatory doses of fungicide active ingredient in amended agar. After three days incubation in the dark, fungal growth was scored as either present (resistant isolate) or absent (sensitive isolate).

Further details are in the Curtin report section 9.4.3.

5.1.5. Genotypic tests

5.1.5.1. Powdery mildew (*Erysiphe necator*)

Fungal DNA was extracted from infected leaf material using a CTAB extraction method (Cubero et al. 1999). The subsequent DNA extractions were used as templates to amplify the complete *cyp51* gene (Délye et al. 1997) and the *cytb* region associated with the G143A mutation (Grasso et al. 2006). Amplification was carried out using high-fidelity Phusion polymerase (New England Biolabs) according to the supplier's protocol. Un-purified amplified DNA was sent to Macrogen Inc. (Korea) for sequencing. *Cyp51* sequences were then aligned to a reference sequences (GenBank no. U72657.2) while *cytb* sequences from sensitive and resistant isolates were aligned to identify any mutations.

Further details are in the SARDI report section 9.4.2., Curtin report section 9.4.4. and AWRI report section 9.4.6.

5.1.5.2. Downy mildew (*Plasmopara viticola*)

Fungal DNA was extracted from leaf tissue infected with downy mildew using a DNeasy® Plant Mini Kit (Qiagen) and the presence and frequency of the G143A mutation was determined using next generation sequencing of a 180 bp amplicon that surrounded the G143A mutation.

Further details are in the Curtin report section 9.4.4. and AWRI report section 9.4.6.

5.1.5.3. Botrytis (*Botrytis cinerea*)

B. cinerea DNA was extracted from fungal cultures using a Qiagen biosprint method (Qiagen), sequenced and aligned to reference sequences to assess for known mutations. Alternatively a cleaved amplified polymorphic sequence (CAPS) method using restriction enzymes Taq I (Oshima et al. 2006) and Sma I was used to genotype a large number of isolates resistant to iprodione.

Further details are in the Curtin report section 9.4.3.

5.1.6. Resistance populations

Isolates of each of the three pathogens from the infected samples were tested using the phenotypic and genotypic methods described above.

For *E. necator*, sensitivity of each fungicide was defined by calculating the EC_{50} value for each isolate and the result correlated with the presence of the respective mutant.

For *P. viticola*, sensitivities were determined by a previously defined discriminatory doses (Wicks *et al*. 2005, Gisi et al. 2007). No mutations in the genome conferring resistance to phenylamides have been mapped and therefore there are no molecular methods for detection of resistance (FRAC, 2014).

For *B. cinerea*, the sensitivity level was first established in a subset of isolates, followed by the molecular characterisation of sensitive and resistant strains.

Further details are in the various reports section 9.4.

5.1.7. Sampling strategies

Next generation sequencing was used to determine a statistically significant estimate of the number of samples needed within a vineyard to adequately detect the presence of the known mutations linked to resistance.

Powdery mildew samples were collected from five vineyards located in Victoria and South Australia as follows; Yarra Valley (Victoria), Cape Jaffa, Langhorne Creek, Lenswood and Urrbrae (South Australia). Samples were obtained by wiping the end of a sterile cotton tipped swab in sporulating colonies of mildew growing on either on leaves or bunches. Up to one hundred swab samples throughout each vineyard were collected at random. Swabs were stored at 4^{o}C until DNA was extracted following the manufacturer's protocol using a DNeasy® Plant Mini Kit (Qiagen). The presence and frequency of the G143A and the Y136F mutations were determined using next generation sequencing. The results were then analysed to determine the minimum number of samples needed within a vineyard to adequately detect the presence of the known mutations linked to QoI and DMI resistance.

Further details are in the SARDI report section 9.4.2. and AWRI report section 9.4.6.

5.1.8. Develop and validate spray programs

5.1.8.1. Model existing and project developed data to determine potential fungicide strategies

Dr Frank van den Bosch is a Principal Investigator at Rothamsted Research (http://www.rothamsted.ac.uk/about), UK. He leads a research group whose work focuses on the development and application of generic models combining population genetics and epidemiology to study the population dynamics and evolutionary ecology of plants and their pathogens, including the effect of a range of resistance management strategies on the evolution of fungicide resistance.

Through his association with Curtin University, Dr van den Bosch agreed to assist with the development of models to assist with fungicide choice in viticulture. The data required to develop this model included in-field fungicide efficacy of the various actives, efficacy of current spray programs and levels of phenotypic resistance to the various actives.

Dr van den Bosch provided the project with two models. The first analysed the risk of resistance development in *B. cinerea*, *Erysiphe necator* and *Plasmopara viticola*. This gives an overview of what is known about the management of fungicide resistance, applies it to the current grapevine situation in Australia, and provides a decision tree (adapted from Walker et al. 2013) that can be used to determine the appropriate action.

The second was a model for the economics of fungicide application programs when resistance is developing, to provide a mechanism to calculate the cost and benefit of fungicide treatment programs. The model calculates the optimal dose of fungicide treatments as well as the optimal mixtures of fungicides. Optimal here is defined as minimal cost due to disease. The cost due to disease is the sum of the costs of the fungicide application program and the costs due to disease induced yield loss incurred under the fungicide treatment program. Grape powdery mildew on grapevine was used as the key example and as a contrast was compared with septoria blotch on wheat to build insight into the factors affecting optimal application programs.

Further details are in the Curtin report section 9.4.3.

5.1.8.2. Develop field efficacy levels of single actives

This work was required to assist with providing data to develop the model discussed previously. Unfortunately due to delays in greenhouse development, and then internal administrative issues with the planned experiments, most of this work was not able to be completed. Industry was not willing to allow the work to be undertaken on research areas in a viticulture region, as higher than recommended rates of the fungicides would be applied and may lead to increased resistance risk in the district.

5.1.8.3. Evaluate variable efficacy of fungicides through phenotypic studies

A detached berry assay was developed for *Botrytis* and the efficacy of iprodione on sensitive and resistance isolates of *Botrytis* was evaluated. Ripe berries (Cabernet sauvignon) were washed in a 5% bleach and 10% ethanol mixture, rinsed in distilled water and air dried. The washed berries were then soaked overnight in iprodione 500SC (Imtrade®) in final concentrations of 0, 100 μg/mL and 500 μg/mL. The berries were then air dried, and placed individually in a 70 mL container alongside damp cotton wool. The berries were wounded eight times with an 18G needle and 10 μL of a spore suspension of either the resistance or sensitive isolate (10^6 spores/mL in 0.03% tween) added to the wound site. The berries were kept in the dark at room temperature and the percentage of diseased berries was recorded at day 7.

Further details are in the Curtin report section 9.4.3.

5.1.8.4. Evaluate spray programs

Due to the delay in developing the model, the field trials of spray programs were not completed. Interest was shown by various agrochemical companies to include products and provide the funding required for these trials, and it is still intended that they be undertaken either in the 2017/18 or 2018/19 seasons.

5.1.9. Additional work

Additional work was proposed to be undertaken in a six month extension of the project.

5.1.9.1. Detect shifts in resistant populations (*Botrytis*)

An *in planta* technique was developed to investigate the fitness and competitive abilities of sensitive and resistant isolates. Vines in the Curtin University field trial area experimental vineyard are managed to replicate commercial vineyard management. Bunches were inoculated at flowering and when the *B. cinerea* started to express, DNA was extracted from whole infected berries using a CTAB extraction method. Sequencing was carried out to investigate the presence of each resistance or sensitive haplotype.

This technique was confirmed using iprodione resistant isolates. Bunches were inoculated at flowering with four ratios (1:1:10, 1:10:1, 10:1:1, 1:1:1) of three isolates: Bc-7 (iprodione sensitive; Os-1 - WT), Bc-143 (iprodione resistant; Os-1 - Q369P + N373S) and Bc-179 (iprodione resistant; Os-1 - I365S). Bunches were also inoculated with isolate Bc-7 only as a positive control. Spores of each isolate were harvested and diluted in water containing 0.5% gelatin to concentrations between 10^6 – 10^7 spores/mL for each isolate. In each ratio for each treatment, 1 equalled 10^6 spores/mL and 10 equalled 10^7 spores/mL.

The remaining actives with resistant isolates can now be tested using the same technique.

Further details are in the Curtin report section 9.4.3.

5.1.9.2. Sample strategy for resistance detection (*Botrytis* and powdery)

Using the minimum number of samples determined in previous work to detect resistant alleles in a field population, 10 samples have been collected from one of the already tested vineyards. Sampling occurred late due to the late development of powdery mildew in the vineyard. Phenotypic testing is being carried out, and once completed genotypic testing of the isolates will be completed to correlate the results. It is still not clear whether *Botrytis* will be able to be collected. Very little was detected at flowering so harvest bunches will be collected for testing.

5.1.9.3. Relationship of mutant allele detection to field resistance (powdery mildew)

Four preliminary trials have been undertaken *in planta* using young grape plants in the greenhouse. Rooted cuttings of cv. C. sauvignon, ~ 20 cm high, were placed in 100 mL conical flasks containing tap water. When 3-4 almost fully expanded leaves had emerged from at least one bud on the cutting, leaves were sprayed until runoff with Cabrio® at various concentrations and air dried. The following day, leaves were inoculated with spore suspensions of various isolate combinations to provide increased levels of the mutant allele from 20 to 100%, based on the % G143A allele frequency as determined by NGS (there was no wild type isolate i.e. 0% mutant allele, viable at the time of these studies). Leaves were air dried and PVC cylinders placed over the plantlets with fine mesh material placed over the top to prevent cross contamination. After two weeks the inoculated leaves were assessed for incidence and severity of powdery mildew.

Two trials were also undertaken with detached leaves in the laboratory using the phenological testing method previously described, inoculating with mixtures of dried spores using the spore settling tower.

Further details are in the SARDI report section 9.4.2.

5.1.9.4. Relationship between resistant populations of the different DMIs (powdery mildew)

A heterologous yeast expression system (HYES) (Groeneveld et al. 2002. Revankar et al. 2004) was developed with *E. necator* which could be used to explore how the presence of the Y136F (the main mutation conferring resistance in DMIs), and other potential mutations, affects the sensitivity of powdery mildew strains to different DMI fungicides.

A construct formed by the ligation of a synthesised wild-type *E. necator Cyp51* gene into the pYES3/CT expression vector was transformed into a yeast (*S. cerevisiae* strain YUG37:*erg11*). The yeast strain containing the *E. necator Cyp51* was phenotyped against five DMIs registered in Australia for grape powdery mildew: myclobutanil, penconazole, tebuconazole, tetraconazole and triadimenol.

Further details are in the Curtin report section 9.4.3.

5.2. Industry reference group

Participants for the industry reference group (consultants, growers and wine company technical staff) were proposed by members of the project team and Wine Australia. Other members of the group, including those from the agrochemical companies and research organisations, were part of the initial project proposal but not as principal investigators.

- Leonard Russell, Watershed Wines (grower, WA)
- Di Davidson, Davidson Viticulture (consultant SA) (withdrew part way through due to time commitments)
- Warren Birchmore, Accolade Wines (SA)
- Suzanne McLoughlin, Treasury Wine Estate (SA)
- David Sanderson, Wine Tasmania (Industry Development Officer - replaced by Paul Smart)
- Liz Riley, Vitibit (consultant, NSW)
- Colin Bell, AHA Viticulture (Consultant, WA)
- David Braybrook, Research Solutions (Consultant, Victoria)
- Doug Wilson, NuFarm (retired part way through the project)
- Scot Paton, NuFarm
- Scott Matthew, Syngenta (replaced Richard Lillingstone)
- Shane Trainer, Bayer CropScience
- Hugh Armstrong, Bayer CropScience
- Prof Eileen Scott, University of Adelaide
- Dr Mark Sosnowski (SARDI)
- Dr Trevor Wicks (Private consultant ex SARDI)
- Marcel Essling (AWRI)
- Dr Markus Herderich (AWRI)
- Prof Richard Oliver, Curtin University

During the course of the project five meetings were held, all in Adelaide. The general format was an initial face to face meeting of the project team to present results and discuss issues arising. This was followed by a meeting of the reference group either in attendance or by teleconference, where the team would present the current results and request input in specific areas identified by the team. Then the project team would reconvene to discuss the next year's work following the input from the reference group.

The reference group provided valuable information on fungicide/pathogen combinations for testing, avenues for obtaining samples and sampling methods, the best forums for interaction with growers, provision of local knowledge of fungicide programs, identifying gaps in knowledge.

Meetings were held on 21 February 2013, 22/23 August 2013, 6/7 August 2014, 18/19 May 2015 and 10/11 November 2016.

In addition to input from the reference group, issues were canvassed more widely at presentations and grower meetings to understand the grower perspective.

5.3. Extension

Updates to industry on testing outcomes and possible management strategies were provided through over 24 presentations at grower days, chemical supplier agronomist training, Agrochemical Reference Group meetings and fungicide resistance forums and industry workshops.

At least 11 articles were written for industry journals and grower group newsletters. In addition issues of resistance in Australian viticulture including results of the project were presented at three international and three national conferences, with full peer reviewed papers provided for journal style proceedings for three of these conferences.

A full list of these presentations and articles is included in the Communication section (Appendix 9.1). No changes have been recommended to be included in the resistance management strategies in the publication 'Agrochemicals registered for use in Australian viticulture 2013/2014' (also known as the 'Dog book'), as the current strategies are still considered the best available. However further work to correlate the results of laboratory testing to field performance will assist in providing better advice to growers.

6. RESULTS AND DISCUSSION

6.1. Identify and quantify resistant populations in Australia

6.1.1. Vineyard sampling

A total of 185 samples of powdery mildew were submitted to the laboratory, of which 108 isolates were able to be established for testing. Samples were collected from 27 regions in South Australia, NSW, Victoria, WA and Tasmania (Table 1), with 17 different cultivars represented.

For downy mildew, 64 samples were received from 10 regions in South Australia, NSW, Victoria, WA and Tasmania. Of these only 25 were successfully subcultured and able to be tested (Table 2), with seven cultivars represented.

Botrytis was collected from 95 sites over six states, with one site from Queensland (Table 3). 745 isolates were established and tested from these sites.

Table 1. Powdery mildew samples received from 2013 to 2016, and the number of single spore isolates successfully established.

State	Region	No. samples received	No. isolates established
South Australia	Adelaide Hills	17	17
	Adelaide Plains	13	10
	Barossa Valley	13	12
	Langhorne Creek	13	9
	McLaren Vale	1	1
	Riverland	25	11
	South East	5	3
NSW	Hilltops	2	1
	Hunter	2	2
	Orange	3	2
	Riverina	1	1
	Wagga Wagga	8	3
	Murray Darling	2	0
Tasmania	Tamar Valley	4	3
	South-east	2	1
Victoria	Bellarine Peninsula	1	1
	Mornington Peninsula	4	4
	Goulburn Valley	1	1
	Yarra Valley	6	6
	Strathbogies	8	3
	Myrrhee, King Valley	5	3
Western Australia	Geographe	3	0
	Great Southern	14	2

	Margaret River	20	10
	Swan District	4	1
	Perth Hills	7	0
	Frankland	1	1
TOTAL		**185**	**108**

Table 2. Downy mildew samples received from 2013 to 2016, and the number of isolates successfully established.

State	**Region**	**No. samples received**	**No. isolates established**
NSW	Hunter	30	12
	Wagga Wagga	2	2
Tasmania	Tamar Valley	5	4
	Kayena	1	0
Victoria	Yarra Valley	5	3
	King Valley	3	0
Western Australia	Margaret River	8	5
South Australia	Adelaide Hills	2	0
	Limestone Coast	6	0
	Southern Vales	2	0
TOTAL		**64**	**25**

Table 3. *Botrytis* samples received from 2013 to 2016, and the number of isolates tested.

State	**Number of isolates tested**	**Number of sites tested**
Western Australia	371	47
New South Wales	110	13
Victoria	123	15
South Australia	100	18
Queensland	20	1
Tasmania	21	1
Total	745	95

6.1.2. Sampling methods

In the first season, five sites per sampling area were collected with the intention of testing the five separately. While this proved to be possible for *Botrytis*, the difficulty in obtaining viable cultures from material infected with powdery and downy mildew, and the time involved with culture maintenance and the phenological testing resulted in only one sample being used from each site rather than all five.

Viable cultures of powdery and downy mildew were not able to be obtained from many of the samples submitted in the first year of the project. Either the sample had no viable spores for regrowth, or was not in a suitable condition when it arrived at the laboratory. For example from the samples sent for powdery mildew testing in 2013/14, cultures were obtained from 73% of the young bunches with fresh infection, compared to 38% from older bunches (Table 4).

Table 4. Samples received for powdery mildew testing and the percentage success rate of successful isolation and culture establishment.

Sample type	**Successful isolation (%)**
Young, open bunches 	73%
Older closed bunches 	38%
Young leaves 	50%
Old end of season leaves 	18%

In the second year of the project, following advice from the reference group, a ‘how to sample’ sheet with example pictures was produced and sample packs made up to send out to collectors (see examples in section 9.4.1.). This ensured they could collect ‘on the spot’ when they saw the issue and return the samples to the laboratory immediately, rather than having to either wait until they

returned to their office, or return to the vineyard later to resample. In addition a letter to growers was produced outlining the project and the reason for sampling.

With these new measures, samples were received in much better condition. For example in 2014/15, the successful isolation rate for powdery mildew had risen from 45% to 75% of the samples.

To ensure biosecurity conditions were met, the three laboratories obtained permits from their respective biosecurity agencies to enable samples to be received from all areas of Australia.

6.1.3. Pathogen maintenance and storage techniques

6.1.3.1. Powdery mildew (*Erysiphe necator*)

Isolate maintenance

Single spore isolates were established by transferring a single conidium or conidial chain from diseased samples to detached surface sterilised leaves on water agar amended with pimaricin (aTWA). Each isolate was grown in an incubator at 22^{o}C and maintained in duplicate by transferring sporulating colonies to freshly prepared sterilised leaves every 2-3 weeks.

To maintain a fresh supply of suitable leaves, grapevines (cv. Cabernet sauvignon) were grown in pots in a controlled environment room (CER), pruned and fertilised regularly, sprayed weekly with a mixture of potassium bicarbonate and emulsifiable botanical oils, and biological control predators were applied to the potting media when required.

Storage methods

No viable powdery mildew was recovered from spores at either 1 day, week, 1 month or 3 months after storage using any of the three storage methods. The amount of spores used by Stummer et al. (1996) was 5 mg fresh weight or 2 mg dry weight. In their experiments, only one isolate was used and they used sterile tissue culture plants to bulk up this isolate to obtain sufficient conidia; a process which took over a year to achieve (T. Zanker pers.comm.). In our experiments, the yield of spores from one infected leaf ranged between 20 and 70 µg fresh weight, so to reach the desired amount of 5 mg FW required for viable recovery would require at least 70 leaves per isolate. The feasibility of obtaining enough fresh leaves to bulk up a large number of isolates, as well as the time and resources available in the project were the major limitations to achieving successful storage. Anecdotally, results of many discussions with other researchers throughout the world also working with *E. necator*, indicated that they had also not managed to successfully store this fungus and retrieve viable cultures from stored spores or leaf material.

6.1.3.2. Downy mildew (*Plasmopara viticola*)

Isolate maintenance

Leaf discs covered in *P. viticola* were stored at -20^{o}C or freeze-dried until required for further experiments.

To maintain a fresh supply of suitable leaves, grapevines (cv. Sultana) were propagated from cuttings and grown in pots in a greenhouse with drip irrigation and fertilised fortnightly. During the autumn and winter months, active shoot growth was maintained by providing a 1h photoperiod using cool white fluorescent bulbs positioned above the plants. Grapevines were treated by burning sulfur powder, applying predatory mites and using sticky aphid and white fly traps.

Storage methods

The greatest recovery of viable downy mildew spores was achieved from infected leaf field samples that were air dried at room temperature and then stored at room temperature. Recovery was also achieved from leaf samples, both field and pure sub-cultured spores onto leaves, stored at -20^{o}C. The samples stored in water at -20^{o}C and at -80^{o}C meant for long-term storage were not tested due to time constraints. Anecdotally, spores can remain viable for up to 12 months frozen at -20^{o}C, however recovery is sporadic and not reliable.

6.1.3.3. Botrytis (*Botrytis cinerea*)

Storage methods

Botrytis was able to be retrieved from all storage methods, however while water and glycerol storage had a retrieval rate of 99% at four years, sclerotical storage was only 50% successful at four years.

6.1.4. Resistant populations

Resistant populations were determined using the results of the phenotypic tests, and with *Botrytis* correlated with the genotypic data. Unlike *Botrytis*, with powdery mildew there was little correlation between the phenotypic and genotypic test results.

6.1.4.1. Powdery mildew (*Erysiphe necator*)

One hundred and eight isolates were successfully established out of the total of 185 samples, and of these, 94 were suitable for fungicide sensitivity testing over the course of the project. The others were lost during the maintenance phase from contamination.

Of the 94 available isolates, 42 were phenotyped to Topas®, 66 to Mycloss™ and 21 to Domark®. The ranges of EC_{50} values and RFs for these isolates are: Topas® 0.0004-0.7 µg/mL and RF 0.001-3.2; Mycloss™Xtra 0.2-6.6 µg/mL and RF 0.28-9.7; and Domark® 0.0008-0.05 µg/mL and a RF of 0.8 -5.0 (Table 5).

Thirty-eight isolates of *E. necator*, obtained in 2013/14, were tested against Cabrio® and a subset of 11 tested against Amistar® using a full bioassay with 6-7 concentrations of each fungicide. Fifty-six isolates from samples received in late 2014 season and 2015/16 season were tested for sensitivity using a discriminatory dose assay. The range of EC_{50} for Cabrio® for the isolates tested using the full bioassay was 0.008 to 14.4 and the RFs ranged from 0.004 to 6.85.

Reduced sensitivity was indicated where the isolated had an EC_{50} greater than 1.0 µg/mL, or for the discriminatory dose growth was greater than 20% at 1.0 µg/mL. Testing showed that 53% of isolates tested against pyraclostrobin had reduced sensitivity, 36% with EC_{50} > 1.0 µg/mL and 63% with a 1.0 µg/mL discriminatory dose of pyraclostrobin. Myclobutanil was the only DMI to have reduced sensitivity, with 14% of the samples having an EC_{50} > 1.0 µg/mL.

High levels of the Y136F mutation in the *cyp51* gene (68-84%) and the G143A mutation in the *cytb* gene (86%) were detected in the samples, irrespective of their sensitivity to the fungicides (Table 5). Further details are in the SARDI report 9.4.2.

Table 5. Percentage of samples with reduced sensitivity of powdery mildew to fungicides, either by EC_{50} > 1.0 µg/mL[1] or with a discriminatory dose of pyraclostrobin with >20% growth at 1.0 µg/mL[2].

Fungicide	No sites tested	Reduced sensitivity (%)	% sites with mutant present
pyraclostrobin (Cabrio®)[1]	36	36	86
pyraclostrobin (Cabrio®)[2]	58	63	
penconazole (Topas®)[1]	38	0	68
myclobutanil (Mycloss™ Xtra)[1]	66	14	84
tetraconazole (Domark®)[1]	20	0	82

6.1.4.2. Downy mildew (*Plasmopara viticola*)

Pyraclostrobin: The highest concentration used in the test was 0.1 µg/mL. This was chosen because the initial testing samples had an EC_{50} much lower than this. However a higher rate should have been used, as most of the isolates had an EC_{50} higher than 0.1 µg/mL. Resistance has been reported from 3 µg/mL (Giraud et al. 2013) and 390 µg/mL (Ward-Gauthier and Amsden 2014). Baseline sensitivities to pyraclostrobin of up to 0.94 µg/mL were reported by Wong and Wilcox (2000).

Nine of the samples tested had an EC_{50} lower than 0.1 µg/mL and can be classified as sensitive. However the remaining 14 samples tested had an EC_{50} greater than 0.1 µg/mL so would need to be retested to accurately determine their sensitivity. Unfortunately that is not possible, as the samples were unable to be successfully retrieved from storage. Fresh samples from the same areas will need to be sourced and tested in future.

Three of the 24 samples genotyped had the G143A allele in the *cytb* gene, two from the Hunter Valley and one from the Yarra Valley (Table 6). However only one from the Hunter Valley had high levels of the allele present (91.6% compared to 7.2 and 3.5%). The samples with low levels of the allele also had very low EC_{50} values (0.02 and 0.04 µg/mL), much lower than many with 100% wild type (G143). As with powdery mildew, the presence of the allele does not mean the sample will be phenotypically resistant, however it would be unusual for a sample with 100% wild type to be phenotypically resistant. Therefore it is proposed that although the EC_{50} values for many of the isolates were higher than 0.1 µg/mL, they would have been phenotypically sensitive as no mutation was present.

Following the detection of the G143A mutation, 15 additional samples were collected from the Hunter Valley in early 2016. These were genotyped using NGS to determine the presence and frequency of the G143A allele. Of the eight samples from the same area as the original detection, only one was 100% wild type, the other seven ranged from 66 to 98% G143A. Of the remaining seven samples, four were wild type, and the others varied from 10 to 60% (Table 6).

Table 6. Detection of G143A allele in samples of *Plasmopara viticola*

Sample location		**No samples tested**	**G143A allele detection**	
State	**Region**		**No samples**	**Frequency**
NSW	Hunter Valley	15	5	7, 10, 47, 60, 92
	Hunter Valley (resample +ve site)	8	7	66, 69, 69, 74, 85, 91, 98
	Wagga Wagga	2	0	-
Tasmania	Tamar Valley	6	0	-
Victoria	Yarra Valley	6	1	3.5
Western Australia	Swan Valley	1	0	-
	Margaret River	6	0	-

Metalaxyl: Six of the 10 samples tested from Hunter Valley were resistant to metalaxyl, with EC_{50} values of 10 µg/mL or greater. Of the three samples tested from Yarra Valley, only one was sensitive with an EC_{50} of 0.94 µg/mL. Metalaxyl resistance is known to be present in both the Hunter Valley (Wicks et al. 2005) and the Yarra Valley. New regions now with resistance detected include Wagga Wagga (1/1), Tamar Valley (2/2) and Margaret River (2/2).

No mutants are known for metalaxyl so genotyping was not undertaken. Further work in this area using NGS would be useful to investigate any aberrations between sensitive and resistant isolates to see if there are any genetic traits of the resistant populations that can be detected.

Mandipropamid: Of the 24 samples tested, only one from Wagga had an elevated EC_{50} of 4.95 µg/mL. Studies by Gisi et al. (2007) suggested that isolates with EC_{50} values below 3.6 µg/mL were considered sensitive to Mandipropamid. Therefore all other isolates could be considered sensitive, with EC_{50} values ranging from 0.06 – 2.62 µg/mL. Only 11 samples from WA were genotyped and no mutant alleles were detected.

Further details are in the CSU report section 9.4.5., Curtin 9.4.4. and AWRI 9.4.6.

6.1.4.3. Botrytis (*Botrytis cinerea*)

Of the 745 isolates tested from the 95 sites, 633 isolates (84.9%) were classified as sensitive and 56 (59%) or the sites had no resistance detected to any of the fungicides tested (Table 7).

For all fungicides except iprodione, isolates with an EC_{50} <0.5 µg/mL were classified as sensitive, and this threshold was supported by genotyping results. The iprodione sensitivity threshold was higher at <2.05, as this was the EC_{50} of the least sensitive isolate (Bc-279) that was wildtype for *os-1* (data not shown). For boscalid, fenhexamid, iprodione and pyrimethanil, 3.6%, 5.6%, 16.7% and 13% of isolates were classified as resistant, respectively (Table 7).

Table 7. Number of isolates from sampling sites tested with at least one isolate resistant to boscalid, fenhexamid, iprodione and pyrimethanil.

State	No. isolates (sites) tested	Number of isolates (sites) with resistance				
		Sensitive	Boscalid	Fenhexamid	Iprodione	Pyrimethanil
Western Australia	371 (47)	326 (26)	14 (11)	-	35 (15)	22 (14)
New South Wales	110 (13)	107 (8)	-	2 (1)	2 (2)	2 (2)
Victoria	123 (15)	92 (8)	1 (1)	11 (1)	26 (3)	21 (7)
South Australia	100 (18)	82 (13)	6 (3)	3 (3)	14 (5)	11 (4)
Queensland	20 (1)	20 (1)	-	-	-	-
Tasmania	21 (1)	6 (0)	1 (1)	-	14 (1)	1 (1)
Total (number)	745 (95)	633 (56)	22 (17)	16 (5)	91 (25)	57 (30)
Total (%)	-	85 (59)	3.0 (17.9)	2.1 (5.2)	12.2 (26.3)	7.7 (31.5)

Of the 95 sites tested, 17 had resistance to one of the four modes of action (MOA) tested, nine to two MOA, 12 to three MOA and only one to all four MOA (Table 8).

Table 8. Sampling sites characterised in terms of the isolate/s at each site resistant to the most MOAs (1-4).

State	No. isolates tested	No. sites tested	Number of sites with resistance to 0 – 4 fungicide MOA				
			Sensitive	1 MOA	2 MOA	3 MOA	4 MOA
Western Australia	371	47	26	8	7	6	-
New South Wales	110	13	8	3	1	1	-
Victoria	123	15	8	4	1	2	-
South Australia	100	18	13	1	-	3	1
Queensland	20	1	1	-	-	-	-
Tasmania	21	1	-	1	-	-	-
Total	745	95	56 (59%)	17 (18%)	9 (9.5%)	12 (13%)	1 (1%)

A selection of resistant isolates identified in the microtitre assay and high throughput agar screen were screened for the presence of mutations. The expected mutations were detected as well as two novel mutations found in pyrimethanil resistant isolates (Table 9). A number of pyrimethanil resistant isolates lacked any mutations in the *cgs* gene and were candidates for investigating the potential role of multi-drug resistance (MDR) in resistance to pyrimethanil. Sequencing of the *mrr1* gene in one highly resistant pyrimethanil isolate showed a number of indels and non-synonymous mutations commonly found in MDR1 characterised isolates (Kretschmer et al. 1999). No candidate isolates were found to contain a transposon in the promoter of the *mfsM2* transporter (MDR2) (Kretschmer et al. 1999).

Table 9. Mutations identified in the target genes; *sdh*B, *erg*27, *bos*-1 and *cgs* in a selection of resistant isolates. (CAPS) = CAPS results for the *os-1* gene.

Fungicide	Target Gene	Non-synonymous mutation	RF (mean)	Number of isolates
Boscalid	***sdhB***	H272R	55	9
		H272Y	188	1
Fenhexamid	***erg27***	F412S	256	6
Iprodione	***bos-1***	I365S	8	9
		I365N/R/S (CAPS)	-	18
		I365S + D747N	23	1
		Q369P + N373S	27	6
		Q369H/P (CAPS)	-	11
Pyrimethanil	***cgs***	L8P (novel)	16	1
		D416E (novel)	15	5

Further details are in the Curtin report section 9.4.3.

6.1.4.4. Discussion

From the samples collected in the main viticultural regions in Australia, it is clear that resistance to all of the commonly used fungicides is present, albeit at low levels in some instances, in most of the

regions. However this is a small sample snapshot, and it is difficult to determine at this stage whether there is loss of field performance resulting from this resistance.

Results from this study have confirmed that there is a good correlation between the detection of the mutants conferring resistance and the phenotypic resistance detected with *Botrytis*. Therefore the repertoire of well described and novel mutations identified in this study can be used for the future development of in-field molecular high throughput screening methods. The identification of novel mutations in the *cgs* gene will also be important for elucidating the mechanisms behind pyrimethanil resistance, which are currently not well defined. The identification of an isolate with the classic MDR1 genotype is of interest as it is the first report of this mechanism in Australia. The role of MDR and its development alongside target site mutations in resistant isolates could be of interest for investigating resistance development under current fungicide management practices. A small scale screen against azoxystrobin (QoI) and fludioxinil (phenylpyrrole) was also completed in this study and the frequency of resistant isolates was 5% and 0% respectively (data not shown). Further screening and monitoring of sensitivity to fludioxonil may be worthwhile as significant resistance to this fungicide has yet to be identified. Further research is needed to understand the relationship between the *in vitro* results in this study and field performance.

There has been work to correlate resistance detected in laboratory testing in *Botrytis* with field performance for some of the older fungicides (e.g. iprodione, pyrimethanil) (e.g. Beever et al. 1989). However there are limited studies in powdery and downy mildew that discuss the relationship between laboratory results and field performance (e.g. Rallos et al. 2014). In addition, the results from this study have shown that unlike *Botrytis*, with downy and powdery mildew there is a poor correlation between the detection of the mutants conferring resistance and the phenotypic resistance detected. This is an area that needs significant research before resistance testing for the mildews can become a useful predictive tool for growers.

6.1.5. Sampling strategies

Each of the five data sets for both Y136F and G143A differed both in the mean of the distribution and the distribution shape. Data were not normally distributed and confidence intervals based on normal distributions are therefore not reliable. Simulations used to estimate quantiles were the best method to estimate the required number of samples.

Based on our data sets, if we are to be 95% confident that the mean allele frequency falls within a range of between e.g. 20-40 or 75-95, with a 10% SE, then we need 10 samples to achieve this. However the simulations showed that the greater the confidence interval and smaller SE required, the more samples are needed.

Several issues arose from this study. There was considerable variability in the amount of mildew on each swab depending on who collected the sample and how much powdery mildew was available. Detection of the Y136F mutant allele with NGS required a higher quantity and quality of DNA to be extracted than for G143A and this was not possible from all of the swabs.

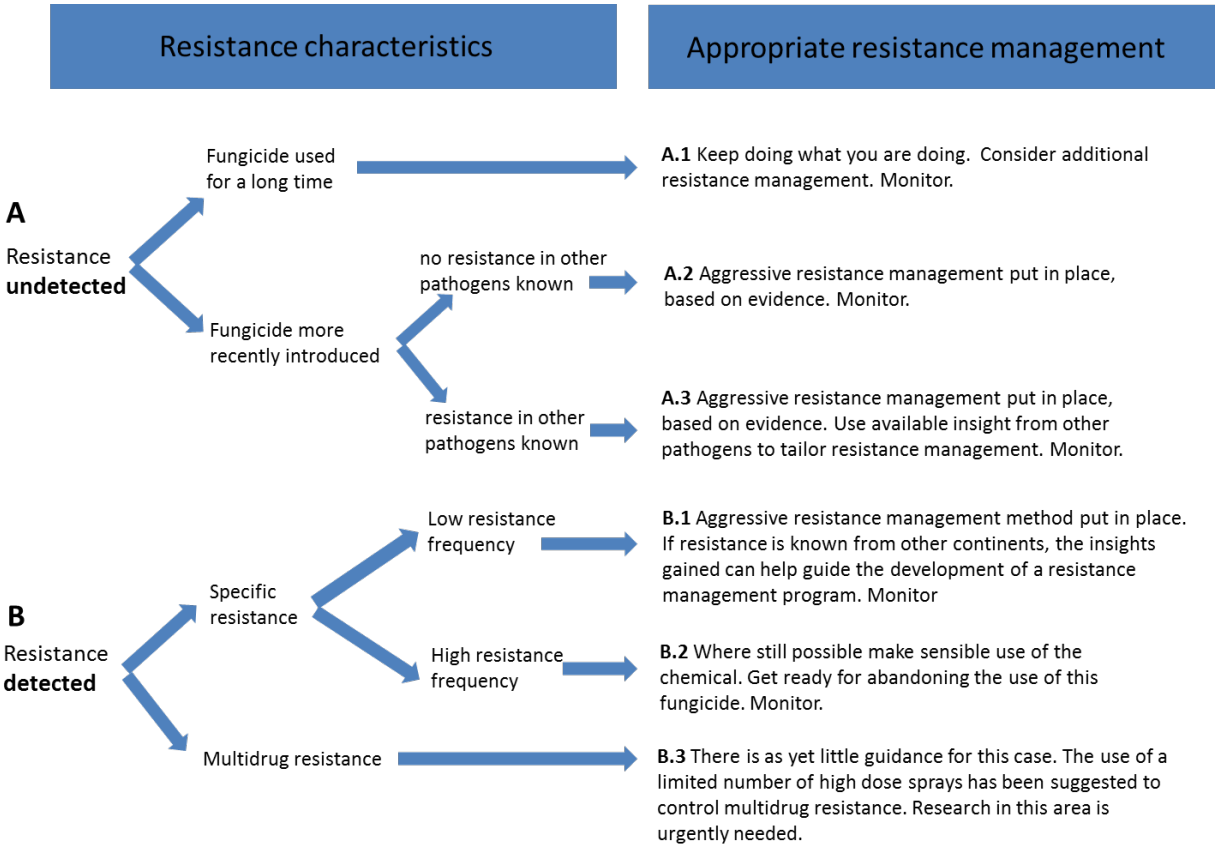
In addition, the patchy distribution of powdery mildew needs to be taken in to account when designing sampling strategies to understand the distribution and pattern of resistance in a vineyard.

Further details are in the SARDI report section 9.4.2. and AWRI 9.4.6.

6.1.6. Develop and validate spray programs

6.1.6.1. Model existing and project developed data to determine potential fungicide strategies

From the modelling based on the available evidence, a decision tree has been developed to help guide the approach to developing a resistance management program (diagram adapted from Walker et al. (2013)).



The work leads to five key conclusions:

Conclusion 1: Although the situation with resistance development in *Botrytis* is the most pressing problem at this moment in time, it would be a failure not to invest in the development of anti-resistance strategies for powdery mildew and downy mildew.

Conclusion 2: We now have a simple governing principle that predicts whether a change in a fungicide application program increases or decreases the rate of selection for fungicide resistance. This governing principle together with an exhaustive literature review has led to a set of clear-cut advice about resistance management. The list can be found in the Curtin report on pages 18-21. This list will help develop resistance management programs.

Conclusion 3: Research into the management of multidrug resistance is urgently needed because of its potential importance in the development of effective resistance management methods. Further work is needed to investigate whether multi-drug resistance plays an important role in the Australian pathogen populations.

Conclusion 4: Abandoning the use of a fungicide MOA to allow the pathogen population to regain sensitivity, after which the MOA is re-introduced is unlikely to be a practical resistance management tool.

Conclusion 5: Research is needed on non-fungicidal disease control methods. If such a method or a combination of these methods significantly reduces the rate of epidemics. These methods will also significantly reduce the selection of fungicide resistance. This leads to the possibility to manage fungicide resistance without any change in the fungicide application program.

The second model for the cost and benefit calculation of fungicide treatment programs showed how to calculate the optimal dose of fungicide treatments as well as the optimal mixtures of fungicides. The model and analytical method are quite general and can be applied to a wide variety of pathosystems and fungicides.

There were several key conclusions from the study:

1. For risk averse growers the optimal dose will always be considerably higher than for growers aiming only at the long term mean economic gain.

2. When resistance is developing to the fungicide we found that:

    - When the disease-yield loss relation is linear or concave, the optimal fungicide application dose decreases with increasing frequency of resistance in the pathogen population.
    - When the disease-yield loss relation is convex there are cases where the optimal fungicide treatment dose increases with the frequency of resistance and there are cases that the optimal dose decreases with frequency of resistance.
    - The balance between the steepness of the disease-yield loss relation and the fraction of the years developing a high disease severity in untreated crops determines whether optimum dose increases or decreases with the frequency of resistance in the pathogen population.
3. For fungicides to which partial resistance develops we found that the optimal dose increases with increasing frequency of the resistant strain in the pathogen population.

4. The optimal dose combination in a mixture depends on (i) the cost of the mixing partner relative to the cost of the at-risk fungicide, (ii) the efficacy of the mixing partner relative to the efficacy of the at-risk fungicide and (iii) on the frequency of resistance in the pathogen population.
    - When the cost of the mixing partner is higher than that of the at-risk fungicide, and their efficacies are comparable, the optimal economic solution is not to use a mixture when no resistance is present in the pathogen population. When in this situation resistance develops it can become economically sensible to use mixtures. Whether a mixture is the economically optimal solution is again determined by costs and efficacy of the mixing components.
    - When the mixing partner is less expensive than the ‘at risk’ fungicide and their efficacies are comparable, mixtures are economically sensible in all cases.
    - The frequency of resistance in the pathogen population determines the optimal dose combination in the mixture.

The model developed in this project has, for the first time, enabled a systematic study of the costs and benefits of fungicide application programs which can now be quantified, and analysed for different application programs.

Further development of the model in conjunction with appropriate field experimentation will make it possible to apply the analysis to actual fungicide application programs for multiple diseases threatening crop yield. The further developments needed to this end are:

- Develop and apply an efficient method to measure the dose response curves of the pathogens to the fungicide applied in the programs to be analysed.
- Including multiple diseases in the model and enabling combined analysis of the costs and disease induced yield losses for multiple diseases. This is still a considerable development that needs to be done but it would open the way to a full analysis of practice cases.
- Further development of the evolutionary model and establishing its relation to the economics. This will need the development of a quantity to measure the success of a fungicide application programme during the entire time frame where resistance in emerging and spreading in the pathogen population. This quantity will measure the economic gain to be analysed application program from the moment the application program is introduced to the moment the costs of application exceed the benefits in terms of reduced yield loss.

Further details are in the Curtin report section 9.4.3.

6.1.6.2. Evaluate variable efficacy of fungicides through phenotypic studies

The results of the *in vitro* detached berry assay showed that with the berries soaked in the highest concentration of fungicide, the resistant isolate (Bc-128) was responsible for a 40% disease increment compared to the sensitive strain (Bc-7) (Figure 3).

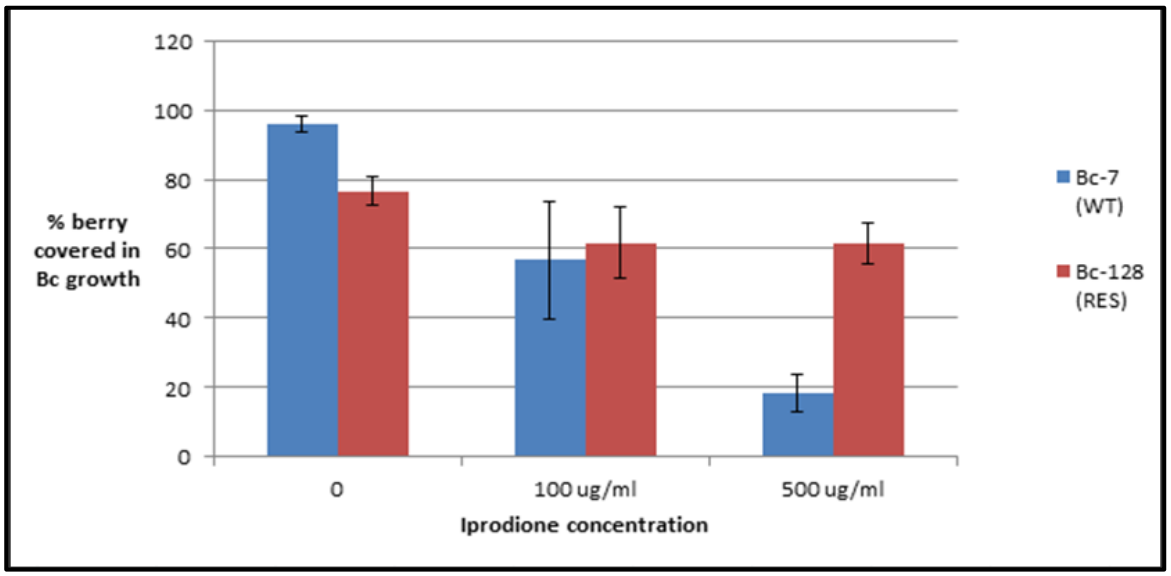


Figure 3. Percentage of berry area diseased for sensitive (Bc-7) and iprodione resistant (Bc-128) isolates on detached berries soaked in different concentrations of iprodione 500SC at day 7.

This detached berry assay is a suitable method to investigate efficacy of fungicides instead of using more complex *in planta* systems. This method will be used to test further actives with *Botrytis* to give additional information to the model.

Further details are in the Curtin report section 9.4.3.

6.1.7. Additional work

Additional work was proposed to be undertaken in a six month extension of the project.

6.1.7.1. Detect shifts in resistant populations (*Botrytis*)

The *in planta* investigation into the fitness and competitiveness of sensitive and resistant isolates has shown that bunches can be inoculated at flowering with different strains and, once disease expresses, molecular tools based on specific polymorphisms can be used to assess the presence of resistant genotypes. Further experiments and alternate molecular tools are required to investigate the relative amounts of each of these resistant genotypes within the treatments.

The number of replicates sampled was highly variable, as not all four replicates expressed *B. cinerea* for analysis (Table 10). One to three of the haplotypes were identified across four replicates in the 1:1:1 treatment. For the 1:10:1 and 1:1:10 treatments, the isolate at the highest inoculation concentration were the only haplotypes identified in those treatments, which probably indicate a higher fitness of these genotypes.

Table 10. Presence of each haplotype for each treatment where *Botrytis cinerea* DNA was extracted from berries in an *in planta* vineyard experiment

Ratio of Bc-7:Bc-143:Bc-179	Replicate (Bunch)	WT (Bc-7)	Q369P + N373S (Bc-143)	I365S (Bc-179)
1:1:1	1	Yes	No	Yes
1:1:1	2	Yes	Yes	Yes
1:1:1	3	No	Yes	No
1:1:1	4	Yes	Yes	Yes
1:10:1	1	No	Yes	No
1:1:10	1	No	No	Yes
1:1:10	2	No	No	Yes
Bc-7 only	1	Yes	No	No
Bc-7 only	2	Yes	No	No

The remaining actives with resistant isolates can now be tested using the same technique.

Further details are in the Curtin report section 9.4.3.

6.1.7.2. Relationship of mutant allele detection to field resistance (powdery mildew)

In both the *in planta* preliminary trials and detached leaf assays the powdery mildew infection rate was variable. In addition, there was evidence of a low level of cross contamination in two of the experiments, with mildew in the un-inoculated controls. No isolates of powdery mildew grew, even those containing 100% G143A allele, with Cabrio® applied at the full label rate. When applied at ¼ and ½ label rate, mildew growth was patchy and inconsistent.

The lack of available sensitive isolates for use as the control and to compare the effect of the presence of the resistant allele was also a significant limitation. Although samples were obtained from areas unexposed to fungicides, genotyping by either Sanger sequencing or NGS revealed that

few isolates were wild type i.e.G143. Furthermore, it was very difficult to keep isolates alive for long periods, while awaiting genotype results, so that any wild type isolates were dead by year 3 of the project and therefore unavailable for these experiments.

New methods are currently being evaluated to address these issues and obtain more consistent results.

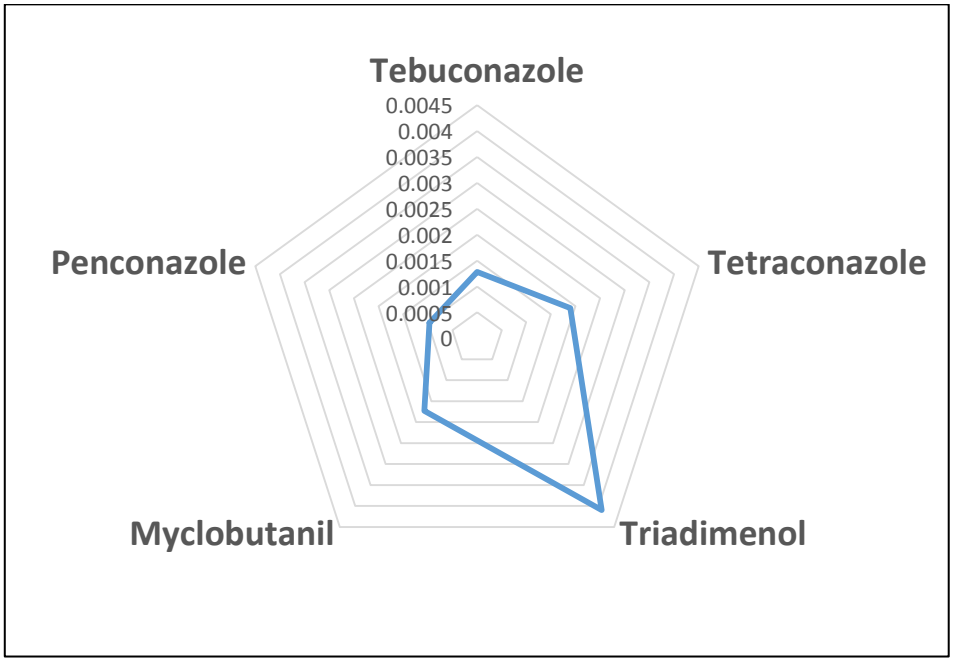
Further details are in the SARDI report section 9.4.2.

6.1.7.3. Relationship between resistant populations of the different DMIs (powdery mildew)

Expression of the *E. necator Cyp51* protein in a heterologous yeast expression system (Groeneveld et al. 2002. Revankar et al. 2004) provided a simple, reliable and high-throughput phenotypic test of the wild-type *E. necator* Cyp51 against demethylation inhibitors (DMIs).

The yeast expression system provided a means to test the sensitivity of WT *E. necator Cyp*51 against a number of DMIs registered for powdery mildew in Australia. Future work will include introducing mutations into the *E. necator Cyp*51 construct which would allow insights into what effect specific mutations (e.g. Y136F) have on the sensitivity to specific DMIs. This will provide a better idea of which DMI is best to use depending on the presence of the mutant alleles.

Figure 4. Sensitivity of the yeast strain YUG37:*erg11* expressing WT *E. necator Cyp*51 against the DMIs tebuconazole, tetraconazole, triadimenol, myclobutanil and penconazole



Further details are in the Curtin report section 9.4.3.

7. OUTCOMES AND CONCLUSIONS

This project has provided a significant bank of knowledge for both the wine industry and the research community. Techniques have been developed and refined for studying fungicide resistance in these three pathogens. A significant collection of *Botrytis* cultures, DNA of the three pathogens and sequencing data have been collected. In addition, new mutations and the first Multi Drug Resistant genotype have been detected in *Botrytis* populations. The repertoire of well described and novel mutations identified in this study can be used for the future development of in-field molecular high throughput screening methods.

Most of the planned outputs and performance targets have been achieved. Resistant populations in Australia have been identified and quantified to the fungicide/pathogen combinations determined by industry as the most critical. Effective sampling and phenotypic testing methods for each fungicide/pathogen combination have been optimised and samples tested. Next generation sequencing has been utilised and the method validated to provide a quantitative measure of the proportion of each sample population that contain resistant alleles. Methods such as *in planta* and *in vitro* tests have been developed to evaluate various parameters required to fill the knowledge gaps identified, including fitness of resistant populations and understanding of the effect of fungicide on resistant populations. Two models were developed which with further development in conjunction with appropriate field experimentation will make it possible to apply the analysis to actual fungicide application programs for multiple diseases threatening crop yield.

An Industry Reference Group was formed and remained active throughout the project. They provided invaluable advice and an industry perspective on the results and directions for the project. All have indicated they would be more than willing to continue to be involved in further research in this area.

Information was disseminated to industry with over 24 presentations to growers and industry personnel, 11 industry journal articles and three scientific conference presentations. In addition information was disseminated internationally with three conference presentations at scientific forums.

The two areas where outcomes were not fully met were in testing for resistance to populations of downy mildew, and developing and validating spray programs.

Downy mildew, like powdery mildew, is an obligate parasite and must be grown on living grape tissue. It proved to be more difficult than anticipated (given past experiences) to effectively culture the downy mildew from samples submitted for testing. Many of the samples did not arrive in a condition suitable for testing: the organism would not sporulate and grow. The original funding requested for this aspect and provided by AGWA was limited compared to powdery and *Botrytis*, as we did not anticipate the difficulties. However while the 60 samples originally proposed did not get tested on plant material, all samples have been genotyped and DNA is in storage for further testing of new mutations if they are identified. In addition, testing is continuing, albeit at a reduced level. As the mutant allele conferring resistance to QoIs was detected, we wish to determine whether there is also phenotypic resistance present or whether (like the powdery mildew results) the relationship between phenotypic sensitivity and presence of the allele is not clear.

The models of fungicide use developed by the UK associate Dr Frank van den Bosch were provided much later than originally anticipated, as the data required to be inputted into the models proved more difficult to obtain than originally anticipated. Dr van den Bosch was not funded for the development of these models, although some of the data collection required for the model was. Therefore the development and validation of spray programs was not completed. This part of the work was to be funded by industry and the agrochemical companies, and some have expressed interest in the trials and are willing to provide funding in the future once the proposed spray program has been developed. It is proposed that these will be completed in the 2017/18 or 2018/19 seasons.

Significant gaps in scientific knowledge about resistance mechanisms were identified from research within this project. These gaps need to be addressed before more effective and sustainable resistance management strategies can be provided to industry.

As previously stated, the main area of uncertainly is the correlation between the laboratory testing results and field performance of the fungicide. To understand this relationship, a better understanding is needed in many areas of the resistance mechanisms at play in the chosen pathogen/fungicide combinations. Some preliminary work has been undertaken to develop techniques required to do this, but these need to be further refined and completed for all of the chosen pathogen/fungicide combinations.

Until this work is completed and a better understanding is achieved, current resistance management strategies are the best available tools. The results of this work have highlighted the importance of adhering to these strategies, and ensuring that at-risk fungicides groups are used effectively and sparingly to maintain their efficacy into the future.

8. RECOMMENDATIONS

Future directions from this research are aimed at filling the gaps identified in the scientific knowledge of the mechanisms of fungicide resistance.

These include, but are not limited to:

- competitive ability of resistant isolates and fitness costs associated with mutations
- how fungicide applications affect resistance development and the frequency of resistant isolates in the field
- the importance of multi-drug resistance in the pathogen populations
- the relative sensitivity of resistance and sensitive *E. necator* populations to the various DMI actives
- testing of additional pathogen/fungicide combinations for pathogen sensitivity
- detecting new mutations in resistant populations that may impact on the fitness of the pathogen populations

Once these are elucidated, they will provide a better understanding of the relationship between laboratory testing and field performance.

Future directions should also include concurrent development and validation of effective in-field resistance testing methods. With the improved understanding of the test result/field performance relationship, the ability to utilise in field testing methods that will provide a rapid understanding of the resistance status of a vineyard will assist with future management strategies.

Further development of the models of fungicide use will improve knowledge of the effect of actual fungicide application programs for multiple diseases threatening crop yield. As well as including multiple diseases in the model to enable combined analysis of the costs and disease induced yield losses for multiple diseases, there needs to be development of a quantitative measure of the success of a fungicide application program during the entire timeframe where resistance in emerging and spreading in the pathogen population. Additional input into the model from field experimentation is also required to measure the dose response curves of the pathogens to the fungicide.

Once achieved, these identified areas of research will provide a more solid scientific basis for a sustainable and effective strategy for resistance management of fungicides in Australian viticulture.

9. APPENDICES

9.1. Communication

Industry Presentations:

- Nov 2016: 4th Australian Agrichemical Resistance Meeting
- Nov 2016: Crop protection forum NSW
- Oct 2016: AWRI roadshow, Geelong Victoria
- Aug 2016: Sydney Uni fungicide resistance meeting
- May 2016: Agrochemical Reference Group
- Apr 2016: EE Muir annual viticulture conference
- Nov 2015: 3rd Australian Agrichemical Resistance Meeting
- Sept & Oct 2015: NSW Spring Vine Health Field days in Griffith, Mudgee, Hunter Valley, Orange, Tuggeranong and Tumbarumba (reported in local newspaper and on TV).
- Aug 2015: AWRI Webinar
- Aug 2015: Landmark Webinar
- Aug 2015: EE Muir (SA)
- July 2015: Margaret River grape growers (WA)
- June 2015: Farmer Johns (SA)
- June 2015: Landmark (SA)
- May 2015: New Zealand wine group
- May 2015: Agrochemical Reference Group
- Nov 2014: Margaret River grape growers (WA)
- May 2014: Agrochemical Reference Group
- Nov 2013: 2nd Australian Agrichemical Resistance Meeting

Conferences & Proceedings

2016: 16th Australian Wine Industry Technical Conference, Adelaide: Abstract and published article in proceedings

Fungicide resistance in Australian viticulture (B.H. Hall, S.F. McKay, F. Lopez, L. Harper, S. Savocchia).

2016: 18th Reinhardsbrunn Symposium: 'Modern fungicides and anti-fungal compounds', Germany. Abstract and published article in proceedings

Hall BH et al., Fungicide resistance in Australian Viticulture. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), 'Modern Fungicides and Antifungal Compounds', Vol. VIII, pp. 51-xxx. © 2016 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-xx-x

2015: Australasian Plant Pathology Conference, Perth. Abstract.

S.F.McKay, L. Harper, F. Lopez and B.H. Hall. Resistance of Erysiphe necator to QoIs and DMIs

2014: Grape Downy Mildew and Powdery Mildew International Symposium, Spain. Abstract.

Barbara Hall, Suzanne McKay, Doug Wilson, Trevor Wicks. Testing fungicide resistance in grapevine powdery mildew: how to work with biotrophs.

2014: Crush, Adelaide. Abstract.

Suzanne McKay, Lincoln Harper, Fran Lopez, Barbara Hall. Sensitivity of powdery mildew to Cabrio® and Topas® in Australian vineyards.

2013: 17th Reinhardsbrunn Symposium: 'Modern fungicides and anti-fungal compounds', Germany. Abstract and published article in proceedings

Wicks TJ et al., Development of grape powdery mildew strains resistanct to QoI fungicides in Australia. In: Dehne, HW.; Deising HB.; Fraaije, B.; Gisi, U.; Hermann, D.; Mehl, A.; Oerke, E.C.; Russell, PE.: Stammler, G.; Kuck KH. and Lyr H. (Eds), 'Modern Fungicides and Antifungal Compounds', Vol. VII,, pp. 52-xxx. © 2013 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-13-6

Journal articles

- Jan 2017: Australian & New Zealand Grapegrower & Winemaker: Research finds some 'red warning flags' in the vineyard
- Sept 2015: WA Wine industry newsletter: Fungicide resistance and new management guidelines
- Jul 2015: Wine Australia RD&E News: Research identifies growing fungicide resistance
- March 2015: Australian & New Zealand Grapegrower & Winemaker: International research on downy and powdery mildew
- 2013: NSW DPI Grapevine Management Guide 2013-14: Managing fungicide resistance in Australian vineyards
- Oct 2013: Wine & Viticulture journal: Understanding fungicide resistance in Australian vineyards
- Sept 2013: Australian & New Zealand Grapegrower & Winemaker (with Nufarm): Still some sting in the tail of strobilurins
- Sept 2013: SA Grower: Spray group diversity
- June 2013: WIC-e-Links June 2013: New WIC Projects. Understanding fungicide resistance in powdery and downy mildew
- June 2013: Australian & New Zealand Grapegrower & Winemaker: Fungicide resistance research fueled by international knowledge
- March 2013: WA Wine industry newsletter: Fungicide resistance in Australian vineyards

Outlines of the project have been included in industry newsletters in WA, Victoria and Tasmania and at several Grapevine Trunk Disease Workshops (Qld, Tasmania and Victoria) with requests for assistance in sampling. A radio interview for the ABC Goulburn Murray and Riverina Rural Report was held on Thursday January 16 2014 asking for samples.

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9.3. Staff

This project was a joint undertaking of the project team, advisors from research and agrochemical organisations, and advisory team members from the viticulture industry.

The main project team:

- **SARDI:** Mrs Barbara Hall and Dr Suzanne McKay with technical assistance from Lee Bartlett, Ian Bogisch, Anna Child, Chammi Wijayagunawardana
- **Curtin University:** Dr Fran Lopez, Professor Richard Oliver, Lincoln Harper, Dr Frank van den Bosch (Rothamsted Research, UK)
- **National Wine & Grape Industry Centre** (Charles Sturt University): Dr Sandra Savocchia, Lindsay Greer, Helen Waite, Jennifer Bannister.
- **AWRI:** Dr Markus Herderich, Dr Anthony Borneman
- **Department of Agriculture & Food, Western Australia:** Andrew Taylor.

Scientific and Agrochemical advisors:

- Dr Mark Sosnowski (SARDI)
- Prof. Eileen Scott (University of Adelaide)
- Marcel Essling (AWRI)
- Hugh Armstrong (Bayer Crop Science)
- Shane Trainer (Bayer Crop Science)
- Gavin Heard (BASF)
- Doug Wilson (NuFarm)
- Scott Paton (NuFarm)
- Scott Matthew (Syngenta)
- Dr Trevor Wicks (Ex SARDI)
- Prof Wayne Wilcox (Cornell University)

Industry advisors:

- Warren Birchmore (Accolade Wines, SA)
- Suzanne Mcloughlin (Treasury Wine Estate, SA)
- David Sanderson (Wine Tasmania)
- Colin Bell (AHA Viticulture, WA)

- Liz Riley (Vitibit, NSW)
- Leonard Russell (Watershed Wines, WA)
- David Braybrook (Vitisolutions, Victoria)

9.4. Reports

9.4.1. ***Sampling protocols***

Wanted: Samples for fungicide resistance testing

Are you experiencing problems controlling powdery mildew, downy mildew or Botrytis bunch rot? Samples of these diseases are needed from the main wine regions of Australia.

A project to investigate the extent of resistance of fungicides to these three diseases is underway, funded by GWRDC. To improve knowledge of resistance development we will also need access to details of spray programs in the sample area. Confidentiality of information will be maintained at all times and no grower will be identified individually. However the data collected with and from the samples and spray information will be used to inform the industry of issues and to develop improved management strategies.

Results from your samples will be provided once all testing has been completed. As this is research, we cannot guarantee the turn-around time of results. Interpretation of results will not be provided until the research is complete and we can relate the levels of resistance detected with potential control failure.

Before sending samples to the laboratory, please notify the contact person below by phone or email. They will be able to answer any queries about sample handling for particular diseases and can provide sample kits if required.

Downy Mildew: Sandra Savocchia, 02 6933 4341; ssavocchia@csu.edu.au, or Helen Waite, 0419 522 875 hwaite@csu.edu.au

Botrytis: Fran Lopez, 08 9266 3061 fran.lopezruiz@curtin.edu.au

Powdery Mildew: Suzanne McKay, 08 8303 9759 Suzanne.mckay@sa.gov.au or Barbara Hall, 08 8303 9562 barbara.hall@sa.gov.au

Any leaf or bunch sample from a PIZ or PRZ into South Australia or NSW must be accompanied by a plant health certificate from the state authorities declaring it free from symptoms of Phylloxera. Samples from PEZ require a letter of approval which can be supplied on request by the laboratory.

All leaf or bunch samples into Western Australia may need certification – consult the laboratory before sending (swabs do not need certification).

Fungicide Resistance Testing – sample form

DISEASE: **Botrytis** **Powdery mildew** **Downy mildew**

GROWER CONTACT DETAILS:

Name: .....

Address: .....

Suburb/Town: ……..**State:** ……. **Postcode:** .....

Telephone: (…..)…… **Facsimile :** (….) .....

Email: .....

SAMPLE SUBMITTED BY: *(if not as above)*.....

Name: .....

Location/Address: .....

Telephone: (…..)…….. ..... **Facsimile :** (….).....

Email: .....

SAMPLE DETAILS:

Date collected: …../……/…..

Location (State, region)……..

Block ID: …… **Variety:** ……..……..…..

Phenological stage:…….....

Severity of disease (slight, moderate, severe):……...

Sprays applied for this disease 2013/4:……...…..

……..…..

……..……..

Number years spray program available for if required…….…….

Previous issues of control failure? If yes provide details (eg spray application, suspect resistance)……

……..…..

……..…..

SARDI South Australian Research and Development Institute · Curtin University · Department of Agriculture and Food, Government of Western Australia · National Wine & Grape Industry Centre · The Australian Wine Research Institute · The University of Adelaide · GWRDC Grape and Wine Research and Development Corporation

ID NO: …… ***(For Laboratory Use ONLY)***

Date Received: …../…../…..

Dear……

Following the discovery of resistance of *Erysiphe necator* (powdery mildew) to the strobilurin group of fungicides, a project has been funded by Grape and Wine Research and Development Corporation (GWRDC) to investigate the extent of fungicide resistance in the viticulture industry.

This project involves collaboration between industry and scientists from organisations within South Australia, Western Australia, New South Wales, Victoria and Tasmania.

The causal agents of the three main diseases will be targeted:

- *Botrytis cinerea* (botrytis bunch rot or grey mould)
- *Erysiphe necator* (powdery mildew)
- *Plasmopara viticola* (downy mildew).

Samples will be collected from the main wine regions of Australia. To understand the development of resistance to fungicides and the impacts on industry and disease management, details of spray programs will need to be available.

Confidentiality of information will be maintained at all times. Any data collected in this project will be managed and no grower will be identified individually. However the data collected with and from the samples and spray information will be used to inform the industry of issues and to develop improved management strategies.

All results from grower samples will be made available to those growers, but this will not occur until all testing has been completed. As this is for research, we cannot guarantee the turn-around time. In addition, we are not able to provide any interpretation of the results at this stage, as more research is needed before we can relate the levels of resistance detected in the tests with potential control failure.

If you have any queries and require further information about this project, please contact:

Barbara Hall, Barbara.hall@sa.gov.au, 08 8303 9562 or 0418 552 520.

We appreciate your cooperation and hope that this project will provide information on fungicide resistance management that will benefit the industry into the future.

Yours Sincerely

Barbara Hall,
Project Leader, SARDI

Resistance Sampling Protocol. 2014.

Samples sent to South Australia or NSW must include the SA Plant Health Import Certificate or the NSW Permit.

In addition, any leaf or bunch sample from a PIZ or PRZ into South Australia or NSW must be accompanied by a plant health certificate declaring it free from symptoms of Phylloxera.

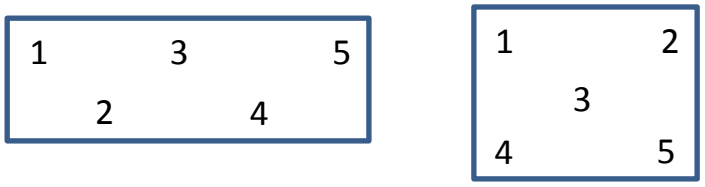
Before sending samples to the laboratory, please notify the contact person by phone or email. They will be able to answer any queries about sample handling for their particular disease.

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

Contact person: Sandra Savocchia, 02 6933 4341; ssavocchia@csu.edu.au, Lindsay Greer, 02 69334079, lgreer@csu.edu.au

Postal address: National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678

Courier address: National Wine and Grape Industry Centre, Charles Sturt University, Central Store, 252 Binya Way, Wagga Wagga, NSW 2650. Att. Sandra Savocchia (Please mark as Urgent Delivery)

- At each of the 5 sites within a block, select 2-3 leaves with downy mildew infection, active and as young as possible.
- 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.

Botrytis

Contact person: Fran Lopez, 08 9266 3061/ 0468 766 584

Postal address: Department of Environment and Agriculture, Faculty of Science and Engineering, Curtin University, Kent Street, Building 303, Room 152, Bentley, Western Australia 6845

Courier address: Department of Environment and Agriculture, Faculty of Science and Engineering, Curtin University, Kent Street, Building 311, Bentley, Western Australia 6845

Bunches with sporulating *Botrytis*:

- At each of the 5 sites within the block, select 3 - 5 bunches with sporulating *Botrytis.*
- Rub the end of the sterile swab into the spores, 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.

Powdery Mildew.

Contact person: Suzanne McKay, 08 8303 9759 Suzanne.mckay@sa.gov.au

Postal address: SARDI, Plant Research Centre, GPO Box 397, Adelaide SA 5001

Courier address: SARDI, Plant Research Centre, Gate 2B, Hartley Grove, Urrbrae, SA 5064

- At each of the 5 sites within the block, select 2-3 leaves or bunches with sporulating powdery mildew, as young as possible.
- 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.

Sampling procedure - bunches





1. Collect 2-3 bunches from each site
2. Place each bunch into a separate paper bag
3. Place the paper bags into a labelled plastic bag
4. Each of the 5 sites should be in a separate sealed bag





5. Place the completed sample sheet in a separate sealed plastic bag
6. Place all 5 site bags into a box labelled with the Block ID and place in **express post** bag

Remember to sign the declaration

Sampling procedure - leaves





1. Collect 3-5 leaves from each site and place in a paper bag
2. Place all 5 site bags into a sealed bag labelled with the block number

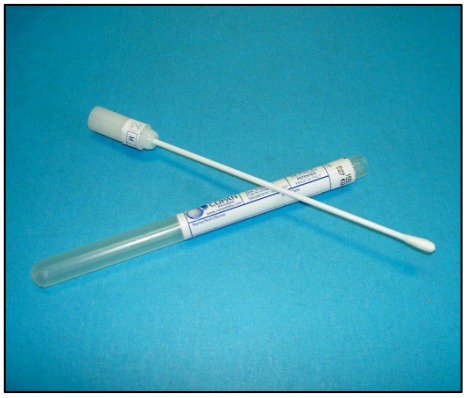


3. Place the completed sample sheet for the matching block in a separate sealed plastic bag
4. Send via express post

Remember to sign the declaration

Sampling procedure - swabs

Sampling procedure - swabs for sporulating *Botrytis*



- Remove swab by twisting the end of the tube with “sterile” written on paper label to break the paper seal
- Rub the cotton bud end of the swab into the sporulating *Botrytis*
- Place swab back into sterile container and press shut
- Use one swab per bunch
- Label swab container with block and site (eg block #/site 1)
- Place in plastic bag with sample sheet
- There should be 15-25 swabs per block (3-5 swabs per site, 5 sites per block)
- Send to laboratory by **express post**



Remember to sign the declaration

9.4.2. SARDI: Powdery mildew

Project SAR 1204: Grapevine Powdery Mildew, *Erysiphe necator*

Suzanne McKay, SARDI

Methods

The methods for establishing and maintenance of *Erysiphe necator* isolates and the fungicide sensitivity bioassays are adapted from previous work carried out by Evans et al. (1996), Gubler et al. (1996) and Erickson and Wilcox (1997).

Maintenance of potted vines

Grapevines (cv. Cabernet sauvignon) were grown in pots in a controlled environment room (CER) to provide year-round source of young, disease free leaves. To maintain vigorous and healthy vine growth, plants were pruned and fertilised regularly, sprayed weekly with a mixture of potassium bicarbonate and emulsifiable botanical oils and, biological control predators were applied to the potting media when required.

Establishment and maintenance of E. necator isolates

Leaves or berries, infected with powdery mildew, were obtained during the growing seasons 2013-2016 from 185 vineyards, in South Australia, Victoria, Western Australia, New South Wales and Tasmania (Table 1). Samples were sent via express-post to SARDI or collected and delivered directly to the laboratory by researchers, viticulturists or consultants. If possible, spray history for the two years immediately prior to the sample collection was obtained. We aimed to obtain samples with different levels of exposure to fungicides, including those with no history of exposure but it was difficult to obtain unexposed samples due to the widespread use of fungicides to control powdery, in particular the use of QoIs and DMIs. A total of five isolates were considered to be unexposed with no known exposure to any synthetic fungicides. If the spray history was unknown then the isolate was considered 'exposed'. Most samples received were from Chardonnay, followed by Shiraz, Cabernet sauvignon and Pinot Noir (Table 2).

To establish powdery mildew cultures, young glossy leaves were collected from plants grown in the CER, surface sterilised in bleach for 3 mins, then washed in sterile distilled water (SDW). Excess moisture was removed by blotting with sterile paper towel then leaves were placed on the surface of tap water agar amended with pimaricin (aTWA), contained in 100 x 20 mm Petri dishes (Evans et al. 1996).

Powdery mildew was viable on 108 out of the 185 samples and single spore isolates were established by transferring a single conidium or conidial chain from diseased samples to detached surface sterilised leaves on aTWA. Each isolate was grown in an incubator at 22^{o}C and maintained in duplicate by transferring sporulating colonies to freshly prepared sterilised leaves every 2-3 weeks. Extreme care was taken during subculturing to avoid cross contamination. Each isolate was kept separate by containment within a plastic tray and sealed

within a plastic bag to avoid cross-contamination. Due to the experimental protocols and by using uninoculated leaves in the incubator as a check for cross-contamination, it is considered that the chances of cross contamination were low.

Table 1. Grapevine powdery mildew samples received from 2013 to 2016, and the number of single spore isolates successfully established.

State	Region	Number of samples received	Number of isolates established
South Australia	Adelaide Hills	17	17
	Adelaide Plains	13	10
	Barossa Valley	13	12
	Langhorne Creek	13	9
	McLaren Vale	1	1
	The Riverland	25	11
	The South East	5	3
New South Wales	Hilltops	2	1
	Hunter	2	2
	Orange	3	2
	Riverina	1	1
	Wagga Wagga	8	3
	Murray Darling	2	0
Tasmania	Tamar Valley	4	3
	South-east Tas	2	1
Victoria	Bellarine Peninsula	1	1
	Mornington Peninsula	4	4
	Goulburn Valley	1	1
	Yarra Valley	6	6
	Strathbogies	8	3
	Myrrhee, King Valley and surrounds	5	3
Western Australia	Geographe	3	0
	Great Southern	14	2
	Margaret River	20	10
	Swan District	4	1
	Perth Hills	7	0
	Frankland	1	1
TOTAL		**185**	**108**

Table 2. The percentage of samples received per grape variety out of a total of 185 samples

Variety	Samples (%)	Variety	Samples (%)
Chardonnay	54	Tempranillo	2
Shiraz	7	Sauvignon blanc	2
Cabernet sauvignon	7	Grenache	1
unknown	7	Prosecco	<1
Pinot Noir	6	Viognier	<1
Verdelho	4	Crouchen	<1
Merlot	2	Harslevelu	<1
Riesling	2	Nebbiolo	<1
Pinot Gris	2	Semillon	<1

As the project commenced in May 2013, the samples that were received this late in the growing season meant that powdery mildew colonies were old and in cleistothecial stages. Attempts were made to obtain viable cultures from cleistothecia from a selection of these samples by following the protocol of Gee et al. (2000) as follows: Leaves, with visible cleistothecia, were cut in to segments, soaked in SDW for 2-3 hours then the leaves were shaken vigorously in SDW to dislodge cleistothecia. The solution was filtered using filter paper in a Bucher funnel and the filter paper with retained cleistothecia was placed on the inside lid of a petri dish, facing downwards, with a surface sterilised fresh leaf (placed on aTWA) facing upwards on the bottom of the dish. Dishes were sealed with Parafilm, filter papers kept moist by the addition of SDW every 48 hours whilst incubated at room temperature (RT). After 6 days, the fresh leaves on aTWA were air-dried and incubated for 12-15 days at RT. There was no evidence of ascospore release from cleistothecia resulting in powdery mildew colonies on the fresh leaves. Due to the time consuming and unreliable nature of this method, it was decided not to pursue this method to obtain cultures from late season samples.

Storage of E. necator isolates

Three methods were trialed to store spores from *E. necator* as follows:

Conidia were harvested using a cyclone separator into 1.5ml Eppendorf tubes. The collected conidia were dried leaving tubes open inside a laminar flow cabinet overnight for approximately 18 hrs then the tubes were sealed and snap frozen in liquid nitrogen and stored at -80^{o}C (Stummer et al. 1999).

Conidia were harvested using a cyclone separator into 500 µL tubes with no lid contained in 1.5ml Eppendorf tubes. Two - three sterile silica beads were added to the 1.5 mL tubes, the lids closed and sealed with Parafilm. After approximately 10 hours at room temperature the tubes were stored at -80^{o}C (adapted from method used by James Brown, John Innes Centre UK for storage of *Blumeria graminis*, barley powdery mildew).

Infected leaves were cut leaves in to strips (~5 x 15 mm) and placed in sterile 1.5 mL Eppendorf tubes. The conidia on leaves were dried leaving tubes open inside a laminar flow cabinet overnight for approximately 18 hrs. The tubes were sealed and snap frozen in liquid nitrogen and stored at -80^{o}C (Stummer et al. 1999).

For all three methods, spores were removed from storage at 1 day, 1 week, 1 month and 3 months and tested for viability by placing them on to the upper surface of a young grapevine leaf on aTWA and incubated for up to 2 weeks (as described in the establishment and maintenance of *E. necator* section above).

Bioassay for fungicide sensitivity: 'Phenotyping'

A total of 94 single-spore-derived isolates were tested in various combinations for fungicide sensitivity to commercial formulations of the DMI fungicides, Topas® 100 EC (penconazole 100g/L ai, Syngenta), Mycloss^{TM} Xtra (myclobutanil 200g/L ai, Dow AgroSciences) and Domark® (tetraconazole 40g/L ai, Sipcam), and the QoI fungicides Cabrio® (250g/L ai pyraclostrobin, Nufarm) and Amistar® (azoxystrobin SC 250g/L ai, Syngenta). Fungicides were freshly prepared immediately prior to each assay and were diluted in sterile type 1 water (SMQ). The following concentrations were used for testing Topas®, Domark®; Cabrio® and Amistar® 0.001, 0.01, 0.1, 1.0 and 10.0 µg/mL and 0.002, 0.02, 0.2, 1.0, 4.0 and 16.0 µg/mL for Mycloss^{TM} Xtra. Twenty µg/mL Cabrio® was used in preliminary experiments but phytotoxicity was observed so this concentration was not used in subsequent experiments. SMQ was used for the controls. For Cabrio® only, isolates established from samples collected in late 2014 and 2015/16 season were tested in a leaf disc assay using a discriminatory doses of 0, 0.1, 1.0 +/- 10 µg/mL.

Preliminary experiments assessed several media/substrates as well as inoculation methods for suitability in our bioassays. The following media/substrates were tested (i) TWA + filter paper (Shabi and Gaunt 1997, Steva and Clereau 1990), (ii) TWA amended with pimaricin and (iii) two layers of paper towel and two layers of Miracloth (Gubler et al. 1996). The following inoculation methods were assessed (i) droplet of spore suspension (ii) spore settling tower (SST) (Gubler et al. 1996) and (iii) single conidium (Erikson and Wilcox 1997). Amended TWA and the use of the SST was chosen to use in the fungicide bioassays as these gave the most consistent results (data not shown). A SST was used for the bioassays for samples from season 2013/14 (refer to Final report 5.1.4.1, Fig 1). However, due to the issues using the SST with having to clean it several times after each isolate and the number of infected leaves required to provide sufficient inoculum for consistent infection, another method, 'the cotton tip' method was tested. Spores were collected by touching the surface of a 14-day-old sporulating colony of *E. necator* with a sterile cotton tip and depositing the spores on the centre of a detached leaf disc by touching once with the tip (refer to Final report 5.1.4.1, Fig 1). Approximately 300 spores were deposited with each touch (data not shown). This method was much faster, required less starting inoculum and was more reliable than using the SST, so it was used in all bioassays for samples from obtained from seasons 2014/15 and 2015/16.

The full bioassay method is as follows (refer to Final report, 5.1.4.1, Fig 1): Young, glossy, disease-free leaves from the third or fourth node were collected from grapevines grown in the CER. The leaves were surface sterilised as described previously and 10-mm-diam discs were cut from each leaf using a sterile cork borer. Discs were randomised and placed upper surface downwards in a 140-mm-diam glass petri-dish containing 9 mL of given concentration of fungicide solution. Discs were soaked for 30 mins for each fungicide except for 60 mins for Mycloss^{TM} Xtra (Wong and Wilcox 2002, Frenkel et al. 2015). Discs were blotted dry with sterile paper towel, then placed upper surface uppermost on to the surface of aTWA in 50 mm-diam petri-plates. There were three discs/plate and three replicate plates for each fungicide concentration/isolate combination. The discs were inoculated the following day with *E. necator*. Plates from each concentration were kept in a separate plastic tray sealed in a clear plastic bag to prevent any vapour effect of the fungicides, in particular for the DMIs. Fourteen days after inoculation, the percentage of each disc covered in sporulating powdery mildew was assessed under a dissecting microscope at x 20 magnification. The mean of the six percentages (discs) were calculated and the EC_{50} µg/mL was determined for each isolate by Probit analysis using Genstat (18th edn, VSN International Ltd).

Genotype analyses (refer to Curtin report 9.4.4.)

Fungal DNA was extracted from infected leaf material using a CTAB extraction method (Cubero et al. 1999) and the DNA from each isolate was used as a template to amplify the complete cyp51 gene (Délye et al. 1997) and the *cyt*B hotspot region (Grasso et al. 2006). Amplification was carried out using high-fidelity Phusion polymerase (New England Biolabs) according to the supplier's protocol. Un-purified amplified DNA was sent to Macrogen Inc. (Korea) for Sanger sequencing. Cyp51 sequences which were then aligned to a reference sequence U72657.2 and cytb sequences were aligned to a Western Australian wild type sequence from this study to identify any mutations. Sequences were deemed wild type, mixed or mutant based on the chromatogram peaks. Mixed genotypes were found for both cyp51 and for cytb genes. To determine the frequency of alleles, which is unable to be determined accurately by Sanger sequencing, an allele specific qPCR method was designed to investigate this phenomenon. Primers were designed and tested; but the specificity for either WT or mutant was unable to be optimised using the designed primers. Due to time and financial constraints, it was determined that Next Generation Sequencing (NGS) may provide a better option to investigate relative ratios of WT vs mutants for the isolates from this project. DNA from 57 available isolates were processed using NGS to determine allele frequency as follows: Three rounds of nested PCR were used to amplify the relevant genetic locus (cytB or cyp51) while adding both Illumina-compatible sequencing adaptors and barcodes for sample multiplexing. The presence and frequency of the G143A and the Y136F mutations were determined using NGS of a 180 bp amplicon that surrounded the G143A mutation and a 431 bp amplicon that surrounded the Y136F mutation. Amplicon sequences were then aligned to a reference sequence consisting of the wild type gene, with overall allele frequencies determined from the proportion of each nucleotide variant detected at either the G143A codon of cytB or the Y136F codon of cyp51.

Population studies

The aim of these studies was to determine a statistically significant estimate of the number of samples needed to provide a useful description of the incidence and severity of resistance in a vineyard, by using quantification of known mutations linked to resistance to QoIs (G143A) and DMIs Y136F) using high throughput NGS carried out by AWRI. Powdery mildew samples were collected from five vineyards located in Victoria and South Australia as follows; Yarra Valley (Victoria), Cape Jaffa, Langhorne Creek, Lenswood and Urrbrae (South Australia) (Table 3). Samples were obtained by wiping the end of a sterile cotton tipped swab in sporulating colonies of mildew growing on either on leaves or bunches. Up to one hundred swab samples throughout each vineyard were collected at random. Swabs were stored at 4^{o}C until required. DNA was extracted from the mildew on the swabs following the manufacturer's protocol using a DNeasy® Plant Mini Kit (Qiagen) and NGS was performed as described in the 'Genotype analyses' section.

Table 3. Powdery mildew samples used for genotype analyses to quantify the resistance alleles for the cyp51 and cytb genes using high throughput Next Generation Sequencing (NGS), performed by AWRI.

Area	**Year**	**Variety**	**Number of swabs collected**	**Number of swabs successfully sequenced by NGS for:**	
				Y136F	**G143A**
Yarra Valley (Vic)	2014	Chardonnay	97	28	97
Cape Jaffa (SA)	2014	Chardonnay	100	86	100
Langhorne Creek (SA)	2015	Verdelho	94	49	94
Lenswood (SA)	2015	Chardonnay and Pinot Noir	93	92	93
Urrbrae, Adelaide Plains (SA)	2015	Chardonnay, Sultana and Grenache	41	32	41

To obtain a statistically significant estimate of the number of samples needed to provide a useful description of the incidence and severity of resistance in a vineyard, statistical analyses was carried out by Dr Ray Correll from Rho Environmetrics, on the 10 data sets obtained by NGS for both Y136F and G143A results. Simulations of differing sample sizes were carried out to estimate the percentiles of the distributions of the means for each data set. Standard errors and confidence intervals were calculated to obtain the required number of samples required to obtain a given standard error and at the desired confidence interval.

Relationship of mutant allele frequency to field resistance - Preliminary studies

Rooted cuttings of cv. Cabernet sauvignon, ~ 20 cm high, were placed in 100 mL conical flasks containing tap water. A cylinder of clear PVC sheet (0.25mm thick), covered on the top with two layers of muslin cloth, was placed around each flask to prevent potential cross-contamination among plants. When 3-4 almost fully expanded leaves had emerged from at least one bud on the cutting, the expanded leaves were marked with a texta and sprayed until runoff with Cabrio®. Leaves were air dried and PVC cylinders replaced. The following day, leaves were inoculated with spore suspensions of various isolate combinations until runoff. Isolates were selected based on % frequency of G143A as determined by NGS (Table 4). The 'sensitive' isolate, # 160, had the lowest % G143 frequency out of all isolates in the collection. No isolate in our collection was true wild type i.e. 100 % G143. Leaves were air dried and PVC cylinders replaced. Water was added to each flask as required and shoots were grown in a greenhouse for ~ 2 weeks then each marked leaf was assessed for incidence and severity of powdery mildew. Experiments were a randomised complete block design with between two and four replicates and controls were 1. Plus fungicide/minus powdery, and 2. Minus fungicide/plus powdery. A total of four experiments (Table 5) were carried out to develop methodology as follows; Experiment 1. Cabrio® applied at dilute label rate of 40 mL/100 L, and inoculation with individual isolates 147, 151 and 160 (5 x 10^4 spores/mL), Experiment 2. Cabrio® applied at dilute label rate, 40 mL/100L and inoculation with individual isolates of 160, 147, 148, 149, 150, 154, 158 and 186; Experiments 3 and 4. Cabrio® at ¼, ½ ¾ and full dilute label rate and inoculation with isolates 160, 158, 164 and 186. In addition, two experiments using same treatments for experiments 3 and 4 were carried out on detached leaves, with the addition of inoculation using dry spores in a spore settling tower in Experiment 4.

Table 4. The isolates of *Erysiphe necator* used in the preliminary experiments to determine the relationship between genotype and field resistance. The percentage the isolate that contained the G143A mutation was determined by Next- Generation Sequencing.

Isolate number	G143A %	G143 %
160	19	81
147	50	50
148	50	50
151	87	13
149	100	0
150	100	0
154	100	0
158	100	0
186	100	0

Table 5. Experimental methods used for the four preliminary experiments to determine the relationship between genotype and field resistance. [1]The full dilute label rate of Cabrio® (pyraclostrobin 250 g/L ai) is 40 mL/100L.

Experiment	Isolates used	Inoculation method	Rate of Cabrio®[1]	Use of shoots	Use of detached leaves
1	147, 151, 160	Spore suspension	Full	yes	no
2	147, 148, 149, 150, 154, 186, 160	Spore suspension	Full	yes	no
3	158, 164, 186 & 160	147, 148, 149, 150, 154, 186, 160	Full, ¾, ½, ¼,	yes	yes
4	158, 164, 186 & 160	Spore suspension & Spore settling tower	Full, ½, ¼,	yes	yes

Statistical analysis

The EC_{50} values (ug/mL) were determined for each isolate by Probit analysis using Genstat (18th edn, VSN International Ltd). The range, mean, median and standard deviation of the EC_{50} values for both the wild type, mixed genotype and mutant groups were calculated using Excel. The resistance factor (RF) values were calculated for each isolate as follows: RF=EC_{50} selected isolate/mean EC_{50} wild type isolates. RF is often used to define whether an isolate is resistant or not. However they vary considerably between studies, and are dependent on having unexposed isolates which are true wild type. As our exposure records are often only for recent years, it is difficult to be sure that we have isolates with no exposure. Therefore we have used EC_{50} as the determination for resistance not the RF, but have included it in this report as it is a commonly used classification of resistance.

Cross resistance between DMI fungicides was analysed using either Pearsons correlation coefficient if $\log_{10}$ EC_{50} values were normally distributed or Spearmans rank correlation coefficient if distributions were not normally distributed (Wu et al. 2014). The following number of isolates were used in analyses: Mycloss™ Xtra and Domark® (n=16); Mycloss™ Xtra and Topas® (n=14); and Topas® and Domark® (n=15).

Results/Discussion

Sample collection, isolate maintenance and storage

A total of 185 samples were collected from the commencement of the project in May 2013 to the end of the 15/16 season. One hundred and eight isolates were successfully established out of the total of 185 samples, and of these, 94 were suitable for fungicide sensitivity testing over the course of the project. Many of the samples received during the 2013/14 season were in poor condition or the powdery mildew was old and in the cleistothecial stage of the life cycle so that viable isolates of powdery could not be established and available for fungicide sensitivity testing. Improvements in methodology for sample collection were implemented for the two subsequent seasons, resulting in much improved isolation rate of powdery (refer to Final Report, section 6.1.1, Table 1). These included: the preparation of sampling kits and sending them to viticulturists, consultants and growers with clear instructions for collection and postage; targeting specific areas for sample collection; and a sample collection field trip to regions of Victoria, in particular to PEZ and PIZ areas where powdery isolation was carried out in the field to reduce the issues involved in bringing samples back to the laboratory at SARDI. As many isolates as possible out of the available 94 were tested for fungicide sensitivity. It was not possible to test all isolates to every selected fungicide because of the obligate biotrophic nature of *E. necator*. Cultures of powdery mildew need to be maintained on live grapevine leaves all year round and storage of this pathogen is extremely difficult. Isolates slowly become non-viable over time and/or overwhelmed with other fungal contamination which renders them either dead or not suitable for testing.

Storage testing

No viable powdery mildew was recovered from spores at either one day, one week, one month or three months after storage using any of the three storage methods. The amount of starting spore material needed by Stummer et al. (1999) was 5 mg FW or 2 mg dry weight and in our experiments, the yield of spores from one infected leaf ranged between 20 and 70 µg fresh weight, so to reach the desired amount of 5 mg FW required for viable recovery would require, at least, 70 leaves per isolate. In additions, Stummer et al. (1999) only used one isolate and they used sterile tissue culture plants to bulk up this isolate to obtain sufficient conidia; a process which took over a year to achieve (T. Zanker pers. comm). To obtain enough fresh leaves to bulk up a large number of isolates, as well as the time and resources for this that we had in this project is not feasible and is a major limitation to achieve successful storage for multiple isolates as we had in this project (>60 at any one time). Anecdotally, results of many discussions with other researchers throughout the world also working with *E. necator*, indicated that they have also never managed to successfully store this fungus.

Phenotype and genotype analyses **(all raw data is in a Table at the end of this document)**

DMIs

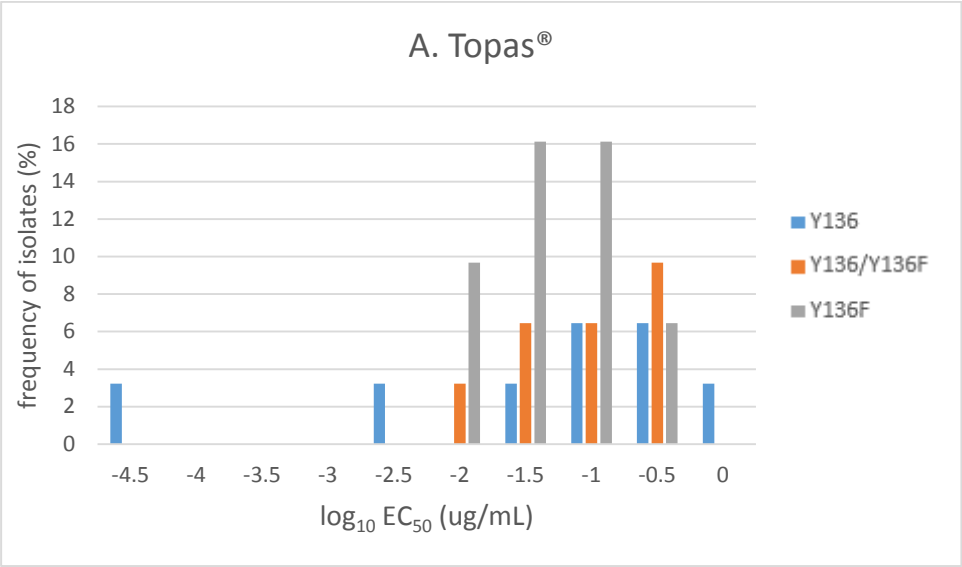
Of the 94 available isolates, 42 were phenotyped to Topas®, 66 to Mycloss™ and 21 to Domark®. The ranges of EC_{50} values and RFs for these isolates are: Topas® 0.0004-0.7

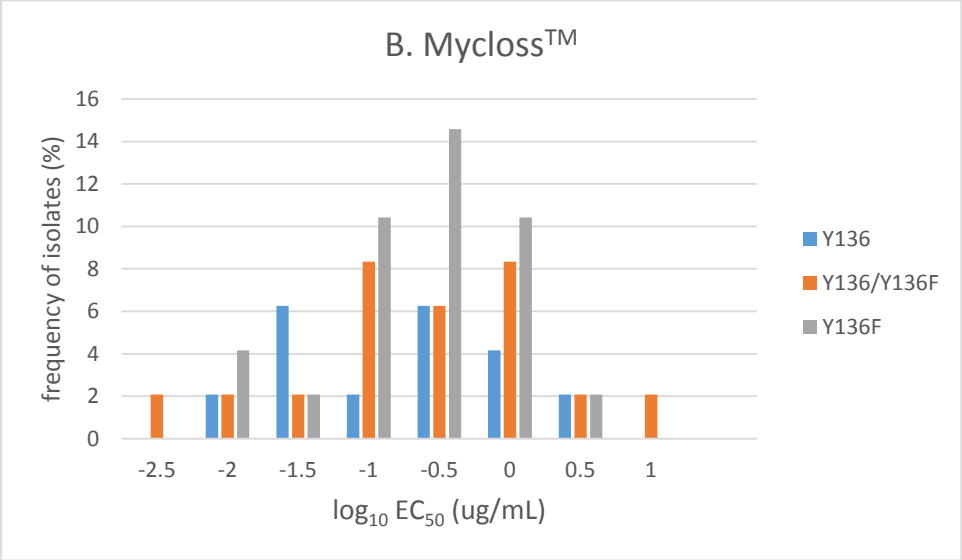
µg/mL and RF 0.001-3.2; Mycloss™ Xtra 0.2-6.6 µg/mL and RF 0.28-11.1; and Domark® 0.0008-0.05 µg/mL and RF of 0.8 -5.0 (Table 6). Detection of the Y136F allele was carried out by Sanger sequencing for 72 of the 94 isolates. From the 72 isolates genotyped, 19 were Y136/wild type, 21 were mixed Y136/Y136F and 32 were Y136F (Table 6 and 11).

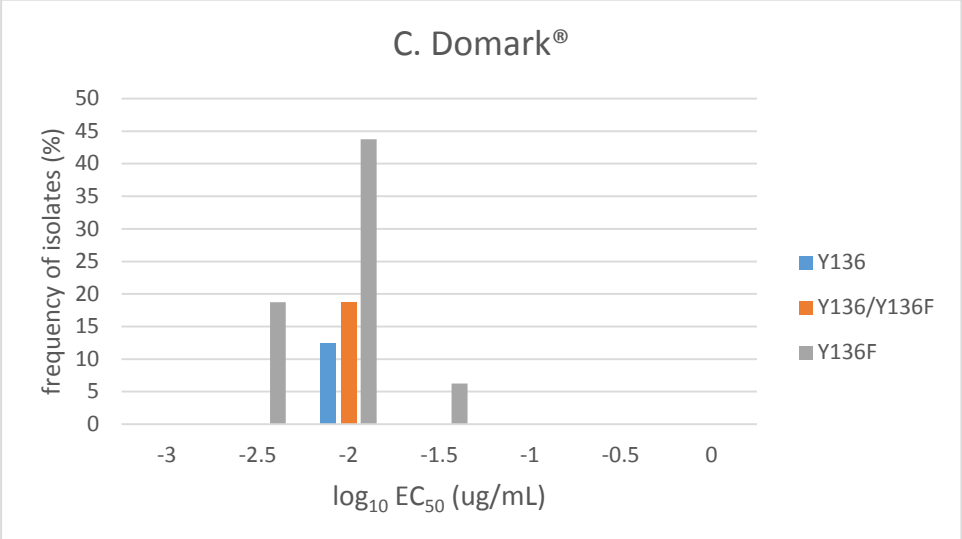
Table 6. The mean, median and range of EC_{50} values (µg/mL) for isolates tested to the DMI fungicides, Topas®, Mycloss™ and Domark®. Isolates are categorised by genotype as either wild type (Y136), mutant (Y136F) or mixed Y136/Y136F genotype.

Isolate group (number of isolates)		**Topas®**	**Mycloss™ Xtra**	**Domark®**
Y136 (19)	mean	0.22	0.6	0.01
	median	0.25	0.3	0.01
	range	0.0004-0.7	0.01-4	0.01
Y136/Y136F (21)	mean	0.15	0.9	0.01
	median	0.15	0.4	0.01
	range	0.01-0.5	0.002-6.6	0.006-0.02
Y136F (32)	mean	0.12	0.5	0.01
	median	0.05	0.3	0.008
	range	0.009-0.5	0.01-3.4	0.0008-0.05
RF	mean	0.739626	1.9	1.0
	median	0.31	2.6	0.7
	range	0.001-3.2	0.28-11.1	0.08-5

The majority of isolates were phenotypically sensitive to the three DMIs tested, with the EC_{50} <1.0 µg/mL for all isolates tested to Topas® and Domark®, and 58 out of 67 isolates with Mycloss™. However, based on RFs a small number of isolates showed a small decrease in sensitivity, in particular for Mycloss™, with six isolates from SA, Vic and NSW, having a RF >5. Several of these vineyards were concerned about field failure to DMIs which is reflected by our phenotype results. Although, phenotypic resistance to DMIs does not appear to be widespread, the Y136F allele was present in over 60% of isolates. Y136F is considered a precursor mutation for the potential resistance development in *E. necator*, and other yet to be elucidated mechanisms are thought to be involved including the overexpression of cyp51 (Jones et al. 2014, Frenkel et al. 2015). Our results found there was no obvious relationship between EC results for any DMI fungicide tested and the absence/presence of Y136F for either Topas®, Mycloss™ or Domark® (Figs 1A-C), nor for EC values and allele frequency determined by NGS. These results are consistent with other studies which have found that resistant isolates do not always carry the Y136F mutation and neither are isolates always resistant when they have the Y136F mutation (Dèlye et al. 1997, Miazzi et al. 2011).


A. Topas®
frequency of isolates (%)
18
16
14
12
10
8
6
4
2
0
-4.5
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
log10 EC50 (ug/mL)
Y136
Y136/Y136F
Y136F


B. Mycloss™
frequency of isolates (%)
16
14
12
10
8
6
4
2
0
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
log10 EC50 (ug/mL)
Y136
Y136/Y136F
Y136F


C. Domark®
frequency of isolates (%)
50
45
40
35
30
25
20
15
10
5
0
-3
-2.5
-2
-1.5
-1
-0.5
0
log10 EC50 (ug/mL)
Y136
Y136/Y136F
Y136F

Figs 1 A-C. The frequency distributions (%) of the $\log_{10}$ EC_{50} (µg/mL) values for isolates of genotypes Y136, mixed Y136/Y136F and Y136F tested with A. Topas®, B. Mycloss™ and C. Domark®.

The finding of mixed Y136/Y136F genotypes was initially unexpected. It could be a results of cross contamination of isolates and/or DNA, however, extreme care was taken to avoid cross contamination in both culturing and in DNA handling, so although possible, it is unlikely. Multiple copies of the cyp51 gene occur (Jones et al. 2014) but these authors found that isolates containing multiple copies were mostly those with the mutant allele Y136F and not mixed genotype. However, recent work by Rallos et al. (2014) found mixed Y136/Y136F genotypes of *E. necator* using qPCR. Further work is needed to understand this phenomenon.

Sanger sequencing gives presence or absence of a particular allele where as NGS can reveal the allele frequencies. NGS for the cyp51 alleles were performed for 57 out of the 72 isolates that were sequenced using Sanger methodology. The results for Sanger and NGS sequencing were broadly consistent with one another, with the mean and median allele frequency being highest for Y136F and mixed genotypes and lowest for Y136 isolates (Table 7). However, four isolates found to be Y136 wild type using Sanger were found to be >80% Y136F by NGS.

Table 7. The percentages (mean, median and range) of Y136F genotype determined by Next-Generation Sequencing (NGS) and the genotypes determined by Sanger sequencing.

Genotype determined by Sanger sequencing	**Y136F % determined by NGS**		
	range	**mean**	**median**
Y136	0-100	44.12308	41
Y136/Y136F	39-100	69.68333	75
Y136F	59-100	91.63077	91

The relationship between previous exposure to DMIs and presence or absence of the Y136F allele was not consistent. Of the three out of five unexposed isolates one was wild type, one mixed Y136/Y136F, one Y136F and two unknown. Nine of the 32 'exposed' were also wild type. One of the unexposed isolates was from a 20 yr+ organic vineyard yet was mixed Y136/Y136F and another unexposed isolate was from a CER at SARDI where DMIs had never been applied yet was Y136F. In both these cases it is likely that powdery mildew spores have come in from neighbouring vineyards which have used DMIs. The organic vineyard was close, <100 m, to a vineyard which uses DMIs and likewise, although no DMIs were used in the research CER, other vines are located within the Waite Campus and powdery could have easily have been dispersed from them to the CER. These results indicate that the history of exposure is not necessarily useful to determine if isolates are likely to be sensitive or not.

Detailed spray records for the 1-2 seasons prior to sample collection were obtained for 67 vineyards and revealed that Topas® and Mycloss™ Xtra were the most commonly used fungicides, at 45 and 39% respectively. These were followed by unidentified DMI products (12%) then fenarimol (Rubigan®) (5%) and Domark® (1%). Triadimenol (Bayfidan®) and tebuconazole were also used in a couple of vineyards but isolates from these samples were not established, therefore not tested against DMIs. The range of the number of DMIs used per season in commercial vineyards was between 0-3 applications/season, with 33% of vineyards not using a DMI, 30% using them twice then 27% using them once.

A selection of isolates was also phenotyped with two or more of the DMIs tested. Statistical analyses revealed that there were no significant correlations between the $\log_{10}$ EC_{50} values for isolates tested with either Mycloss™ Xtra and Domark® (n=16), Mycloss™ ®and Topas® (n=14) or Topas® and Domark® (n=15). Although, cross resistance has been demonstrated among some DMI fungicides (Ypema et al. 1997, Savocchia et al. 2004), in our study, those isolates showing a decreased sensitivity to Mycloss™ did not show a corresponding decrease in sensitivity to either Topas® or Domark®.

QoIs

Forty-nine isolates of *E. necator*, obtained in 2013/14, were tested against Cabrio® using a full bioassay with 6-7 concentrations of each fungicide (Fig 2) and a subset of six tested with Amistar®. The range of EC_{50} values and RFs for the isolates tested with Cabrio® was 0.008 to 14.4 and 0.004 to 6.85 respectively (Table 8). For Amistar®, the EC_{50} values ranged from 0.01-1.1 but RFs were not calculated as no wild type isolates were available; all were mixed G143/G143A genotype. Overall, for Cabrio®, 27 % of isolates had an EC_{50} >1.0 µg/mL and a RF > 3 and were considered phenotypically resistant. Isolates came from regions in SA, WA, Vic, NSW and Tas.

Cross resistance between Cabrio® and Amistar® was not demonstrated possibly because only a small number of isolates were tested with Amistar®.

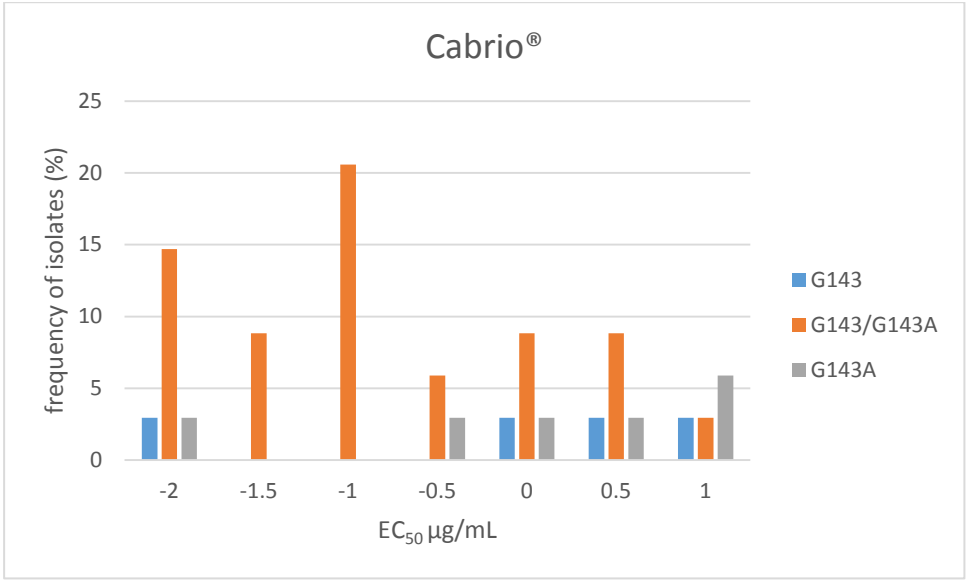


Fig 2. The frequency distributions (%) of the $\log_{10}$ EC_{50} (µg/mL) values for isolates of genotypes G143, mixed G143/G143A and G143A tested with Cabrio® (pyraclostrobin 250g/L ai).

Table 8. The mean, median and range of EC_{50} values (µg/mL) for isolates tested to the QoI fungicide, Cabrio® (pyraclostrobin 250g/L ai). Isolates are categorised by genotype as either wild type (G143), mutant (G143A) or mixed G143/G143A genotype.

	mean	median	range
G143(4)	0.0095	1.5	0.01-5.8
Mixed (24)	2.550833	0.1	0.008-14.4
G143A (6)	0.021667	1.9	0.01-10
RF	0.859776	0.14	0.004-6.85

Sixty isolates obtained during 2014-2016 were tested for sensitivity to Cabrio® using a discriminatory dose. Assessment of sensitivity was based on the following (Beever et al. 1983):

- Sensitive-=no growth or growth <20% of control at 1.0 µg/mL
- Weakly resistant=growth at 1.0 µg/mL is between 20 and 60% of control
- Moderately resistant=growth at 1.0 µg/mL is >60% of control but no growth at 10.0 µg/mL
- Resistant= growth at 10.0 µg/mL

Based on this classification, 23 isolates were sensitive, 14 weakly resistant, 12 were moderately resistant and 11 resistant. Twenty-one isolates out of 66 were genotyped by NGS. The results did not support any relationship between phenotype and genotype (Table 9) as each sensitivity classification, including sensitive, had a high frequency of the G143A allele.

Table 9 The percentage of G143A allele, mean and range, determined by Next Generation Sequencing (NGS) and the isolates classified as sensitive, weakly resistant, moderately resistant and resistant using a discriminatory dose assay for Cabrio® (pyraclostrobin 250g/L ai).

Sensitivity classification (number of isolates)	G143A % using NGS	
	mean	range
Sensitive (23)	83	25-99
Weakly resistant (14)	53	8-99
Moderately resistant (12)	93	87-97
Resistant (11)	85	52-100

Thirty-four of the 49 isolates tested for sensitivity with Cabrio® from 2013/14 were sequenced using Sanger methodology and of these 34, 20 were sequenced for allele frequency by NGS. Sanger results revealed four were G143, six were G143A and the remaining 24 were mixed G143/G143A genotype (Fig 2) however, there was a poor relationship between these Sanger and NGS results as shown by the mean allele frequencies (from NGS) were all >70% for each Sanger genotype category i.e. G143, mixed and G143A (Table 10). However, being only based on 20 isolates, it is premature to make any firm conclusion about this lack of relationship apart from saying it is more desirable to understand allele frequency than just presence or absence of an allele. Following on, an attempt to develop qPCR as a method to determine allele frequency as an alternative to the relatively expensive method of NGS was made. However, qPCR was unable to be optimised within the timeframe.

For all samples obtained during 2014 to 2016, genotyping was carried out using NGS only. All isolates were successfully sequenced using NGS for the cyp51 gene to detect the allele associated with DMI resistance (Y136F) however, despite 3 attempts, about one third of isolates failed to yield any cytb sequence data. Currently, we are re-extracting DNA from any isolates that failed to amplify and these will be sequenced by NGS and data will be available in the future.

Table 10. The percentages (mean, median and range) of G143A genotype determined by Next Generation Sequencing (NGS) and the genotypes determined by Sanger sequencing.

Genotype as determined by Sanger sequencing	**G143A % using NGS**		
	mean	**median**	**range**
G143	79.2	na	68.3-90.1
G143/G143A	74.6	87.1	2.8-100
G143A	89.3	86.5	84.2-100

In general, isolates with an EC_{50} >1.0 µg/mL are considered resistant and are commonly associated with high frequencies of the G143A mutation (Rallos 2014, Colcol and Baudoin 2016) however, our results are not always consistent with this, as we found some G143 isolates to have an EC_{50} >1.0 µg/mL and some G143A isolates to have low EC_{50} values as low as 0.01 µg/mL (Table 8) and mean G143A frequency was 83% for those isolates deemed sensitive in the discriminatory dose assay (Table 9). Colcol and Baudoin (2016) also found some isolates that lacked G143A to be phenotypically resistant and some that were phenotypically resistant to have G143. Whilst to some extent the inconsistencies between phenotype and genotype in these data illustrate the natural variability seen with in a small population (Colcol and Baudoin 2016), it highlights that a potential diagnostic test based on either just either phenotype or genotype alone as a reliable and accurate test may not always be easy to interpret. Also, understanding the phenotype/genotype results and linking these to field efficacy is critical.

Spray diary records from 67 vineyards showed that Cabrio® was the most commonly used QoI (55%), followed by the use of unidentified QoIs (23%), then trifloxystrobin (Flint®)(20%) and lastly azoxystrobin (Amistar®)(2%). The range of number of QoIs applied for the season was between 0 and 2, (mean number of sprays 0.7) with 50% of vineyards not using a QoI at all.

Population studies

DNA extracted from each swab with powdery mildew was subjected to NGS and the results show the % of both wild type and mutant allele for each swab collected within a particular block (Figs 3A-D). The results give a snapshot of the range of allele frequencies distributed throughout a vineyard or block, with four examples shown in Figs 3A-D. Each of the five data sets for both Y136F and G143A differed both in the mean of the distribution and the distribution shape (Fig 4). Data were not normally distributed and confidence intervals based on normal distributions are therefore not reliable. Simulations used to estimate quantiles were the best method to estimate the required number of samples. If the following were considered acceptable for research purposes, a standard error of 10% and a 95% confidence interval that the mean resistance allele frequency is within a range of 20%, then 10 samples would be adequate, based on our data sets. For example, if we are to be 95% confident that the mean allele frequency falls within a range of between e.g. 20-40 or e.g. 75-95, with a 10% SE, then we need 10 samples to achieve this. The greater the confidence interval and smaller SE required, the more samples are needed (Correll 2016). Previous work on detection of fungicide resistance in *Botrytis* carried out by our lab at SARDI has found that 30 bunches are needed to get an adequately detect resistance. Our results, here, found that 10 samples may be sufficient to adequately detect resistance for powdery mildew in a vineyard.

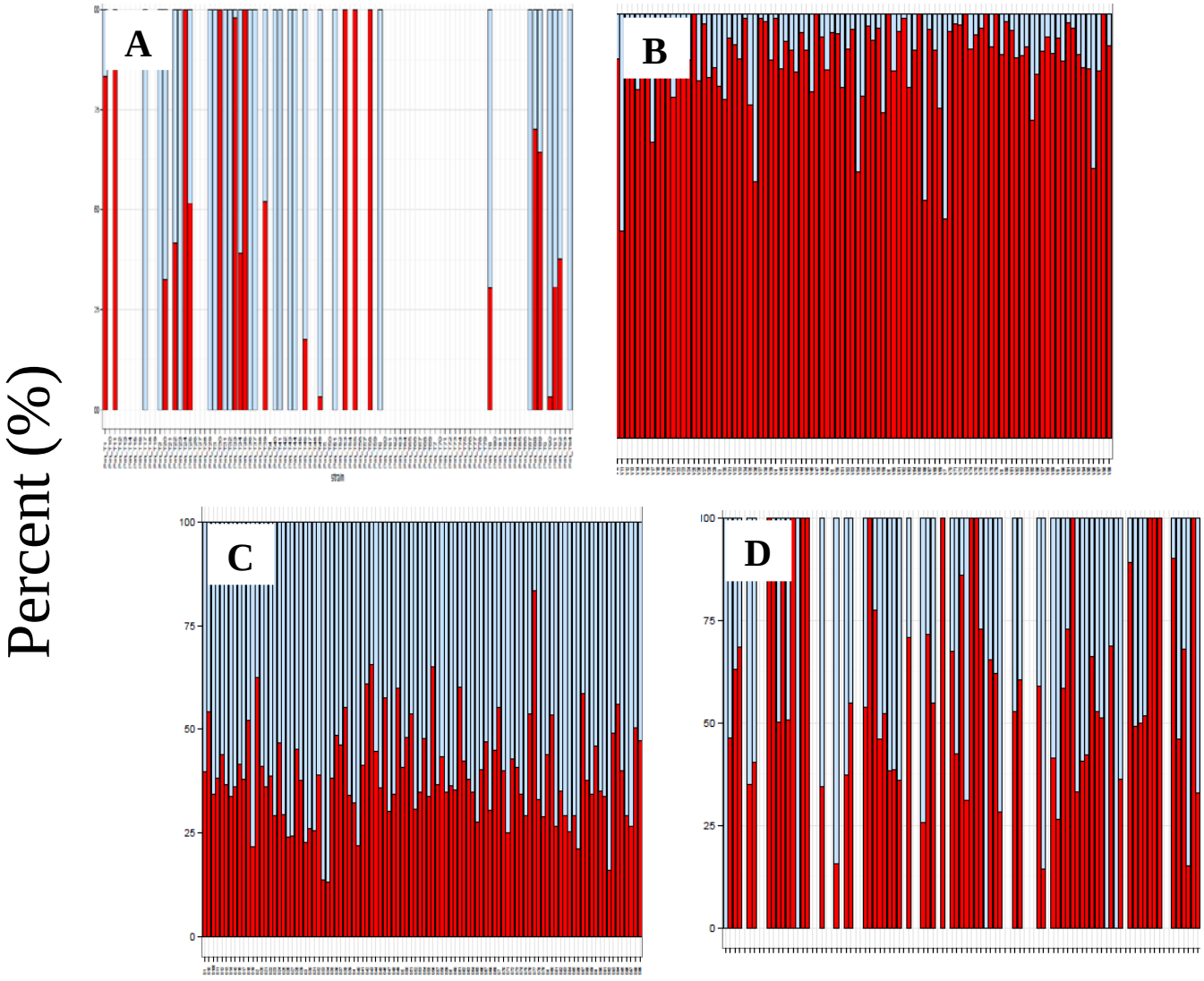


Fig 3. Wild type and mutant allele frequencies distributed in four vineyards in South Australia and Victoria for **A.** Y136/Y136F, **B-D** G143/G143A . Each figure shows one vineyard/block in which ~100 samples of powdery mildew were collected on sterile cotton swabs. Each vertical bar represents one swab of powdery mildew obtained from a leaf or bunch with a vineyard. DNA was extracted from the powdery mildew on each swab and Next Generation Sequencing carried out to determine the percentage of wild type allele (Y136 or G143) and mutant allele (Y136F or G143A). The red part of each bar shows the % mutant allele (either Y136F or G143A) and the blue part of each bar shows the % wild type allele (Y136 or G143). The white areas or absence of bars show where there was insufficient powdery mildew on a swab to enable DNA of sufficient quality or quantity to be obtained for NGS to be carried out.

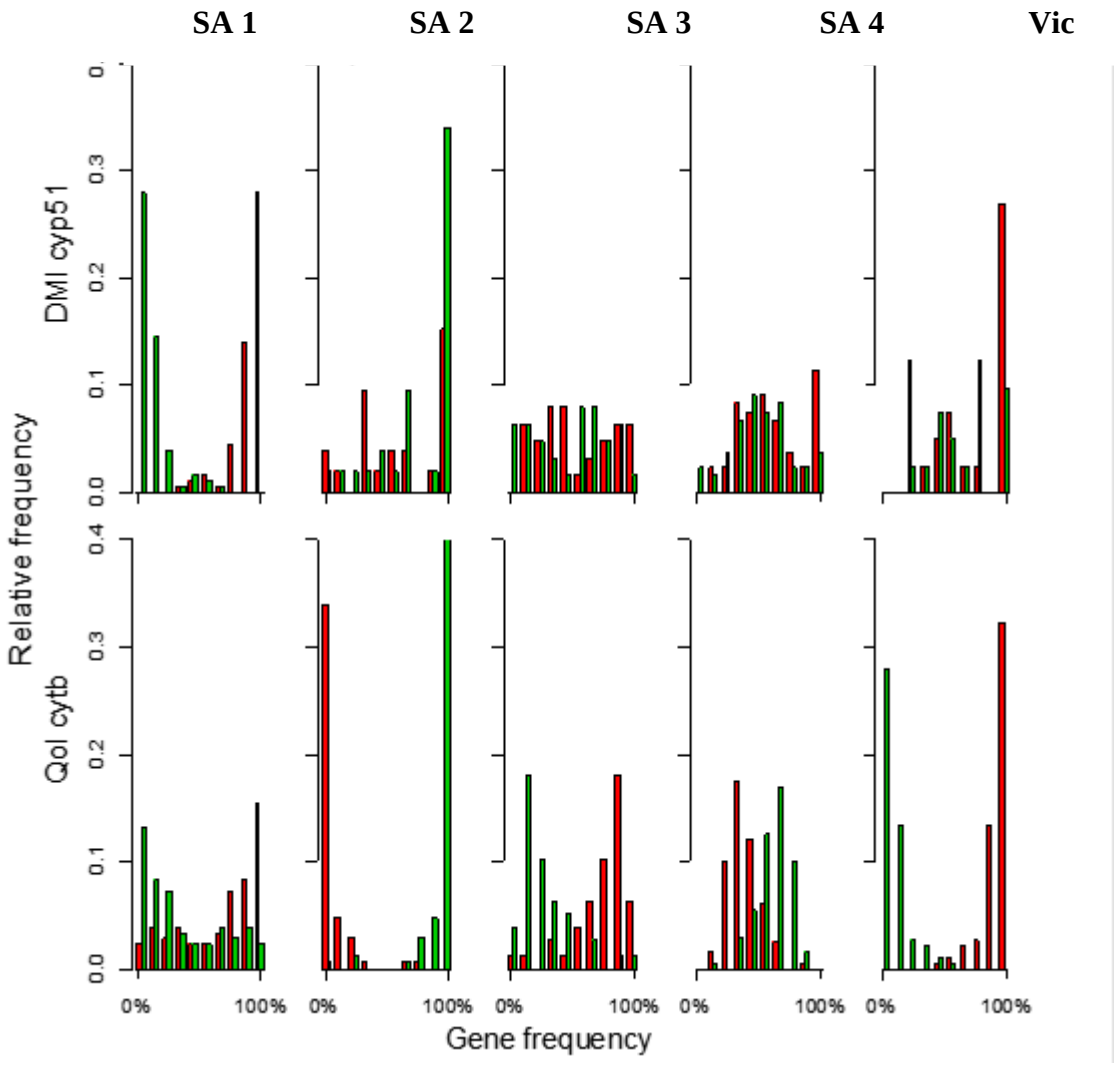


Fig 4. Relative frequencies of the G143A and Y136F alleles (in red) and wild type alleles G143 and Y136 (in green) from the cytb and cyp51 genes, obtained by Next Generation Sequencing for ~ 100 samples taken from five vineyards (four South Australian, SA and one Victorian, Vic) (Ray Correll, Rho Environmetrics, SA).

Other important findings that need to be considered for the future:

- Consistency of sampling technique is essential for enough powdery mildew to be collected on each swab to enable sufficient, good-quality DNA to be extracted for use

in NGS. For example, 97 swabs were collected from the vineyard in Victoria, but only 28 were suitable for NGS to detect Y136F (Fig 3A), whereas all were suitable for detection of G143A (Fig 3B).

- The distribution of powdery in most vineyards is often patchy affecting the chosen sampling strategy e.g. stratified vs random, which then affects subsequent data analyses and interpretation. Future studies should include assessment of powdery mildew incidence and severity at the same time as sample collection, to be carried out by selected personnel to decrease variability.

Relationship of mutant allele frequency to field resistance-Preliminary studies

No isolates of powdery mildew grew, even those containing 100% G143A allele, with Cabrio® at the full label rate on leaves on shoots in experiments 1 and 2. In exp 3, both resistant and sensitive grew at 0, ¼ and ½ label rates of Cabrio® but mildew growth was patchy and inconsistent. Similar results were found for the same treatments using detached leaves. Results for exp 4 and associated detached leaf experiments, were also similar. There was also some powdery mildew on a few of the uninoculated controls in two of the experiments indicating a low level of cross contamination. Changes to methodology are currently being evaluated for use in further experiments, including

- Using rooted cuttings in potting media in water well pots, to alleviate the need to lift the PVC cylinders to water to decrease cross contamination risks and for ease of handling.
- Increase spore suspension concentration
- Double inoculation to ensure adequate infection.

One of the challenges highlighted by these experiments is the availability of isolates of varying genotype in particular wild type i.e. G143 and Y136 isolates. Despite efforts to obtain unexposed isolates with the wide spread use of fungicides and the mobility of powdery mildew spores, this has proved challenging. In addition, as discussed previously, the challenges in having to constantly maintain cultures on to fresh young grapevine leaves. It can be difficult to keep valuable isolates alive and continuously available for several years. We are currently evaluating the use of tissue culture plantlets, obtained from CSIRO Adelaide, as well as using grapevine seedlings in isolation tubes to maintain isolates without compromising isolate purity by cross contamination.

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Table 11. Origin of *Erysiphe necator* isolates used in this study. The EC_{50} µg/mL of isolates exposed to a range of concentrations of fungicides and genotype data from both Sanger and Next Generation Sequencing for the cyp51 and cytb genes. Selected isolates were tested with Cabrio ® using a discriminatory dose assay only and were classified as follows [1] S=sensitive, WR=weakly resistant, MR=moderately resistant, R=resistant.

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
ID1	May-13	SA	Adelaide Plains	Cabernet	0.1			Y136	35	0.0005			
12a	29-May-13	WA	Swan District	Chard	0.2			Y136	50.4	0.003		G143	35.8
TW1	May-13	SA	Adelaide Plains	Sultana	0.1					0.003			
TW2	May-13	SA	Adelaide Plains	Sultana	0.4			Y136		0.01		mix G143A/G143	
15a	27-Nov-13	SA	Adelaide Plains	Chard	0.04			Y136F		1.4		mix G143A/G143	
15b	27-Nov-13	SA	Adelaide Plains	Chard	0.02	1.2				2.3			
16a	27-Nov-13	SA	Adelaide Plains	Grenache				Y136F		0.09			
16b	27-Nov-13	SA	Adelaide Plains	Chard	0.05			Y136F	58.9	2.3		mix G143A/G143	88.7
17a	27-Nov-13	SA	Adelaide Plains	Chard						0.7		G143A	
24c	13-Dec-13	SA	Adelaide Hills	Chard				Y136F	100	0.03		mix G143A/G143	50.6, 75.9
25a	16-Dec-13	SA	Barossa	Crouchen	0.1				100	1		mix G143A/G143	100, 87.1
25b	16-Dec-13	SA	Barossa	Crouchen						5.4			100, 87.1
25c	16-Dec-13	SA	Barossa	Crouchen						0.7			
27a	16-Dec-13	SA	Langhorne Creek	Verdelho	0.06	0.6		mixY/F	58.5, 44.3	2.2		G143	68.3, 90.1
28a	17-Dec-13	SA	Barossa	Chard	0.3			Y136F/Y136	79.4	0.2			100

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
30a	17-Dec-13	SA	Barossa	Shiraz	0.05					5.8		G143	
32a	17-Dec-13	SA	Barossa	Chard	0.003			Y136		0.1		mix G143/G143A	
33a	17-Dec-13	SA	Barossa	Chard	0.04	0.9	0.01	Y136F	100	14.4	0.9	mix G143/G143A	100
34a	18-Dec-13	SA	Langhorne Creek	Chard					34.3	0.1			100
34b	18-Dec-13	SA	Langhorne Creek	Chard	0.1		0.006	Y136F/Y136		0.4		mix G143/G143A	
35a	9-Jan-14	SA	Riverland	Chard	0.05		0.007			0.4			
40a	15-Jan-14	WA	Margaret River	Semillon	0.01					0.008		G143A	
40b	15-Jan-14	WA	Margaret River	Semillon									
42a	15-Jan-14	WA	Margaret River	Chard									
44a	15-Jan-14	SA	Adelaide Hills	Tempranillo						0.01		mix G143/G143A	
50a	21 Jan 2014	SA	Riverland	Chard	0.06			Y136F	100	0.01		mix G143/G143A	100
53a	21 Jan 2014	SA	Riverland	unknown	0.01			Y136/Y136F	100	0.01		mix G143/G143A	21.3
57a	21 Jan 2014	SA	Riverland	Chard	0.05			Y136/Y136F	38.9	0.01			100
58a	21 Jan 2014	SA	Riverland	Chard	0.5	0.4	0.013	Y136/Y136F	75.7	0.06		mix G143/G143A	73.4
59a	21 Jan 2014	SA	Riverland	Chard					100	0.01		G143A	
62a	21 Jan 2014	SA	Riverland	unknown	0.009	0.6	0.008	Y136F	100	0.03	0.01/0.07	mix G143/G143A	
64a	21 Jan 2014	SA	Langhorne Creek	Chard	0.15	2.4	0.016	Y136/Y136F	49.9	0.5		mix G143/G143A	100

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
67a	21 Jan 2014	SA	Langhorne Creek	unknown	0.2			Y136/Y136F		0.1			
70a	23 Jan 2014	WA	Margaret River	Chard	0.03				100	0.02			67.1
75a	23 Jan 2014	WA	Margaret River	Chard	0.5		0.005	Y136F	92.5	4.1		mix G143A/G143	38.9
76a	23 Jan 2014	WA	Margaret River	Chard	0.1			Y136F	100	2.7		mix G143A/G143	2.8
77a	24 Jan 2014	NSW	Hilltops	Shiraz	0.07		0.006	Y136F	100	0.02		mix G143A/G143	55.4
78a	31 Jan 2014	VIC	Myrrhee	Chard	0.3	0.8	0.006	Y136	16	9.7		G143A	100
78b	31 Jan 2014	VIC	Myrrhee	Chard	0.5			Y136		0.5		mix G143A/G143	
79a	31 Jan 2014	VIC	King Valley	Chard	0.7	0.8	0.01	Y136	100	0.9	0.01	mix G143A/G143	54.9
80a	31 Jan 2014	VIC	King Valley	Chard	0.0004			Y136	41	0.01		mix G143A/G143	100
85a	03 Feb 2014	WA	Margaret River	Pinot Noir	0.03	0.04	0.05	Y136F	100	>10		G143A	84.2, 84.2
86a	03 Feb 2014	WA	Margaret River	Verdelho	0.3			Y136F		0.2		mix G143A/G143	
89a	07 Feb 2014	SA	Padthaway	Chard	0.4			Y136		0.7		mix G143A/G143	
90a	07 Feb 2014	SA	Padthaway	Chard	0.4		0.014	Y136F		0.06		mix G143/G143A	
91a	20 Feb 2014	Tas	Tamar Valley	Sauvignon blanc	0.03			Y136		1.9		mix G143A/G143	
92a	20 Feb 2014	Tas	Tamar Valley	Pinot Noir	0.05	0.8		Y136F		0.1		mix G143A/G143	

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
93a	20 Feb 2014	Tas	Tamar Valley	Riesling	0.07	0.01	0.01	Y136	50	1.6		mix G143A/G143	69.1
94a	20 Feb 2014	WA	Great Southern	Chard	0.01			Y136F	100	0.2		mix G143A/G143	100
100a	20 Feb 2014	WA	Great Southern	Pinot Noir	0.2	0.7	0.0008	Y136F	100	3.9			92.8
108b	11 April 2014	SA	Adelaide Hills	Chard	0.05	1.4	0.003	Y136F		0.03	0.07	mix G143A/G143	
117	18-Nov-14	SA	Coonawarra Sth	Chard		0.13			70	S			
118	Nov-14	NSW	Pokolbin/Hunter	Chard		0.04		Y136/Y136F		S			
122	5-Dec-14	NSW	Wagga	Sultana		0.3			50	S	0.03		
123	18-Dec-14	NSW	Orange	Chard		0.3	0.002		75	WR			
124	17-Dec-14	SA	Adelaide Hills	Chard		0.5	0.01	Y136F	99	WR			
125	17-Dec-14	SA	Adelaide Hills	Chard		0.3	0.005	Y136F	100	WR			
126	17-Dec-14	SA	Adelaide Hills	Chard		0.24	0.006		73	S			
127	17-Dec-14	SA	Adelaide Hills	Chard		0.4	0.007	Y136F	100	WR	1.2		
128	17-Dec-14	SA	Langhorne Creek	Verdelho		0.07			77	MR	1.1		
129	22-Dec-14	NSW	Wagga Wagga	Sultana		0.2		Y136F	100	S			25
130	22-Dec-14	NSW	Wagga Wagga	Merlot		0.1		Y136/Y136F	75	S	0.03		
134	16-Jan-15	SA	Adelaide Hills	Chard		0.04			95	S	na		
135	16-Jan-15	SA	Adelaide Hills	Chard		0.4		Y136F	100	WR			
136	16-Jan-15	SA	Adelaide Hills	Chard		0.6			78	WR			
137	16-Jan-15	SA	Adelaide Hills	Chard		0.1		Y136F	100	S			

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
138	16-Jan-15	SA	Adelaide Hills	Chard		0.03		Y136F	100	S			
139	16-Jan-15	SA	Adelaide Hills	Chard		0.3			68	S			
141	15-Jan-15	SA	Adelaide Plains	Grenache		4		Y136	80	WR			8
143	16.01.15	WA	Frankland River	Chard		0.28		Y136F	90	WR			
144	20-Jan-15	Vic	Bellarine peninsula	Chard		0.11		Y136/Y136F	63	R			96.8
145	20-Jan-15	Vic	Mornington	Pinot		0.06		Y136	85	S			
146	20-Jan-15	Vic	Mornington	Pinot		3.4		Y136F	85	MR			92.5
147	20-Jan-15	Vic	Mornington	Pinot		0.01		Y136F	86	R			51.6
148	20-Jan-15	Vic	Mornington	Chard					69.2	WR			50.9
149	21-Jan-15	Vic	Yarra Valley	Chard		6.6		Y136/Y136F	60.4	R			98.4
150	21-Jan-15	Vic	Yarra Valley	Chard		0.08		Y136F	92	S			98.9
151	21-Jan-15	Vic	Yarra Valley	Chard		0.2			42.6	MR			87
152	21-Jan-15	Vic	Yarra Valley	Chard		0.3			59	WR			98.7
153	22-Jan-15	Vic	Strathbogie Ranges	Chard		0.3		Y136	67	MR			97.3
154	22-Jan-15	Vic	Strathbogie Ranges	Chard		0.07		Y136F	74	R			100
155	22-Jan-15	Vic	Strathbogie Ranges	Chard		0.5			65	S			98.7
156	23-Jan-15	SA	Adelaide Hills	Chard		0.02		Y136	80	WR			
157	22-Jan-15	NSW	Orange	Chard		1		Y136/Y136F	80	WR			
158	29-Jan-15	Vic	Banksdale	Prosecco		0.5		Y136/Y136F	65	R			100
159	29-Jan-15	Vic	Myrrhee	Pinot Gris		0.03		Y136	65	S			100

isolate code	date collected	state	wine region	variety	Topas® EC_{50} µg/mL	Mycloss™ EC_{50} µg/mL	Domark® EC_{50} µg/mL	DMI cyp51 using Sanger sequencing	% Y136F using NGS	Cabrio® EC_{50} µg/mL [1]	Amistar® EC_{50} µg/mL	QoI cytb gene using Sanger sequencing	% G143A using NGS
160	29-Jan-15	Vic	Myrrhee	Pinot Gris		0.06			56				19.4
161	29-Jan-15	SA	Barossa	Chard		0.3			85.3	S			95.8
164	9-Feb-15	TAS	S/E Tas	Chard		0.2			100	R			100
166	27-Feb-15	SA	Langhorne Creek	Chard		0.08		Y136F	65	S			
167	27-Feb-15	SA	Langhorne Creek	Cabernet		0.06		Y136/Y136F	45	MR			
168	4-Mar-15	SA	Adelaide Hills	Chard		0.3		Y136	74	MR			96
170	5-Mar-15	Vic	Yarra Valley	Chard		0.3		Y136/Y136F	85	MR			
172	13.03.15	SA	Langhorne Creek	Merlot		0.2		Y136	80	MR			
173	13.03.15	SA	Meadows	Chard		0.01		Y136/Y136F	90	WR			
174	13.03.15	SA	Meadows	Chard		0.3		Y136F	85	R			56
176	13.03.15	SA	Langhorne Creek	Chard		0.8		Y136/Y136F	70	MR			
177	13.03.15	SA	Barossa	Shiraz		0.02		Y136	95	S			
178	13.03.15	SA	Barossa	Chard	0.05	0.002		Y136/Y136F	75	R			100
179	13.03.15	SA	Barossa	Chard		1				R			
180	13.03.15	SA	Barossa	Chard		0.7		Y136/Y136F	80	R			64
182	20.03.15	SA	Adelaide Plains	Cabernet		0.3	0.025	Y136F	80	S			
183	24.04.15	SA	Barossa	Shiraz		0.07		Y136/Y136F	80	S			
184	07.12.15	NSW	Riverina	Chard		0.9				R			
185	08.12.15	SA	Adelaide Plains	Cab sauv					0	S			
186	10.12.15	SA	Riverland	Chard		1.6				S			
187	10.12.15	SA	Riverland	Chard						S			
188	10.12.15	SA	Riverland	Chard						S			

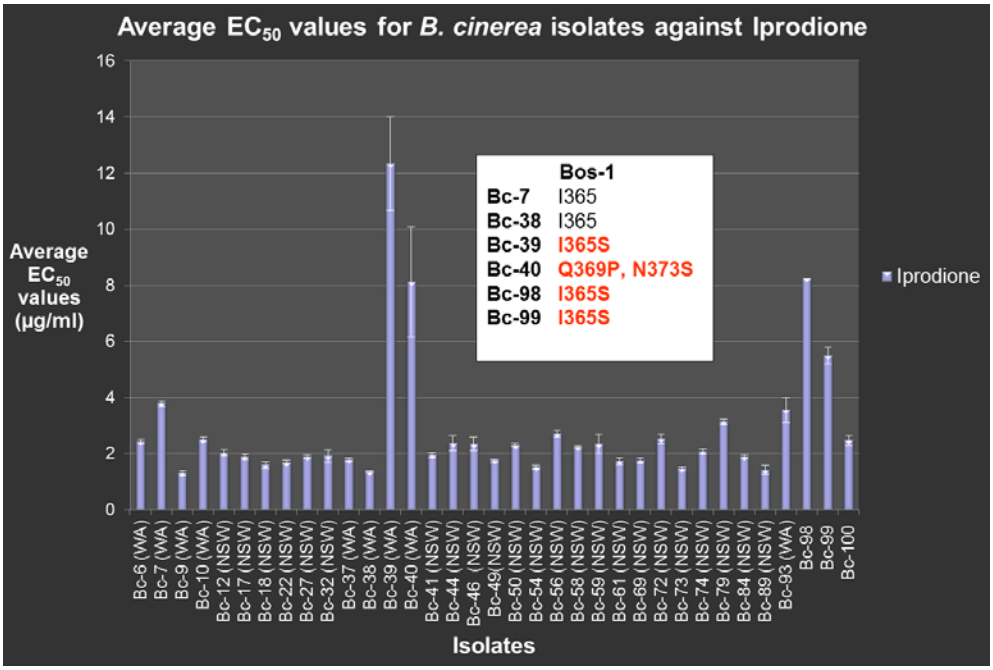
9.4.3. Curtin: Botrytis and modelling

Understanding fungicide resistance in powdery mildew, downy mildew and botrytis

Part A: Fungicide resistance analysis of *Botrytis cinerea* populations and mechanisms of resistance

Part B: Modelling analysis of risk resistance development in *B. cinerea*, *Erysiphe necator* and *Plasmopara viticola*

Part C: A model for the economics of fungicide application programs when resistance is developing



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Fungicide resistance analysis of *Botrytis cinerea* populations and mechanisms of resistance

Abstract

Botrytis cinerea is a necrotrophic fungal pathogen responsible for the most important disease of grapevines in Australia after powdery mildew, with a cost to the grape and wine industry on average of $50M per annum (Scofield et al. 2010). Along with cultural practices, the main approach to control *Botrytis* in vineyards is through the routine application of fungicides beginning at flowering and continuing right through to seven days before harvest. *B. cinerea* is labelled a classic 'high risk' pathogen for resistance development due to its short life cycle and high reproductive rate (Brent KJ, Hollomon DW. 1998). The aim of this research was to provide current data on fungicide sensitivity levels in grapevines to anilinopyrimidines (pyrimethanil) and dicarboximides (iprodione), and the first data on sensitivity to hydroxyanilides (fenhexamid) and SDHIs (boscalid). A population of 745 isolates from across six states was screened against boscalid, fenhexamid, iprodione and pyrimethanil, showing resistance frequencies of 3.0%, 2.1%, 12.2% and 7.7%, respectively. Interestingly, 8.6% and 6.4% of isolates were resistant to one or more than one mode of action fungicide groups, respectively. Out of 95 sampling sites tested, 41% and 23% exhibited at least one isolate resistant to one or more than one mode of actions, respectively. In addition to this, the molecular mechanisms behind the resistance detected were investigated revealing several mutations that could be correlated with resistance. Mutations in target genes were identified for all four fungicide groups; boscalid (H272R, N272Y), fenhexamid (F412S), iprodione (I365S, Q369P + N373S, I365S + D747N) and pyrimethanil (L8P, D416E). There is also genotypic evidence for isolates that besides having target site mutations, they may exhibit multi-drug resistance (Kretchmer et al. 1999). Further research is needed to understand the relationship between these *in vitro* results and field efficacy. Ultimately, prudent management of fungicide applications is needed to ensure that these strains do not become a widespread and significant problem.

Introduction

Botrytis bunch rot (BBR) or grey mould, caused by *Botrytis* species, is one of the most destructive diseases of grapevines (*Vitis vinifera*) worldwide. *Botrytis cinerea* is a necrotrophic fungal pathogen responsible for the most important disease of grapevines in Australia after powdery mildew. *B. cinerea* infections, and to a lesser extent other bunch rots, impact all Australian grape growing regions and cost the grape and wine industry an average of $50M per annum (Emmett RW, Nair NG. 1991; Schofield et al. 2010). Along with cultural practices, the main approach to control *B. cinerea* in vineyards is through the routine application of fungicides during bloom and fruit maturation. In Australia, alongside broad spectrum multi-site (e.g. captan) and broad spectrum single site fungicides (e.g. azoxystrobin), there are also a number of single-site botryticides registered (e.g. anilinopyrimidines) (AWRI Dog book 2016-2017). *B. cinerea* is labelled a classic 'high risk' pathogen for resistance development due to its short life cycle and high reproductive rate (Brent KJ, Hollomon DW. 1998). For this reason, it is recommended that each season application of single site mode of action (MOA) fungicides is limited. In Australia, single site fungicides are limited to a maximum of two sprays per season (AWRI Dog book 2016-2017). To date, through a number of target site modifications and a multi-drug resistance (efflux) mechanism, isolates resistant to the single site MOA dicarboximide, hydroxyanilide, phenylpyrrole, succinate dehydrogenase inhibitor (SDHI), anilinopyrimidine and quinone

outside inhibitor (QoI) classes, have been found in grapevines in a number of countries (Leroux et al. 1999; Barroffio et al. 2003; Kretchmer et al. 2009; Leroch et al. 2011). In Australia, *B. cinerea* isolates resistant to benzimidazoles, dicarboximides and anilinopyrimidines have been previously described in grapevines (Nair et al. 1997; Sergreeva et al. 2002). *B. cinerea* isolates resistant to SDHI, QoI and phenylpyrrole classes, have been identified in vegetable crops in Australia through phenotyping methods (Hall et al. 2011). However, monitoring of resistance in Botrytis isolates from grapevine to these and other more recent fungicide groups has not yet been carried out in Australia. Due to the lack of up to date fungicide sensitivity data on grape *B. cinerea* in Australia, the aim of this research was to provide current data on resistance in grapevines to anilinopyrimidines (pyrimethanil) and dicarboximides (iprodione), and the first data on sensitivity to hydroxyanilides (fenhexamid), and SDHIs (boscalid). The sensitivity levels to all four fungicide MOA groups was first established in a subset of isolates, followed by the molecular characterisation of sensitive and resistant strains. A nation-wide collection of *B. cinerea* isolates was screened via a combination of high-throughput discriminatory dose (DD) and molecular tests. In addition to this, an *in vitro* detached berry assay and an *in planta* vineyard experiment were carried out to provide preliminary data on treatment efficacy, fitness and competitive ability of sensitive and resistant isolates.

Methods

Phenotypic assay

745 *B. cinerea* isolates from across 95 sampling sites were single-spored and maintained as mycelial plugs at 4^{o}C and as spore stocks at -80^{o}C. To establish sensitivity baselines for the fungicide actives boscalid, fenhexamid, iprodione, and pyrimethanil, 53 *B. cinerea* isolates were randomly selected from across the population to determine their EC_{50} (concentration of fungicide inhibiting fungal growth by 50%) values on a 96 well micro-titre assay system. Isolates were grown on a solid nutrient medium (Leroux et al. 1999) and were then placed under near-UV light to induce sporulation. Spores were harvested and washed via centrifugation. Spore density was estimated using a haemocytometer and was adjusted to a concentration of 1x10^{5}/mL. Pure fungicide compounds were dissolved in absolute ethanol and serial dilutions of stocks were prepared so that the total volume of fungicide/solvent added to each well was kept constant. Tween20 was added to the liquid media at a final concentration of 0.05%. The type of media used depended on the fungicide tested. Sensitivity to fenhexamid and iprodione was assessed using a liquid media as described by Leroux et al. 1999. Sensitivity to boscalid and pyrimethanil was assessed using YBA broth and the Leroux et al. 1999 liquid media minus the yeast extract, respectively. For all fungicides, a range of concentrations were used ranging from 0.01 – 5 ug/mL. To investigate significant reduction in sensitivity concentrations between 0.2 – 100 ug/mL were used for each fungicide. A 5µL volume of spore suspension (1x10^{5}/mL) was inoculated into 95µL of the media mixture to a final concentration of 5000 spores/mL; two biological replicates each with two technical replicates were inoculated for each isolate. Immediately following inoculation, optical density (OD) was measured at 450nm wavelength in a Synergy HT microplate reader (BioTek). All plates except those ones containing Pyrimethanil were incubated for 72h at room temperature in darkness, after which the optical density was again measured. Pyrimethanil plates were incubated for 96h. Final OD values were corrected with readings taken immediately following inoculation. The EC_{50} concentrations were estimated by linear regression of percentage reduction in OD (compared to zero fungicide control) against log concentration of fungicides.

High-throughput phenotypic assay

To screen the remaining isolates in the collection, a discriminatory dose agar assay was developed. Minimum inhibitory concentration **(**MIC) values identified in the micro-titre assay were used to design a discriminatory dose agar screen to test the remaining 692 isolates in the collection. The discriminatory doses for the agar assay are as follows; boscalid – 1ug/mL, fenhexamid – 1ug/mL, iprodione – 3 ug/mL, and pyrimethanil – 0.4 µg/mL. The screen was carried out using the respective liquid media as described in the micro-titre assay except agar was added. Isolates were sub-cultured onto each of the solid mediums and incubated for three days. Fresh plugs were taken from these plates and placed on each medium containing each fungicide. Plates were then incubated at room temperature in the dark for 3 days, and then isolates were scored on their ability to grow on each fungicide.

Genotypic assay

To identify mutations related to the reduced sensitivity to specific fungicides, target genes from candidate isolates exhibiting a significant reduction in sensitivity were genotyped. These genes include: *sdh*B and *sdh*D (boscalid), *erg*27 (fenhexamid), *os-1* (iprodione) and *cgs* (pyrimethanil). The primers used to genotype all candidate isolates are described in table 1. Three wild-type strains were genotyped for all target genes for comparative purposes. To test for any potential isolates that appeared to be exhibiting multi-drug resistance (MDR), the *mrr1* transcription factor gene (MDR1) was sequenced and the *mfsM2* transporter promoter (MDR2) was investigated for the presence of a previously described transposon (Kretchmer et al. 1999). To extract fungal gDNA, isolates of interest were sporulated as described above. Spores and hyphae were scraped off each plate, frozen in liquid nitrogen and then ground up. Each ground sample was then used in the Qiagen biosprint instrument for gDNA extraction as per supplier recommendations. All PCR were carried out in an Eppendorf thermocycler. Phusion or MyTaq polymerase were used to amplify the whole target genes using flanking primers. Amplified gene products were checked on a 1% agarose gel and then sent to Macrogen (Korea) for purification and Sanger sequencing. Flanking and internal primers were subsequently used to sequence the whole genes (appendix). Consensus gene sequences were aligned to the following reference sequences: *sdh*B (AY726618), *sdh*D (GQ253439), *os-1* (AF435964), *erg*27 (AY220532), *cgs* (KB707952.1/BcDW1_6954) and *mrr* (KF545938). The cleaved amplified polymorphic sequence (CAPS) test was carried out to identify the I365N/R/S mutant in os-1 using the restriction enzyme *Taq* I (Oshima et al. 2006). To identify the Q369H/P mutation, *Sma* I was used to digest the hotspot fragment containing the mutation.

Detached grape berry assay

To investigate the efficacy of iprodione sensitive (Bc-7; WT – *Os-1* gene) and iprodione resistant (Bc-128; I365S – *Os-1* gene) isolates to infect berries under iprodione exposure, an *in vitro* detached berry assay was developed. The berry method was designed and optimised as it provided an easier and faster approach compared with developing a shoot-let based system. Ripe berries (Cabernet sauvignon) were harvested from the experimental Curtin University vineyard and were washed in a 5% bleach and 10% ethanol mixture. The berries were then rinsed in distilled water and air dried. The washed berries were then soaked overnight in iprodione 500SC (Imtrade) in final concentrations of 0, 100 µg/mL and 500 µg/mL iprodione. The berries were then air dried, and placed individually in a 70ml container alongside damp cotton wool. The berries were then perforated eight times with an 18G needle to create a wound site. Spores of Bc-7 and Bc-128 were harvested as described above, diluted to a concentration of 10^6/mL in 0.03% tween, and

10 µL of the spore suspension was added to the wound site. The berries were kept in the dark at room temp and percentage of berry diseased was recorded at day 7.

In planta bunch assay

To investigate the fitness and competitive abilities of iprodione sensitive and iprodione resistant isolates in an *in planta* scenario, grape bunches were inoculated in the Curtin University field trial area experimental vineyard. The vines were managed in a way to replicate commercial vineyard management. Bunches were inoculated at flowering with four ratios (1:1:10, 1:10:1, 10:1:1, 1:1:1) of 3 isolates: Bc-7 (iprodione sensitive; *Os-1* - WT), Bc-143 (iprodione resistant; *Os-1* - Q369P + N373S) and Bc-179 (iprodione resistant; *Os-1* - I365S). Bunches were also inoculated with isolate Bc-7 only as a positive control. Spores of each isolate were harvested as described above, and diluted in water containing 0.5% gelatin to concentrations between 10^6 – 10^7/mL for each isolate. In each ratio for each treatment, 1 equalled 10^6/mL and 10 equalled 10^7/mL. Once the *B. cinerea* started to express, DNA was extracted from whole infected berries using a CTAB extraction method (Doyle JJ, Doyle JL. 1987). PCR amplification of the *Os-1* region containing the different mutations (table 2, primers os-1 F and os-1 R) and sequencing was carried out to investigate the presence of each haplotype.

Development of a heterologous yeast expression system

To complete a simple, reliable and high-throughput phenotypic test of the wild-type *E. necator* Cyp51 against demethylase inhibitors (DMIs) registered in Australia for powdery mildew, *E.necator Cyp51* protein was expressed in a heterologous yeast expression system (Groenvald et al. 2002. Revenkar et al. 2004). This system allows an exogenous CYP51 protein to be expressed and complement the suppressed native CYP51 protein. If the exogenous CYP51 complements, the transformed strain can be used to investigate the sensitivity of the strain to DMIs of interest. The wild-type *E. necator Cyp51* gene was synthesised by Bioneer Inc., amplified via PCR and ligated into the pYES3/CT expression vector (Invitrogen) using *Hind* III and *Eco* RI restriction enzymes. The construct was then transformed into the *S. cerevisiae* strain YUG37:*erg11*, in which the endogenous *Cyp51* expression can be suppressed by the addition of doxycycline (Groenvald et al. 2002). The *E. necator Cyp51* was able to complement the suppressed endogenous *Cyp51* as the transformed strain was able to grow on doxycycline containing media. The yeast strain containing the *E. necator Cyp51* was phenotyped against five DMIs registered in Australia for grape powdery mildew: myclobutanil, penconazole, tebuconazole, tetraconazole and triadimenol.

Table 1. Primers used for the genotyping of candidate isolates

Target gene and primer name	**Sequence (5’ – 3’)**	**Reference**
***sdh*B (boscalid)** **Flanking**		

sdhB 1F	AGCAGTTTCGCCTTCTCC	This project
sdhB R	TAGCAATAACCGCCCAAAAC	This project
Internal		
sdhB 1R	TAGCGTCTCTTGGAATG	This project
sdhB F	AAGGTATCTGCGGCAGTTGTG	This project
***sdh*D (boscalid)**		
Flanking		
sdhD 1F	GCCAATCAAATCCGTTCC	This project
sdhD 1R	AATGCAATAGGGCAGAGGC	This project
Internal		
sdhD F	CAACAAGCCCGCTGGACG	This project
sdhD R	CTCCCCTCAAGCCCCACC	This project
***erg*27 (fenhexamid)**		
Flanking		
erg27 2F	GCGCTTAGGATAAATAGG	This project
erg27 1R	CATCACCATTATTCTGACC	This project

Internal		
erg27 F	GGATGGGGATTGAGCGGA	This project
erg27 int R	GAATGTTGCTCCAAGAGG	This project
***os-1* (iprodione)**		
Flanking		
os-1 2F	ATTCAAGAGCGTCATGCG	This project
os-1 7R	ATGCCCATCAATCCATGC	This project
os-1 2R	TCCTATTTCCCGTAGAGC	This project
os_1 F	TGGCCGGTGTACATGCTC	This project
os_1 R	GGCGACAGCGGTGGTAAC	This project
os-1 4F	AATTTGCGCGAGAAGTCACG	This project
os-1 4R	CCATCTGGTTGATCTTTCGC	This project
os-1 5F	CACAACGGATGTCAATACC	This project
os-1 5R	CGAAGACGGAATGAATCACC	This project
os-1 6F	ACCGTATGATCATGGAGG	This project
os-1 6R	GTGATACCAAGATCCAAAGC	This project
os-1 7F	GAACCAAGGAGAAGATTGC	This project
***cgs* (pyrimethanil)**		
Flanking		

cgs 1F	TCTGCAAGCGGGAGCG	This project
cgs 3R	GGCTGATGTTCGCCGTATG	This project
Internal		
cgs 1R	TGGGAAAGGGAGGGAGGG	This project
cgs 2F	ACCCCTCCCTCCCTTTCC	This project
cgs 2R	CATTCCCCAGCACCACTC	This project
cgs 3F	GGCACCGCCTACAATCTC	This project
***Mrr*1 (MDR1)**		
Flanking		
mrr1_TF1-1	CCAATCATTCCCAATCATTCA	Kretchmer et al. 1999
Mrr1_TF1-4	GGATAGGGTATTGCGTAGATCG	Kretchmer et al. 1999
Internal		
mrr int1F	CATCCAAGTATGACTCTCC	This project
mrr int1R	TTGTGCATAGTGACGACC	This project
mrr int2F	AAGAGCAGATGATCAAAGG	This project
mrr int2R	ACCAGCCATAACTTGAGCG	This project
***MfsM2* (MDR2)**		
mfsM2-pfor	TAGCCAATGGATCCTACG	Kretchmer et al. 1999
mfsM2-prev	CGAGATGGATGCCATTTCAGAG	Kretchmer et al. 1999

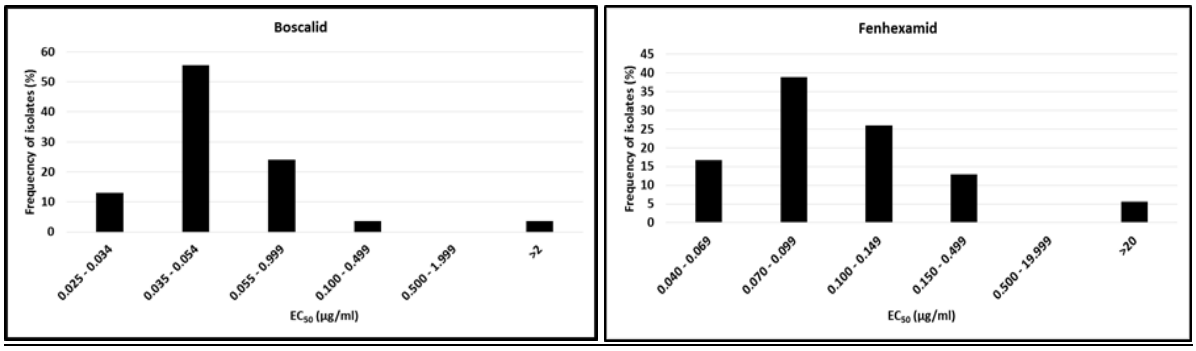
Results

The frequency of isolates for all fungicides tested in the micro-titre assay is reported in figure 1. For all fungicides except iprodione, isolates with an EC_{50} <0.5 µg/mL were classified as sensitive, and this threshold was supported by genotyping results (table 2). The iprodione sensitivity threshold was higher at <2.05, as this was the EC_{50} of the least sensitive isolate (Bc-279) that was wildtype for *os-1* (data not shown). For each fungicide the sensitive EC_{50} range, sensitive EC_{50} mean, resistant EC_{50} range and the resistance factor (RF) range in the subset are shown in table 2. For boscalid, fenhexamid, iprodione and pyrimethanil, 3.6%, 5.6%, 16.7% and 13% of isolates were classified as resistant, respectively (table 2). Additionally, the iprodione resistant isolates were divided into low resistant (LR), medium resistant (MR) and high resistant isolates (HR). The pyrimethanil resistant isolates were also divided into LR and HR isolates.

Table 2. Sensitivity of 53 *B. cinerea* isolates tested against boscalid, fenhexamid, iprodione and pyrimethanil using a micro-titre assay system.

Fungicide	EC_{50} value			no. of isolates		RF range	Resistant frequency (%)
	sensitive range	sensitive mean	resistant range	sensitive	resistant		
Boscalid	0.028 - 0.103	0.051 ± 0.002	2.744 - 2.897	51	2	53.8 – 56.8	3.8
Fenhexamid	0.046 - 0.216	0.103 ± 0.006	25.460 - 27.333	50	3	247.1 – 265.3	5.7
Iprodione	0.753 - 2.050	1.19 ± 0.039	5.160 >50	44	9	4.33 - >42	17.0
Pyrimethanil	0.094 to 0.219	0.14 ± 0.004	0.700 - 5.895	46	7	5 – 42.1	13.2

Figure 1. Distribution of EC_{50} values for the isolates phenotyped in the microtitre assay system.



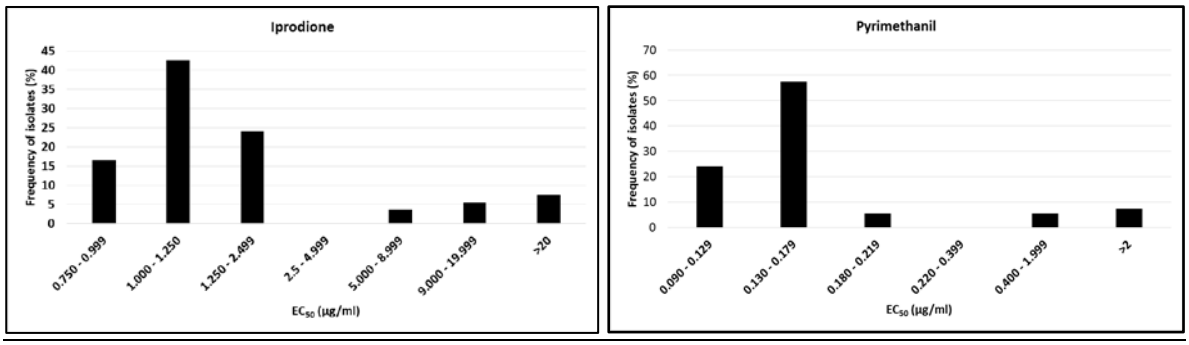


Table 3 outlines the genotyping results for a selection of candidate resistant isolates identified in the micro-titre assay and high-throughput agar screen. Sequencing of the *sdh*B and *sdh*D genes revealed the previously described mutations H272R and H272Y in the *sdh*B gene. Sequencing of the *erg*27 gene showed the presence of the previously described F412S mutation in a number of isolates. Sequencing of *os-1* gene showed the well described I365S and Q369P + N373S haplotypes. The CAPS method cannot distinguish between different mutations in the same codon, so positives are designated as a combination of previously described mutants e.g. I365N/R/S or Q369H/P. It could be assumed that the Q369P/H isolates would also contain the N373S change, which is nearly always found alongside the Q369P mutation. One isolate showed an I365S + D747N haplotype, which has been previously described in *B. cinerea* of strawberries in China (Lu et al. 2016). Two novel mutations were identified in the *cgs* gene, L8P and D416E. A number of pyrimethanil resistant isolates lacked any mutations in the *cgs* gene. These isolates were candidates for investigating the potential role of MDR in resistance to Pyrimethanil. Sequencing of the *mrr1* gene in 1 HR Pyrimethanil isolate showed a number of indels and non-synonymous mutations commonly found in MDR1 characterised isolates (Kretchmer et al. 1999). No candidate isolates were found to contain a transposon in the promoter of the *mfsM2* transporter (MDR2) (Kretchmer et al. 1999).

Table 3. Mutations identified in the target genes; *sdh*B, *erg*27, *bos*-1 and *cgs* in a selection of resistant isolates. (CAPS) = CAPS results for the *os-1* gene.

Fungicide	Target Gene	Non-synonymous mutation	RF (mean)	Number of isolates						
				WA	NSW	VIC	SA	QLD	TAS	Total
Boscalid	***sdhB***	H272R	55	4	-	1	4	-	-	9

		H272Y	188	1	-	-	-	-	-	1
Fenhexamid	***erg27***	F412S	256	-	-	3	3	-	-	6
Iprodione	***bos-1***	I365S	8	4	3	1	-	-	1	9
		I365N/R/S (CAPS)	-	7	1	3	1	-	6	18
		I365S + D747N	23	-	-	-	1	-	-	1
		Q369P + N373S	27	1	-	4	1	-	1	6
		Q369H/P (CAPS)	-	2	-	9	-	-	-	11
Pyrimethanil	***cgs***	L8P (novel)	16	1	-	-	-	-	-	1
		D416E (novel)	15	3	1	-	1	-	-	5

The microtitre phenotyping and genotyping dataset was used to establish a discriminatory dose agar assay to screen the remaining 692 isolates in the collection. The discriminatory doses of 1, 1, 3 and 0.4 µg/mL were used for boscalid, fenhexamid, iprodione and pyrimethanil, respectively. Resistance frequencies from across all isolates and all sites from the microtitre and high throughput agar screen combined data are shown in tables 4 – 7. 633 isolates (84.9%) were classified as sensitive. The number of isolates classified as resistant to boscalid, fenhexamid, iprodione and pyrimethanil was 22 (3%), 16 (2.1%), 91 (12.2%) and 57 (7.7%), respectively (table 2). Victoria and SA had isolates resistant to at least one of the four fungicides tested (table 2). WA, NSW and Tasmania had isolates resistant to only three out of the four fungicides tested, and Queensland has no resistance detected (table 4). Table 5 characterises each isolate in terms of resistance to number of mode of action (MOA) fungicide groups. 64 (8.6%) and 48 (6.4%) of isolates exhibited resistance to one or more than one MOA, respectively (Table 5). All states except Queensland had

isolates resistant to one or two MOA fungicides (table 5). All States except Tasmania and Queensland have isolates resistant to three MOAs (table 5) and only SA had an isolate resistant to 4 MOAs (table 4). 56 sites (59%) exhibited isolates that were sensitive (table 6). The number of sites that showed at least one isolate resistant to boscalid, fenhexamid, iprodione or pyrimethanil was 17 (17.9%), 5 (5.2%), 25 (26.3%) and 30 (31.5%), respectively (table 6). Table 7 shows each site characterised in terms of the isolate exhibiting resistance to the most MOA at that specific sampling site. 17 (17.9%), 9 (9.5%), 12 (12.6%) and 1 (1.1%) sites exhibited isolates with resistance to 1, 2, 3 and 4 MOAs, respectively (table 7).

Table 4. Number of isolates with resistance to boscalid, fenhexamid, iprodione and pyrimethanil.

State	Number of isolates tested	Number of sites tested	Number of isolates with resistance				
			Sensitive	Boscalid	Fenhexamid	Iprodione	Pyrimethanil
Western Australia	371	47	326	14	-	35	22
New South Wales	110	13	107	-	2	2	2
Victoria	123	15	92	1	11	26	21
South Australia	100	18	82	6	3	14	11
Queensland	20	1	20	-	-	-	-
Tasmania	21	1	6	1	-	14	1
Total	745	95	633 (85.0%)	22 (3.0%)	16 (2.1%)	91 (12.2%)	57 (7.7%)

Table 5. Number of isolates with resistance to 0 – 4 mode of action (MOA) fungicides.

Phenotype	Number of MOA	Number of isolates						
		WA	NSW	VIC	SA	QLD	TAS	Total
Sensitive	0	326	107	92	82	20	6	633 (84.9%)
Boscalid	1	4	-	-	2	-	1	7 (0.9%)
Fenhexamid	1	-	-	-	1	-	-	1 (0.13%)
Iprodione	1	19	1	10	4	-	13	47 (6.3%)
Pyrimethanil	1	3	-	5	1	-	-	9 (1.2%)
Iprodione + Pyrimethanil	2	9	-	4	4	-	1	18 (2.4%)
Boscalid + Pyrimethanil	2	3	-	-	-	-	-	3 (0.4%)
Fenhexamid + Pyrimethanil	2	-	1	-	-	-	-	1 (0.13%)
Bosc + Ipro + Pyri	3	7	-	1	3	-	-	11 (1.5%)
Fenh + Ipro + Pyri	3	-	1	11	2	-	-	14 (1.9%)
Bosc + Fenh + Ipro + Pyri	4	-	-	-	1	-	-	1 (0.13%)
Total	-	371	110	123	100	20	21	745

Table 6. Number of sampling sites with at least one isolate resistant to boscalid, fenhexamid, iprodione and pyrimethanil.

State	Number of isolates tested	Number of sites tested	Number of sites with resistance				
			Sensitive	Boscalid	Fenhexamid	Iprodione	Pyrimethanil
Western Australia	371	47	26	11	-	15	14
New South Wales	110	13	8	-	1	2	2
Victoria	123	15	8	1	1	3	7
South Australia	100	18	13	3	3	5	4
Queensland	20	1	1	1	-	-	-

Tasmania	21	1	-	-	-	1	-
Total	745	95	56 (59%)	17 (17.9%)	5 (5.2%)	25 (26.3%)	30 (31.5%)

Table 7. Sampling sites characterised in terms of the isolate/s at each site resistant to the most MOAs (1-4).

State	**Number of isolates tested**	**Number of sites tested**	**Number of sites with resistance to 0 – 4 fungicide** MOA				
			Sensitive	**1 MOA**	**2 MOA**	**3 MOA**	**4 MOA**
Western Australia	371	47	26	8	7	6	-
New South Wales	110	13	8	3	1	1	-
Victoria	123	15	8	4	1	2	-
South Australia	100	18	13	1	-	3	1
Queensland	20	1	1	-	-	-	-
Tasmania	21	1	-	1	-	-	-
Total	745	95	56 (59%)	17 (17.9%)	9 (9.5%)	12 (12.6%)	1 (1.1%)

Figure 2 illustrates the results of the detached berry assay for Bc-7 (iprodione sensitive) and Bc-128 (iprodione resistant) isolates growing on berries soaked in different concentrations of iprodione 500SC. On berries soaked in the highest concentration of fungicide, the resistant isolate (Bc-128) was responsible for a 40% disease increment compared to the sensitive strain (Bc-7).

Figure 2. Percentage of berry area diseased for sensitive (Bc-7) and iprodione resistant (Bc-128) isolates on detached berry soaked in different concentrations of iprodione 500SC at day 7.

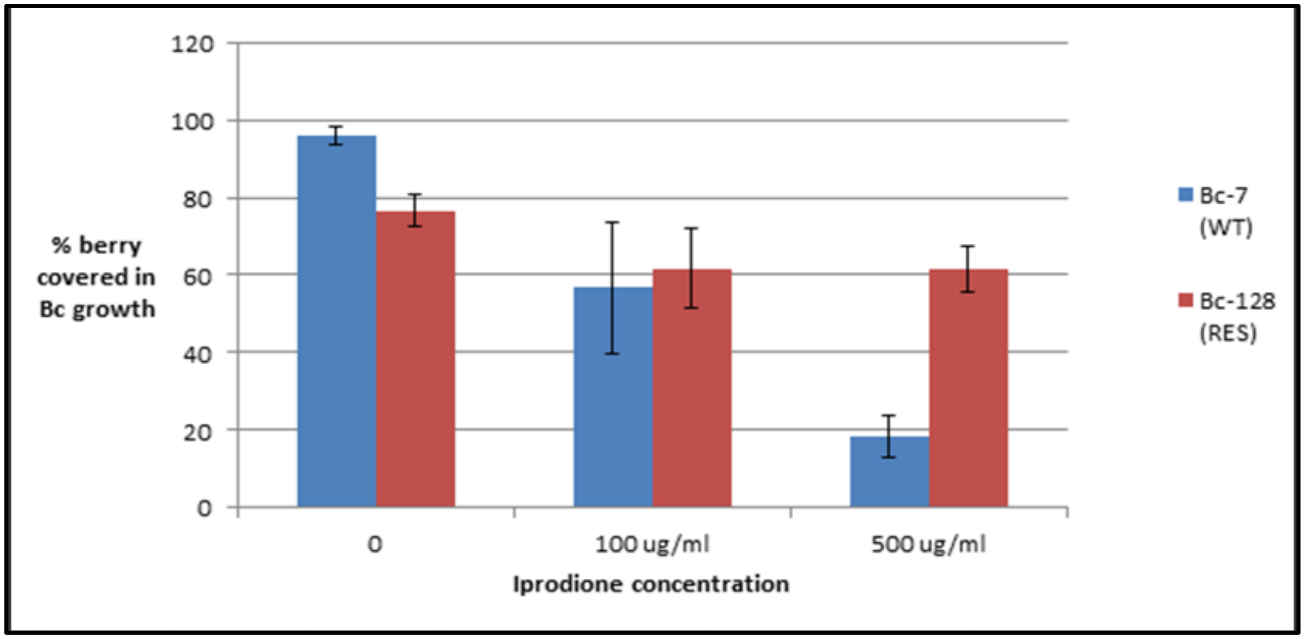


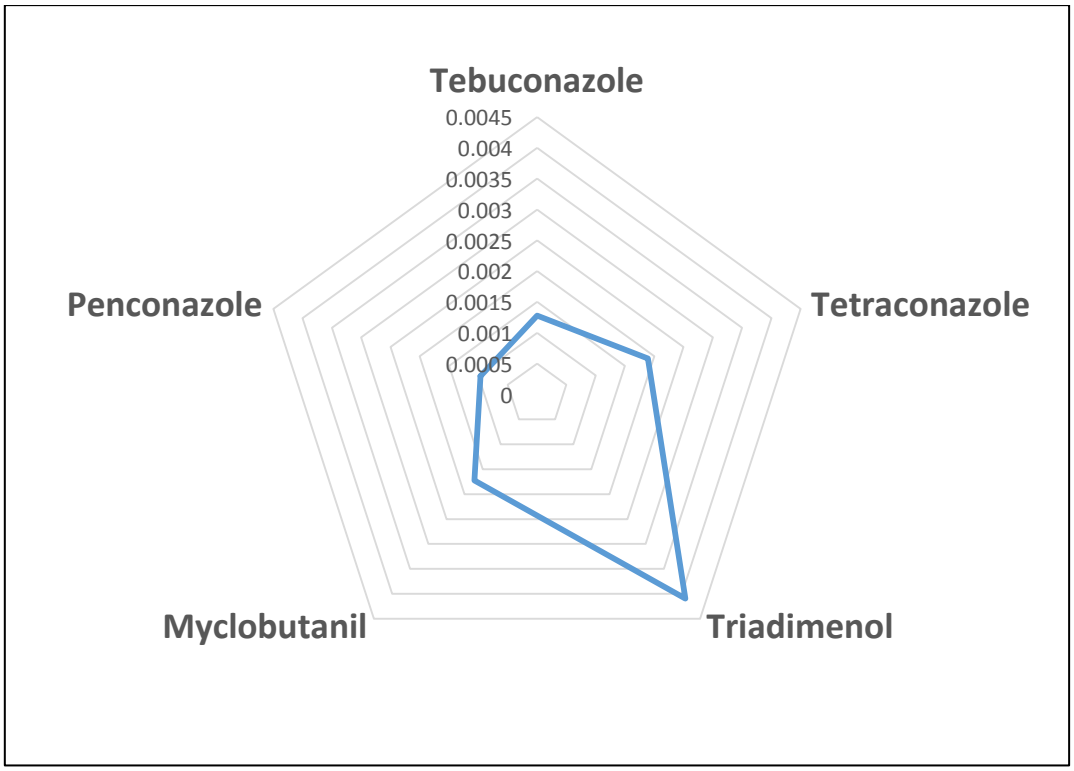
Table 8 show the genotyping results for the *in planta* experiment with respect to the presence or absence of each the haplotypes under each of the treatments. The number of replicates sampled was highly variable ranging from 0 – 4 replicates expressing *B. cinerea* for analysis (table 8). 1 – 3 of the haplotypes were identified across 4 replicates in the 1:1:1 treatment. For the 1:10:1 and 1:1:10 treatments, the isolate at the highest inoculation concentration were the only haplotypes identified in those treatments, which probably indicate a higher fitness of these genotypes.

Table 8. Presence of each haplotype for each treatment where *B. cinerea* DNA was extracted from berries in an *in planta* vineyard experiment

Ratio of Bc-7:Bc-143:Bc-179	Replicate (Bunch)	WT (Bc-7)	Q369P + N373S (Bc-143)	I365S (Bc-179)
1:1:1	1	Yes	No	Yes
1:1:1	2	Yes	Yes	Yes
1:1:1	3	No	Yes	No
1:1:1	4	Yes	Yes	Yes
1:10:1	1	No	Yes	No
1:1:10	1	No	No	Yes
1:1:10	2	No	No	Yes
Bc-7 only	1	Yes	No	No
Bc-7 only	2	Yes	No	No

The yeast system is a quick and easy *in vitro* method to test a range of DMI compounds on the wild type and/or mutant allele. The phenotyping results from an *in planta* testing can be used to support the results. Figure 3 shows the results for the phenotypic testing of WT *E. necator Cyp51* against five DMIs within the yeast heterologous expression system; with an EC_{50} value range from 0.0005 - 0.004 µg/mL. The higher the EC_{50}, the more product is required to inhibit the growth of the yeast strain or theoretically control the disease in the field, than more effective fungicides with lower EC_{50}. By inserting mutant alleles in the yeast, this system could assist by showing which of the fungicides would be more effective in controlling the mildew when the mutation is present.

Figure 3. Sensitivity of the yeast strain YUG37:*erg11* expressing *E. necator Cyp*51 against the DMIs tebuconazole, tetraconazole, triadimenol, myclobutanil and penconazole



Discussion

This report describes the first large scale investigation in Australia into the fungicide sensitivity and resistance mechanisms of *B. cinerea* isolated from grapes against four key fungicide groups. This study has identified isolates with resistance to multiple fungicide groups, as it is the case in previous studies in vineyards in Europe (Leroux et al. 1999, Barroffio et al. 2003, Gulliano et al. 2000, De Miccolis Angelini et al. 2014, Panebianco et al. 2015), Chile (Latorre et al. 2002) and the U.S.A (Saito et al. 2015). The resistance frequencies of 3.0%, 2.1%, 12.2% and 7.7% reported in this study for boscalid, fenhexamid, iprodione and pyrimethanil are lower than in previous *B. cinerea* studies in grapes. Resistance frequencies between 2 – 85%, 13 – 100%, 14 – 65% and 30 - 57% have been found against boscalid, fenhexamid, iprodione and pyrimethanil, respectively (Leroux P, Clerjeau M. 1985, Latorre et al. 2002, Barroffio et al. 2003, Fillinger et al. 2008, Leroch et al. 2011, Latorre et al. 2012, De Miccolis Angelini et al. 2014; Saito et al. 2015, Panebianco et al. 2015). In this study, 8.5% of all isolates were found to have resistance to one MOA, while 2.9%, 3.4% and 0.1% of isolates were resistant to 2, 3, or 4 MOAs, respectively. With the majority of resistant isolates being only resistant to one MOA, this result suggests that a field management approach involving different MOAs can contribute to the mitigation of resistance. Out of 95 sampling sites tested, 41% and 23% exhibited at least one isolate resistant to at least one or more than one MOAs, respectively. The repertoire of well described and novel mutations identified in this study can be used for the future development of in-field molecular high throughput screening methods. The identification of novel mutations in the *cgs* gene will also be important for elucidating the mechanisms behind Pyrimethanil resistance, which are currently not well defined. The identification of an isolate with the classic MDR1 genotype is of interest as it the first report of this mechanism in Australia. The expression of the transporter *atrB*, which is over expressed in MDR1 strains, could be investigated in this MDR1 isolate to further characterise the MDR1 phenotype. The role of MDR and its development alongside target site mutations in resistant isolates could be of interest for investigating resistance development under current fungicide management practices. A small scale screen against azoxystrobin (QoI) and fludioxinil (Phenylpyrrole) was also completed in this study (data not shown). The frequency of resistant isolates against Azoxystrobin and Fludioxinil was 5% and 0% respectively. Further screening and monitoring of sensitivity to Fludioxonil may be worthwhile as significant resistance to this fungicide has yet to be identified. Further research is needed to understand the relationship between the *in vitro* results in this study and field efficacy.

An *in vitro* detached berry assay was designed and optimised in this study. Results showed that the percentage of berry area that was diseased was significantly higher for the resistant isolate than the sensitive isolate on berries soaked in fungicide. These results show that this detached berry method is a potential choice to investigate efficacy of fungicides on berries instead of using more complex systems. A preliminary *in planta* investigation into the fitness and competitiveness of sensitive and resistant isolates was completed in this study. This experiment has shown that bunches can be inoculated at flowering with different strains and, once disease expresses, molecular tools based on specific polymorphisms can be used to assess the presence of resistant genotypes. Further experiments and alternate molecular tools are required to investigate the relative amounts of each of these resistant genotypes within the treatments.

A yeast expression system provided a means to test the sensitivity of WT *E. necator Cyp*51 against a number of DMIs registered for powdery mildew in Australia. Future work could include introducing

mutations into the *E. necator Cyp*51 construct which would allow insights into what effect specific mutations (e.g. Y136F) have on the sensitivity to specific DMIs.

The competitive ability of resistant isolates and fitness costs associated with mutations are also areas requiring additional research to provide understanding on how fungicide applications affect resistance development and frequencies of resistant isolates in the field. Ultimately, prudent management of fungicide applications is needed to ensure that these resistant strains do not become a widespread and significant problem resulting in fungicide field failure.

Modelling analysis of risk resistance development in *B. cinerea, Erysiphe necator* and *Plasmopara viticola*

Introduction

Over the last years several resistance mutations have been found in several grape growing areas in Australia. Resistant strains of the three most economically important pathogens in Australian vineyards, grey mould (*Botrytis cinerea*), grape powdery mildew (*Erysiphe necator*) and grape downy mildew (*Plasmopara viticola*) have been found. The current situation is described in previous sections of this report.

A key question is now how to respond to the current situation. What is possible to achieve with resistance management and what is not possible? This Chapter gives an overview of what is known about the management of fungicide resistance, and applies it to the current grapevine situation in Australia. This leads to a decision tree (adapted from Walker et al. 2013) that can be used to determine the appropriate action.

The dynamics of fungicide resistance selection

When a fungicide is used it reduces the growth rate of the population of fungicide sensitive pathogen strains. That is basically the reason to use a fungicide. Any strains present in the population that are resistant/insensitive to the fungicide will be unaffected or less affected by the fungicide and therefore have a higher growth rate than the sensitive strains. The result of this is that the population size of the fungicide resistant strain increases faster than that of the sensitive strains, resulting in the resistant strain increasing in frequency in the population. That is exactly what selection is about, a phenotype increasing in frequency in the pathogen population. We can thus measure the selection for fungicide resistance by the difference in population growth rate of the sensitive and the resistant pathogen strains. We formalise the selection coefficient, s, as $s = (r_R - r_S)$, where r_R and r_S are the population growth rate of the resistant and the sensitive strain, respectively (Crow and Kimura 1970).

Staub & Sozzi (1984) and Dekker (1986) recognised that the amount of time a fungal population is exposed, *T,* to a fungicide also determines the extent to which the frequency of resistance increases. When a fungicide is applied, the dose of the fungicide on or in the leaf increases sharply. After the application the fungicide dose decreases gradually due to plant metabolism, effects of weather such as UV exposure, rain, etc. until the dose becomes too low to be biologically active. The amount of time, *T*, a fungicide poses a selection pressure thus is limited. Combining this with the selection coefficient we find that a measure of selection for resistance of a fungicide application program is

$$sT = (r_R - r_S)\,T \qquad (1)$$

We refer the reader to van den Bosch et al. (2014) for a more formal mathematical derivation of the selection coefficient and exposure time principle.'' (Citation from van den Bosch et al. 2015a)

The frequency of the resistant genotype on the pathogen population, θ(t), growth logistically with time (van den Bosch et al. 2014). We thus have

$$\theta(t) = \frac{\theta_0 \, e^{(r_R - r_S)t}}{(1-\theta_0) + \theta_0 \, e^{(r_R - r_S)t}} \qquad (2)$$

Figure 1 shows an example of the logistic growth for a situation where (r_R-r_S)=1.5 per year and θ_0=10^{-5}.

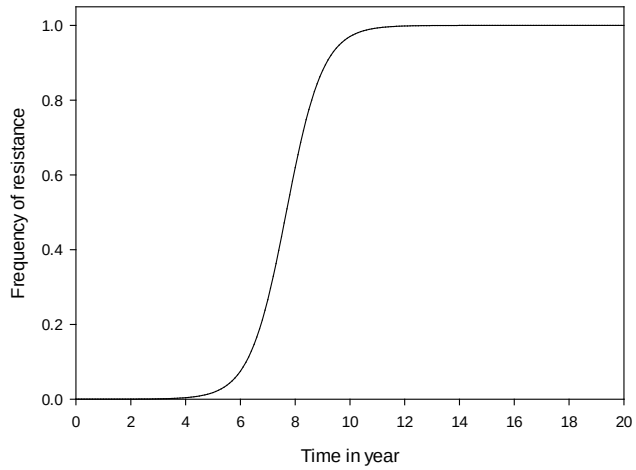


Figure 1. The logistic increase of the frequency of the resistance gene in the pathogen population through time. In this simulation ($r_R - r_S$)=1.5 per year.

In this chapter we will use the equation for sT and the logistic equation for the development of the frequency of resistance in the pathogen population to discuss the use of resistance management methods.

What pathogens and fungicides to concentrate on?

As discussed, the frequency of resistance in a pathogen population growth logistically over time (Figure 1). This implies that when the frequency of resistance is small, as one would expect at the introduction of a new mode of action, the frequency of resistance will initially increase very slowly. When, however, the frequency of resistance has reached 10-20% it will increase in much bigger steps each year.

> For example, with the parameters of figure 2, when the initial frequency of resistance is 10^{-5} the frequency will have increased to $5 \cdot 10^{-5}$ after one year, to $2 \cdot 10^{-4}$ after two years and to 0.018 after five years. However, when the frequency has reached 10% it will be 33% after the next growing season, 69% after two growing seasons and 99.5% after five growing seasons.

This observation extends to cases where resistance management actions are taken. For example, consider the case of figure 1. Assume a mixing partner is introduced to the fungicide to which resistance is developing, and that this mixing partner reduces the rate of growth of both pathogen strains by 50%. Now compare a situation where the new MOA is introduced as mixture, and compare this to a situation where the solo product is introduced and a mixing partner is introduced when the frequency of resistance has reached 10%. The situation is depicted in figure 2. The figure shows that introducing a mixing partner when

the frequency of resistance has reached 10% reduces the buildup of resistance to some extent, but it is far more effective to introduce a mixing partner when the frequency of resistance is still small.

The phenomenon, that the resistance frequency increases slowly when the frequency of resistance is low and increases fast when the frequency of resistance is high, is also observed in more detailed models studies. There are a number of modeling studies that consider the effect of the initial frequency of resistance in the pathogen population. The models range from simple models (Kable & Jefferey 1980; Levy Y, Levi R, Cohen Y. 1983); generalising across many aspects of the biology and epidemiology of pathogens to very detailed models (Milgroom MG, Fry WE. 1988; Hobbelen et al. 2011) modelling canopy dynamics and a detailed pathogen-life-cycle.

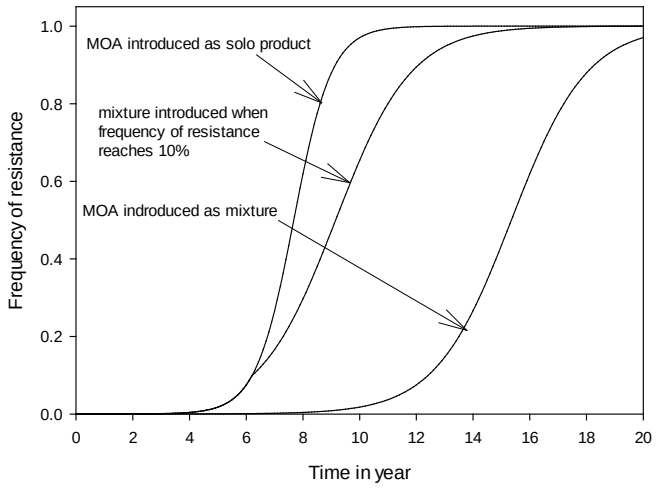


Figure 2. The increase in the frequency of the resistance gene through time. One line is for a situation whether the MOA is introduced onto the market as a solo product and is used as solo product throughout. The second line is a situation where the new MOA is introduced as a solo product, but is replaced by a mixture when the frequency of resistance in the population has increased to 10%. The rightmost line is a situation where the new MOA is introduced as mixture.

Adding to this is the observation that after the introduction of a new MOA resistance is often not observed for several or even many years at all, but once resistance is found its frequency increases so fast that the MOA may lose its ability to control the epidemic in a few years time.

All this evidence leads to the conclusion that a resistance management program is ideally put in place when a new MOA is introduced on the market. If such a resistance management program is not put in place at introduction it should be put in place as soon as possible after the introduction of the new MOA. Failing to do so, and waiting till the resistance is observed in the field, will inevitably lead to a rapid increase in resistance. Resistance management put in place when the frequency of resistance in the field has increased to 10-20% will not help much anymore, and the MOA will need to be classified as ‘lost’. The resistance management program will not be able anymore to prolong the usefulness of the fungicide for effective disease control.

We are not the first to make this very important point, yet discussions about resistance management often start at the moment resistance is found in field populations. The authors are sorry to have to say that the situation for fungicides used to control grapevine diseases in Australian vineyards is an example of this case.

If we use the above discussion to suggest possible actions to be taken in Australian vineyards the following points are important:

- Botrytis: A range of mutants conferring resistance to several MOAs have been detected at high frequency in field populations. Several isolates showed up to 5 resistance mutations. Some isolates showed very high minimal inhibitory concentrations (MICs).
- Powdery mildew: Mutations conferring resistance to QoIs and DMIs have been detected. Some isolates had resistance mutations to both QoI and DMIs.
- Downy mildew: No resistance mutations have been detected so far, but very small numbers have been screened. There is some anecdotal information about metalaxyl field failure.

It is currently not known whether the detected pathogen strains are causing field resistance to fungicide applications. This is however more a case of 'absence of evidence' than of 'evidence of absence'. Moreover, several of the mutations found in the Australian populations have led to the development of field resistance in other places in the world (USA, Europe). We therefore have to assume that field resistance may develop at some stage in the, possibly near, future, if not present yet.

Little is known about the frequency of the various resistance genes in the pathogen populations. One of the first things to be done is thus to further expand the monitoring program to assess resistance frequencies. Although it is logical that the industry is most worried about the Botrytis situation, our above discussion shows that for the powdery mildew and the downy mildew case that implementation of a resistance management program will have a good chance to be effective. The resistance management programs should however be implemented as soon as possible. Waiting till the situation is comparable to the current Botrytis situaton is not advisable. The industry should make use of the opportunities the powdery mildew and downy mildew present at this moment. Further research, including monitoring, should thus not be solely concentrated on the Botrytis case but should include the powdery mildew and downy mildew cases.

Results

Predicting the effect of changes in a fungicide application program on the development of fungicide resistance

Now we have seen which pathogen and fungicides to concentrate on we ask the question 'how do we change a fungicide application program to decrease the rate of buildup of resistance?'. So, what we want to know is whether a specific change in a fungicide application program increase or decrease the rate of selection for fungicide resistance. We will use the governing principle, *sT*, of section II to analyse the effect of changes in application programs on selection for fungicide resistance.

From the governing principle we can derive three basic strategies that reduce the selection for fungicide resistance in an application program (van den Bosch et al. 2014). All anti-resistance measures fit into one or more of these strategies.

> Strategy 1: Selection for fungicide resistance is reduced when the per capita rate of increase of both the sensitive and resistant strains (r_R and r_S, respectively) are reduced.
>
> Strategy 2: Selection for fungicide resistance is reduced when the per capita rate of increase of the resistant strain (r_R) is reduced relative to that of the sensitive strain (r_S).
>
> Strategy 3: Selection for fungicide resistance is reduced when the time span over which selection takes place (exposure time) is reduced.

These three simple strategies can help guide the development of fungicide application programs that minimise the selection for fungicide resistance.

We have used these three strategies to predict whether changes in fungicide application programs will increase or decrease selection for resistance. We refer to van den Bosch et al. (2014) for details. We summarise here that the review of published evidence shows that the governing principle gives an accurate prediction of the effect of a change in an application program. In 87% of the cases the governing principle correctly predicts the effect of a change in an application program. In only 5% the effect measured in the field is opposite to the effect predicted with the governing principle.

We have used the governing principle and an exhaustive literature review to assess what type of changes in an application program increase or decrease the selection for fungicide resistance (van den Bosch et al. 2008, 2011, 2014a,b). We have made a summary of these findings that was published in van den Bosch et al. (2015a). Here we cite, with permission of the editor, the key findings. Note that these conclusions relate mainly to target-site resistance or specific resistance. Multi-drug resistance has little been studied but may have an effect on the development of effective resistance management programs. We will discuss multidrug resistance in the next section of this report.

We have presented the evidence on the effectiveness of a range of resistance management tactics. This chapter combines the information gathered in three recent reviews by van den Bosch et al. (2008, 2011, 2014a,b). From the evidence discussed a set of clear conclusions can be derived. These are:

Managing the application dose:

- The majority of the evidence suggests that an increased dose of fungicide increases selection for fungicide resistance. (but note here that the primary aim of effective disease control may make it impossible to reduce fungicide dosages.)
- A number of possible mechanisms by which an increased dose may reduce selection have, however, not been studied. Partial resistance and multi-gene/multi-mutation cases are the key example of this.

Managing the number of sprays:

- All current evidence suggests that increasing the number of fungicide applications increases selection for fungicide resistance.
- Most evidence suggests that splitting fungicide dosage between two or more applications increases selection.

The use of fungicide mixtures:

- The vast majority of the evidence shows that adding a mixing partner to a high resistance risk fungicide reduces selection for fungicide resistance, even when the dose of the high risk fungicide stays the same in the mixture.
- Adding a mixing component to a high-risk fungicide and reducing the dose of the high-risk fungicide further reduces selection for fungicide resistance.
- There is too little evidence on the use of mixtures of two at risk fungicides and work in this area is needed. The evidence that does exist suggests that mixing two at-risk fungicides is a valid anti-resistance strategy.

The use of fungicide alternations:

- Limited evidence suggests that alternating with a fungicide that has a different mode of action does not alter selection for the high-risk fungicide, if the number of applications of the high risk fungicide remains constant with and without alternation.
- The evidence suggests that replacing part of the fungicide program with a fungicide with a different MOA reduces selection.

Alternations versus mixtures:

- It depends on the balance between increased selection due to dose splitting and decreased selection due to mixing whether mixing reduces selection to a greater or lesser extent than alternation. The experimental and modelling evidence shows that in many cases mixing is the better strategy, but for any single case this needs to be established before conclusions can be reached.

Protective versus curative use:

- There is no evidence that protective or curative use consistently results in a lower rate of selection for fungicide resistance. (but note that protective fungicide applications may be needed for effective disease control.)
- The existing evidence suggests the specific circumstances will determine whether a shift in spray timing will increase or decrease selection for fungicide resistance.''

All these conclusions are well underpinned by evidence, which should guide the development of resistance management methods. As discussed in the chapter

In advocating evidence-based resistance management, the authors recognise that commercial and regulatory decisions about product development and registration cannot wait for the accumulation of all the evidence that might be desirable. Decisions need to be made on basis of the existing evidence. The

review of existing evidence presented in this chapter does, however, lead to a set of conclusions that is well underpinned by the existing evidence. These conclusions are underpinned by experimental as well as modelling research on a wide range of very contrasting modes of action and patho-systems. The upshot of this is that these conclusions can thus be used to underpin the development of resistance management plans.

This issue is most acute where a new mode of action fungicide is being introduced, and the manufacturer, regulatory authorities and advisory bodies have to decide on appropriate resistance management strategies. At that point, there is no history of the resistance behavior, usually no resistant isolates available from the field to help judge how resistance might evolve, and no evidence of the effectiveness of different strategies for that specific mode of action. The only relevant guidance at this point thus is the set of conclusions derived from a review of existing evidence, and the knowledge gained by the company during the development of the fungicide. For example, we find overwhelming evidence that adding a mixing partner reduces selection for fungicide resistance. Given a new MOA about which nothing is known, the only sensible assumption thus is that for this MOA mixing will be a useful resistance management strategy. The same holds for the other conclusions on resistance management.

We thus advocate that the set of conclusions reached in this chapter provides the strongest available basis for decisions on resistance management of new modes of action. The strategies can then be refined by specific information on the new mode of action, as it becomes available - for example from laboratory mutation studies, from specific modelling of resistance evolution based on the known efficacy of the new compounds and their potential mixture partners, and then from experience of resistance development on the first patho-systems on which the new mode of action is used.

Multi-drug resistance

The conclusions about the effect of changes in application programs on selection for resistance are almost all based on experimental work and models for target-site resistance/specific resistance. This type of resistance is typically characterised by (i) mutations leading to changes in the target protein so that it is still functional but less sensitive to the fungicide. There are however another types of fungicide resistance, causing multi-drug resistance, that will affect the development of resistance management methods.

Multidrug resistance (Hayashi et al. 2001, de Waard et al. 2006, Walker et al. 2013, Kretschmer et al. 2009) has been associated with plasma membrane efflux transporters with low substrate specificity. These transporters pump the fungicide out of the cell so that it can do less harm. As the transporters are not very substrate specific a range of fungicides may be pumped out of the cell. Multi-drug resistance is associated with over-expression of transporters.

Multi-drug resistance is an important mechanism in insecticide resistance and herbicide resistance. Until a few years ago it had never been found in plant pathogenic fungi and was considered of little importance. However, since that time multidrug resistance has been shown in *Botrytis cinerea* (Hayashi et al. 2001, de Waard et al. 2006, Walker et al. 2013, Kretschmer et al. 2009). There is some evidence accumulating that multi-drug resistance may also play a role in other fungal plant pathogens.

It is not known whether multi-drug resistance in *Botrytis cinerea* occurs in Australian vineyards. There is an urgent need to look into the occurrence of multi-drug resistance. This is the more important because the presence of multi-drug resistance may have an impact on resistance management methods. The resistance factor of multi-drug resistance Botrytis is low. As resistance factors are low, no loss in field performance due to this multidrug resistance has been reported (Walker et al. 2013). Multi-drug resistance is thus not of direct practical concern, but it can have indirect effects that need to be considered.

For example, mixtures of fungicides used as resistance management method may be far less effective if the multi-drug resistance is present and affects both fungicides in the mixture. The same will hold for alternation programs. Further the effect of dose on the redevelopment of resistance can be different in multi-drug resistant pathogens. If the multi-drug resistant pathogen can be controlled with high dosages, it may help to prevent the evolution of target site resistance to the fungicides used. There is however no evidence on these matters, and research into the effect of multi-drug resistance on fungicide resistance management methods is urgently needed.

The use of a limited number of high dose sprays has been suggested to control multi-drug resistance. There is however no evidence whether this approach does provide control.

Abandoning the use of an MOA temporarily

Suppose we have not implemented resistance management methods in time, resistance has developed and has become widespread. An important question in such a situation is whether anything can be done to reverse this situation?

It has been suggested by several authors that temporarily abandoning the use of a fungicide/MOA could decrease the frequency of resistance against that MOA to a low level due to fitness costs of being resistant, after which the fungicide can be used again to control, disease. Toffolatti et al. (2010) for example conducted a very thorough analysis of field data on downy mildew from a large number of orchards. They saw lower frequencies of resistance in vineyards where no QoIs were applied, and they showed a decrease in the frequency of resistance when the use of QoIs was abandoned. They suggested that this may imply that abandoning the use of QoI may regain the downy mildew population its sensitivity to the QoI fungicides.

If the frequency of resistance to a MOA is to decrease when the use of the MOA is abandoned there must be a fitness cost to the pathogen of being resistant to the fungicide. In this way the population of the resistant isolate will grow slower than the population of the sensitive isolate in the absence of fungicide. This is exactly the opposite case as we have discussed in figure 2. The selection is now AGAINST the resistant strain. This does however not change anything in our reasoning about the selection coefficient and the dynamics of the frequency of resistance. In the present case where the selection is against the resistant strain the selection coefficient takes negative values, $s=r_R-r_S<0$. We can thus use our simple governing principle to assess the effect of abandoning the use of a fungicide on the frequency of resistance in the pathogen population.

Unfortunately, there is no information available on the fitness cost of being resistant to fungicides in Australian vineyards. We will thus have to rely on published evidence from other area of the world. We will concentrate on the QoI fungicides and look into powdery mildew and downy mildew as example cases.

Powdery mildew (Erysiphe necator)

There is one publication studying the fitness costs of resistance to QoI fungicides in the pathogen powdery mildew (Rallos et al. 2014).

To assess the competitiveness of resistant isolates the authors did two types of experiments using (i) pairs of one resistant and one sensitive isolates and (ii) a bulk sample of resistant and sensitive isolates. The inoculum was sprayed onto potted plants, and maintained for four cycles where each cycle new plants were inoculated with inoculum from the previous cycle. In these experiments the frequency of the G134A resistant strains tended to increase over the duration of the four cycles. Only rarely the frequency decreased to some extent. The authors conclude that there is no evidence of a fitness cost in these experiments.

There were also experiments under field conditions. In two successive years a mixture of a sensitive and a resistant isolate was inoculated and the development of the frequency of the resistant isolate followed. The results are contradictory. In one season the frequency of the G134A mutation increased and in the other season the frequency decreased. The result is that this experiment does not provide evidence for a fitness cost of the G134A mutation.

These two sets of experiments thus lead to the suggestion that there is no fitness cost of being resistant to the QoI fungicides used. This implies, in the terminology of the governing principle, that the selection coefficient, $s=r_R-r_S$, is zero (s=0). This in its turn implies that abandoning the use of QoIs will not lead to a decrease in the frequency of QoI resistance.

Field observations were done in a vineyard where the use of QoI was abandoned for a period of four years. In some parts of the vineyard the frequency of the QoI resistant strains decreased, but in some hotspot areas the frequency of resistance remains unchanged. The authors conclude that these findings support the conclusions of the controlled experiments that QoI resistance will persist in the field even without the use of QoI fungicides.

If we ignore the fact that the resistance persisted in the hotspot area of the vineyard without a decrease in the frequency of resistance and only use the data from the rest of the field, we see a decrease in the frequency of QoI resistance over time. The authors state in their summary that the frequency of QoI resistance was still 25% after the four year period. If we assume that the starting frequency is 99%, and assume the frequency decreases exponentially we find that the selection coefficient for selection against the G134A mutation when fungicides are not used is s-0.25. We can now use equation 2 to calculate how many years the use of QoIs has to be abandoned before the resistance frequency has decreased from 99% to the initial frequency of 10^{-5}. Figure 3 shows the development of the frequency of resistance for this case.

From this calculation it is concluded that the use of QoIs needs to be abandoned for more than 60 years before the frequency of resistance has decreased from 99% to the initial 10^{-5}.

Note that this last calculation is an underestimate of the number of years the fungicide needs to be abandoned. All other experiments the authors present suggest that abandoning even for centuries would not bring the frequency of QoI resistance down.

Downy mildew (Plasmopara viticola)

There are two publications studying the fitness cost of being resistant to QoI fungicides in downy mildew (Corio-Costet, et al. 2011; Toffoloatti et al. 2011). We will use the data in these two publications to assess the usefulness of abandoning the use of QoIs to regain population sensitivity to the QoIs.

Corio-Costet et al. used *in planta* (grapevine leaves) experiments with field isolates sensitive and resistant to QoIs. The resistance was caused by the G143A mutation. They measured several life-cycle parameters and compared the resistant with the sensitive strain in the absence of fungicide use.

- Latent period: No significant difference was observed between the latent period of the sensitive and the resistant strains.
- The author also measured spore production rate 7 days after inoculation. They did not find a difference between the sporulation rate of the sensitive and the resistant strains.
- Measuring the infection efficiency they showed that the resistant strain has a significantly higher infection efficiency than the sensitive strain.

These assessments show that the isolates tested do not suffer a fitness cost due to the G134A mutation.

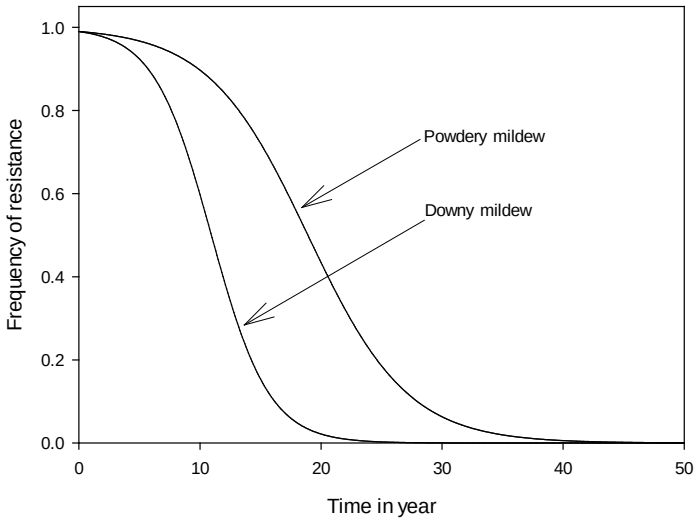


Figure 3. The decrease of the frequency of the fungicide resistance gene through time after abandoning the use of the fungicide. The frequency starts at 99%.

The authors also did competition experiments growing mixtures of resistant and sensitive isolates for eight cycles on vine leaves. Some resistant strains did reduce in frequency over the eight cycles, but other resistant strains did increase in frequency. The increase or decrease in frequency coincided with the trends

in the fitness parameters. The authors conclude that there is no evidence of a fitness cost due to being resistant to QoI fungicides.

Toffolatti et al. (2011) analysed samples from 33 vineyards. Using discriminatory dose techniques the fraction of the pathogen population resistant to azoxystrobin was assessed. The analysis of the data showed that in vineyards where the QoIs were applied around 50% of the population was resistant to QoIs. In vineyards where no QoIs were applied the percentage resistance was lower than 10%. The authors conclude from this that there is potentially a possibility that abandoning the use of QoIs may revert the population from dominated by resistant strains to dominated by sensitive strains, after which the QoI fungicides may be if use again.

To assess the rate at which the frequency of QoI resistance decreases, seven vineyards were monitored for several years. Each year the frequency of resistance was assessed. The data have been reproduced in figure 4. Assuming exponential decay we find that the selection coefficient for selecting against the G134A mutation is s=-0.42. Using this in the same way as done for the powdery mildew case we have plotted the dynamics of the frequency of resistance to QoI fungicides in figure 4. This calculation shows that it will take around 38 years before the frequency of resistance is reduced from 99% to the initial 10^{-5}.

Our calculations thus show that the QoIs have to be abandoned at least for 40 years to regain a fully sensitive population. And we note here again that this is the most optimistic way of looking at the data. Other data suggest that the frequency of resistance will not decrease even when the QoI fungicides are abandoned for centuries.

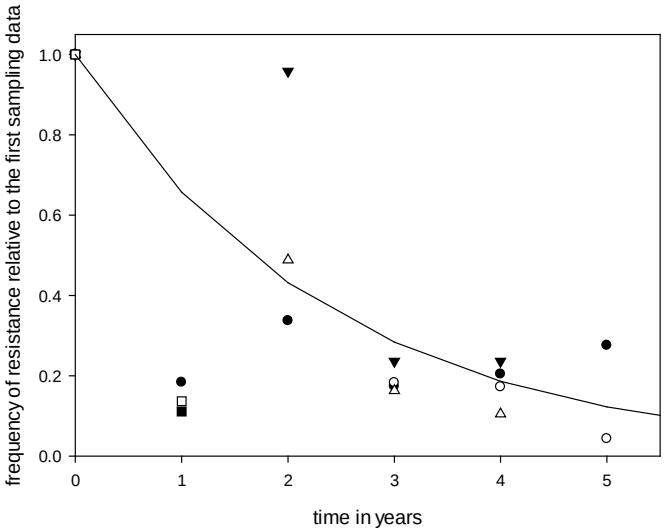


Figure 4. The frequency of the resistance gene, relative to its frequency at the start of monitoring, in four vineyards. Data are from a table in Toffolatti et al. (2011).

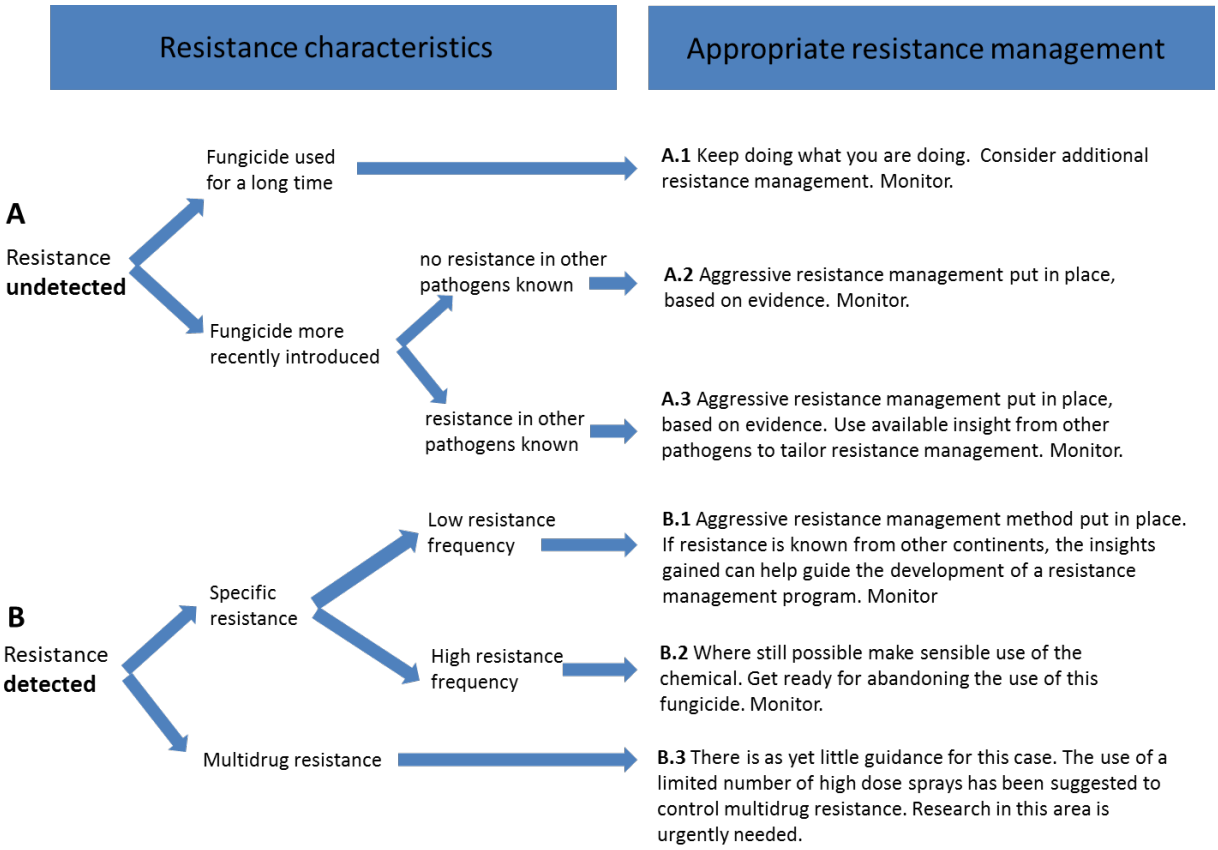
Conclusion: We have discussed here two well-studied examples of fitness costs due to being resistant to QoI fungicides. In both cases the fitness cost was found in some experiments but not in others. Taking the most extreme scenario with fitness cost we calculated the number or years the use of the QoIs need to be abandoned before the population has reverted to fully sensitive and the fungicides can be reintroduced and used for a number of years. In both cases these extreme scenarios showed that use has to be

abandoned for decades before reintroducing the fungicide. This seems to be very long for practical use. Moreover the calculated assumed that no QoI fungicides would enter the vineyards during that whole period. As QoIs are used on other crops or against other diseases it will be difficult to guaranty that no QoI fungicide will enter the vineyard and any time during this long period.

Fitness costs of being resistant to a fungicide have been studied for other pathogens and other fungicides. We have not seen a case where fitness costs seem to be high enough to make abandoning and reintroduction a feasible option. In the insecticide resistance literature, fitness costs are also studied. In these agrochemicals very large fitness costs have been found which may make abandoning and reintroduction an option. But for the fungicides we believe the options are scares.

A decision tree for resistance management actions (diagram adapted from Walker et al. (2013))

Walker et al. (2013) presented a decision tree for the management of fungicide resistance in vineyard pathogens. We have adapted the decision tree based on the information discussed in this report. Each endpoint in the decision tree is discussed in a separate section. The decision tree can help guide the approach to developing a resistance management program as based on the available evidence discussed in this report.



A.1. Keep doing what you are doing. If resistance management methods are in place continue applying these. It may be useful to consider further resistance management methods, but they should only be implemented if it is without doubt that they will further reduce the risk of resistance. Monitoring should be done to assess the situation from year to year.

A.2. Resistance management methods should have been implemented at introduction. If they have not been implemented yet a resistance management program should be developed. Such a resistance management program can be developed on basis of the available evidence as presented in this report and referenced publications. Monitoring is needed to keep track of the resistance situation in the field.

A.3. Resistance management methods should have been implemented at introduction. If they have not been implemented yet a resistance management program should be developed. Such a resistance management program can be developed on basis of the available evidence as presented in this report and referenced publications. Information on resistance development in the pathogens know to have developed resistance can further guide resistance management. Monitoring is needed to keep track of the resistance situation in the field.

B.1. A resistance management program should urgently be implemented. Existing resistance management should be stepped up. If resistance is known from other continents, the insights gained can help guide the development of a resistance management program. Such a resistance management program can be developed on basis of the available evidence as presented in this report and referenced publications. Monitoring is needed to keep track of the resistance situation in the field.

B.2. It is just a matter of time till the use of this fungicide is stopped due to the lack of effective disease control. Where still possible make sensible use of the chemical to bridge the time gap till a new fungicide MOA become available. Monitoring is needed to keep track of the resistance situation in the field.

B.3. There is as yet no evidence that help guide the development of a resistant management program, for multi-drug resistance. The use of a limited number of high dose sprays has been suggested to control multi-drug resistance. Research in this area is urgently needed. Monitoring is needed to keep track of the resistance situation in the field.

Discussion

In this chapter we have given an overview of what is known about the management of fungicide resistance. The results are applied to grape vine pathogens. A decision tree has been developed and presented. The work leads to five key conclusions:

Conclusion 1: Although the situation with resistance development in Botrytis is the most pressing problem at this moment in time, it would be a failure not to invest in the development of anti-resistance strategies for powdery mildew and downy mildew on the argument that these pathogens are not considered a priority by the industry.

Conclusion 2: We now have a simple governing principle that predicts whether a change in a fungicide application program increases or decreases the rate of selection for fungicide resistance. This governing principle together with an exhaustive literature review has led to a set of clear-cut advice about resistance management. The list can be found in the Curtin report on pages 18-21. This list will help develop resistance management programs.

Conclusion 3: Because of its potential importance in the development of effective resistance management methods, research into the management of multidrug resistance is urgently needed. It also needs to be assessed whether multidrug resistance plays an important role in the Australian pathogen populations.

Conclusion 4: Abandoning the use of a fungicide MOA to allow the pathogen population to regain sensitivity, after which the MOA is re-introduced is unlikely to be a practical resistance management tool.

Conclusion 5: Research needs to be done on non-fungicidal disease control methods. If such a method or a combination of these methods significantly reduces the rate of epidemic growth, it will also significantly reduce the selection for fungicide resistance. This opens the possibility to manage fungicide resistance without any change in the fungicide application program.

A model for the economics of fungicide application programs when resistance is developing

Introduction

In this section we develop a model for the cost and benefit calculation of fungicide treatment programs. The model calculates the optimal dose of fungicide treatments as well as the optimal mixtures of fungicides. Optimal here is defined as minimal cost due to disease. The cost due to disease is the sum of the costs of the fungicide application program and the costs due to disease induced yield loss incurred under the fungicide treatment program.

We first discuss risk aversion, which is an essential component of our modelling approach. Previously published cost benefit analysis did not include risk averseness of growers although it is very well known that risk averseness is a key component of grower decisions on fungicide applications. Next we introduce the model and its implementation. We then study the effect of fungicide resistance in the pathogen population on the optimal application dose of fungicides to which absolute resistance develops. To this end the model will be parameterised for QoI type fungicides. We then turn to the question whether the optimal dose is different when partial insensitivity to fungicides develops. To this end we will parameterise the model for azole type fungicides. The final section studies mixtures and their optimal dose combination. We finalise the chapter with an outlook of further developments possible on bases of the results of this project.

We will use grape powdery mildew on grape vine as our key example. As a contrasting case we also discuss septoria blotch on wheat. This comparison was chosen for its contrasting results, so that insight is build up into the factors affecting optimal application programs.

Risk aversion

One of the reasons given by Wiik & Rosenqvist (2010) for the zero or negative returns is the overuse of fungicides. They argue that treatments programs with fewer applications or lower dosages would have resulted in a positive return in years where the achieved returns were negative. For the UK Hardwick et al. (2001) came to similar conclusions.

The economically optimal dose would be that dose that minimises the total cost due to disease. This total cost is the sum of the costs of the fungicide application program and the cost due to disease-induced yield (defined as product value) loss. Since disease pressure differs between years, the optimum dose differs between years. Te Beest et al. (2013) introduced a simple method to calculate the long term average optimal dose in fungicide treatment programs taking the year to year variability in disease pressure into account. They found, in agreement with the cost-benefit studies mentioned above, that dosages used by growers are higher than the long term economic optimum dose.

However, consider a year that by chance develops a high severity and growers used the dose that optimises long term average cost. This dosage may then very well be too low to control the high epidemic pressure. Growers then suffer considerable losses, which in extreme cases may result in total losses. Growers may very well be willing to use the higher dosages they do use to buffer against these infrequent years where a high disease severity will develop that potentially causes a critical economic loss. This is termed ‘risk

aversion' and has been discussed by various authors. Te Beest et al. (2013) were the first to introduce a method to include risk aversion in the calculation of economically optimal dosages. They found that the optimal dosage under risk aversion are much higher, much closer to what is used by growers, than the optimal long term average dose.

We will consider the both the long term average optimal dose and the optimal dose under risk aversion. In both cases we will show how optimal treatment dose varies with the development of fungicide resistance in the pathogen population.

Theory and approaches

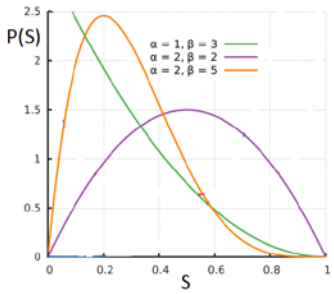
The cost benefit analysis

Disease variability: The severity (fraction of the host (leaf) area infected by the pathogen) of the epidemic at a reference growth stage in the cropping season when no fungicides are used is *S*. The probability distribution describing the probability that in a growing season severity *S* develops is given by *P*(*S*).

Box 1. *The probability density P(S).*
The severity, S, defined as the fraction of the host surface are occupied by the disease, can vary between 0 and 1. (Which means that *P*(*S*)=0 for *S*<0 and *S*>1.) We will use the Beta distribution to model P(S). This distribution is very flexible and can describe a wide range of shapes. The distribution is given by

$$P(S) = \frac{1}{B(a,b)} S^{a-1} (1-S)^{b-1}$$

where *B*(*a*,*b*) is the beta-function $B(a,b) = \int_0^1 S^{a-1} (1-S)^{b-1}\, dS$, *a* and *b* are parameters shaping the Beta distribution. Some examples are given in the figure below.



te Beest et al. (2013) have used an exponential distribution to model P(S). This distribution is given by

$$P(S) = \alpha e^{-\alpha S}$$

And was shown to give a good fit to observed disease severities. For this distribution P(S)=0 for S<0. It does however have a tail to S=∞. When the observed disease severities are small, the exponential distribution can be a useful distribution and the fact that the tail extends beyond S=1 is not an issue because the density is the tail is extremely small.

The cost calculation: We explain the calculation of the total cost due to the fungicide application and due to the disease-induced yield loss. The resulting equation will form the basis of all further calculations in this report.

Assumption: We assume that yield is well correlated with the severity of the epidemic at a key growth stage of the crop. This assumption is an appropriate simplification of reality. For example the grain yield of wheat lost due to the pathogen *Z. tritici* is well known to be correlated to disease severity at growth stage 75 (Zadoks scale). Similarly the grain yield loss of barley due to the pathogen *Rhynchosporium secalis* is known to be correlated with disease severity at growth stage 11.1 (Feekes scale). The same reasoning is expected to hold for grape vine diseases although data on this have not been published to date.

The disease severity S, as we used in the probability density, $P(S)$, that is interpreted as the probability that in a growing season an epidemic develops that reaches severity S if no fungicides are applied, is here further interpreted as the disease severity at this key growth stage.

Definition: Total costs of disease control (of fungicide applications and yield loss due to the disease) is denoted by $C(S, A)$. A is the fungicide application program. See Box 2 for an explanation about the notation on treatment programs.

Cost calculation: The costs of the fungicide application includes the cost incurred to buy the fungicide for application i, C_s, and the, fixed costs of spraying the fungicide in the field (such as labour, tractor fuel, etc.) for application i, C_F. The total cost of the spray programme thus is

$$C_F(A) = M\, C_s + \sum_{i=1}^{M} C_{Ai}$$

The costs due to disease-induced yield loss depend on the severity of the disease when no fungicide is used, S, and the reduction in the severity due to spray program A, giving

$$C_Y(S, A) = P_Y\, Y\; g(S, A)$$

where P_Y is the sale price of the produce (the grape or grain), Y is the yield of the produce given that the crop is disease free, S is the disease severity in the absence of a fungicide treatment program, and A is the application programme. $g(S,A)$ is the fraction of the maximum yield lost under application programme A when the untreated disease would develop severity S.

The disease severity at the reference growth stage is caused by the pathogen population which consists of a strain sensitive to the fungicide and a strain with reduced sensitivity, or even resistance, to the fungicide. The disease severity caused by the sensitive and the insensitive strains under application programme A is given by $S_S(A)$ and $S_R(A)$, respectively. The total severity is the sum of the severities due to the two strains. How the severity of the disease due to the two pathogen strains depends on the fungicide application programme, A, will be discussed later on.

The expression for the cost due to disease is thus

$$C(S, A) = M\, C_F + \sum_{i=1}^{M} C_{Ai} + P_Y\, Y\, (1 - g(S_S(A) + S_R(A)))$$

This is the cost function used to calculate the long term average cost of disease control and the expected cost under risk averseness.

<u>The long term mean cost of disease control</u>: Before we explain the disease epidemiology and the fungicide treatment program we will derive equations for the mean cost of disease control. This shows how cost of fungicides and cost due to disease induced yield loss can be combined with the seasonal difference in disease severity.

The expected cost of control, i.e. the long term average cost, when applying treatment program A, $E(C(A))$, is calculated from

$$E(C(A)) = \int_0^1 P(S)C(S,A)dS$$

The optimal application program is defined as the application program that minimises the long term mean cost due to disease.

The optimal application program is found from $min_A\, E(A)$

The cost of disease control under risk aversion**:** Growers may be willing to pay the additional cost of a higher fungicide dose each year as a risk-reducing investment (i.e. 'insurance') in order to be protected from occasional large costs.

Most studies about risk aversion and the use of pesticides (Pannell 1991) account for the risk aversion considerations of growers' objectives to be

(i) To maximise expected utility. Utility is then assumed to be a function of 'initial wealth' plus the monetary gain.

Box 1. *Application program.*

The application program, A, is the series of fungicide applications in the treatment programme, A={A_1, A_2, A_3……..}. The subscript i=1,2,3,….,M refers to the spray number where M is the total number of applications during the crop growing season. Each of the applications consists of a spray with a fungicide with dosage D_i. We will here describe a two applications programme. It will become clear from this description that other spray programmes can easily be described using the same methods.

After a fungicide application the fungicide affects the pathogen's lifecycle parameters for some time. Because the fungicide is broken down by the plant's metabolism, is broken down by sunlight and/or is washed of leaves by rain the effectiveness of the fungicide decreases over time and finally becomes zero. We will call the time window in which the fungicide affects the pathogen's life-cycle parameters 'the fungicide time window'. Time periods where no fungicide is active are termed 'the non-fungicide time window'.

So, for a two spray programme one season is divided in five time windows that are alternate non-fungicide and fungicide time windows. In time window 1, from the start of the crop growing season till the moment the first spray is applied, is a non-fungicide window of length Δ_1. Time window 2 is a fungicide time window with length Δ_2, time window 3 a non-fungicide time window with length Δ_3, time window four a fungicide time window with length Δ_4, and the final time window till the reference growth stage of length Δ_5. Figure 1 visualises the entire growing season.

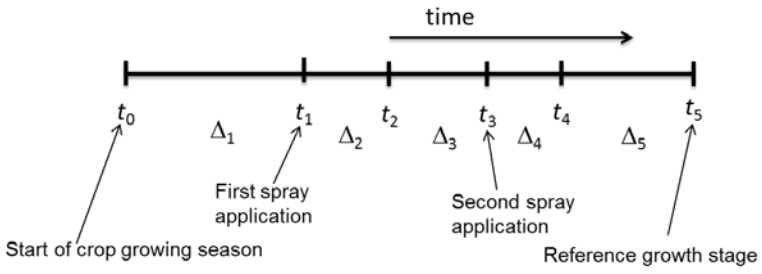


Figure 1.1: The time windows during one crop growing season.

There can of course be cases where the decay of the fungicide's activity is so slow that windows Δ_2 and Δ_4 overlap. In such a case we need to describe the action of the

(ii) Use a 'maximin' principle. Here the grower aims at maximising the minimal gain, which is the same as minimising the maximum cost.

We use the maximin idea as it closely aligns with the idea that a grower aims to reduce the loss they can incur from a severe epidemic. We phrase grower's aim as: *A grower aims to minimise the expected costs incurred from the once each N years where a large epidemic develops during the growing season.* From the probability density, *P*(*S*), we find that once each N years the epidemic severity, *S,* is $S \geq S_N$, and S_N is found from

$$\frac{1}{N} = \int_{S_N}^{1} P(S)dS$$

The expected costs incurred given that the epidemic develops a severity equal to or larger than S_N is calculated from

$$E(C|S \geq S_N) = \int_{S_N}^{1} \frac{P(S)}{(1/N)} C(S,A)\, dS$$

the factor (1/N) is to scale P(S) such that for $S>S_N$ it is a probability density.

The optimal application program is found from $min_A\, E(C|S \geq S_N)$

We will use this minimise the maximum costs to look at risk averseness.

Box 3. Relation with te Beest et al. 2013.

te Beest et al. (2013) use a slightly different way of calculating the cost for risk averse growers. They calculate S_N from equation (I.7) and calculate the costs by substituting S_N in the cost function. The optimal application program then is calculated from $min_A\, C(S_N, A)$.

This leads to a bit cumbersome interpretation of the optimal application program as it is the program that minimises the cost for the treatment of an epidemic developing precisely a severity S_N. And the costs to disease will actually be higher than that when an epidemic develops with the one in *N* years largest severity.

For the linear disease yield relation and exponential P(S) we find that the factor -ln(1/N)/α in their equation (11) needs to be replaced by (–ln(1/N) + 1)/α. This results in larger optimal application dosages than calculated by te Beest et al. I have recalculated all graphs in the paper and found no differences in the qualitative outcomes of the model. The optimal dose is slightly increased in our way of calculating optimal dose under risk aversion.

The epidemic development

We now describe disease dynamics and the calculation of the severity that will develop at the key growth stage when no fungicides are applied, S. Then we introduce the effect of the fungicide on the pathogen dynamics and consequently on disease severity.

The epidemic severity under the fungicide treatment program: We keep track of the disease severity caused by the sensitive and the insensitive/resistant strain, S_S(t) and S_R(t), respectively. The dynamics of the severity caused by these strains is given by

$$\frac{d\,S_S}{d\,t} = r_S(t)\,S_S(t)$$
$$\frac{d\,S_R}{d\,t} = r_S(t)\,S_R(t)$$

where $r_S(t)$ and $r_R(t)$ are the rates of increase in severity of the sensitive and the insensitive strain. These rates are affected by the fungicide applications. The growth rates of the strains depends on fungicide dose and we write $r_S(D)$ and $r_R(D)$ for the growth rate of the sensitive and the resistant strain, respectively, when the fungicide is present. We have assumed that in time windows where the fungicide is not active the growth rates of the two strains have the same value, *r.* Considering here the case of a two spray program (which can straightforwardly be extended to more applications), and referring to box 1, at the end of the last time window the severity of the strains is given by

$$S_S = S_S(0)\; e^{r\,\Delta_1}\; e^{r_S(\mathrm{D})\Delta_2}\; e^{r\,\Delta_3}\; e^{r_S(\mathrm{D})\Delta_4}\; e^{r\,\Delta_5}$$
$$S_R = S_R(0)\; e^{r\,\Delta_1}\; e^{r_R(D)\Delta_2}\; e^{r\,\Delta_3}\; e^{r_R(D)\Delta_4}\; e^{r\,\Delta_5}$$

where $S_S(0)$ and $S_R(0)$ are the severity of the strains at the start of the growing season. The sum of these is the total severity of the primary inoculum, S_0. With the definition of the fraction insensitive, $\theta=S_R/(S_S+S_R)$, we can rewrite $S_S(0)=(1-\theta)S_0$ and $S_R(0)=\theta S_0$. With some further rewriting and reorganising we find

$$S_S = (1-\theta)\,S\; e^{-\left(r-r_S(D)\right)(\Delta_2+\Delta_4)}$$
$$S_R = \theta\,S\; e^{-\left(r-r_R(D)\right)(\Delta_2+\Delta_4)}$$

where *S* is the severity of the epidemic when no fungicide treatment programme is in place. Clearly when the insensitive strain is absolutely resistant to the fungicide $S_R^*=\theta S$.

The growth rates r_S and r_R as function of fungicide dose: What remains is to specify how the growth rates of the fungicide sensitive and fungicide insensitive strains depend on the fungicides dose *D*. As discussed in Hobbelen et al. (2011a,b 2012) a systemic fungicide affects both the pathogen transmission rate, β, (through the infection efficiency and spore production rate) and the length of the latent period, *p*. We use an H-E-I-R model to describe the dynamics of the epidemic. As discussed in Madden et al. (2007) the growth rate of the pathogen population in the H-E-I-R model is given by

$$r = \sqrt{m^2 + \frac{1}{p\,i}\;(\beta\,H - 1)} \;-\; m$$

where

$$m = \frac{p+i}{2\,p\,i}$$

Each strain of the pathogen has its own growth rate, r_S and r_R, and depends on the fungicide applied. The effect of the fungicide is expressed through its effect on the life-cycle parameters of the pathogen strains.

The transmission rate, β: A dose *D* of the fungicide results in a transmission rate

$$\beta_S(D) = \beta_0\left(1 - \beta_{mS}\left(1 - e^{-k_S\,D}\right)\right)$$

for the sensitive strain and

$$\beta_R(D) = \beta_0\left(1 - \beta_{mR}\left(1 - e^{-k_R\,D}\right)\right)$$

for the insensitive strain, where β_0 is the maximum transmission rate when no fungicide is used, β_{mS} and β_{mR} are the maximum fractional reduction of the transmission rate (i.e. at infinite dose) of the sensitive and the insensitive strain respectively under applications of the fungicide. When the insensitive strain is completely resistant we have $\beta_R(D)=\beta_0$ (by taking $k_R=0$).

The latent period, p: In the HEIR models, as used by Peter Hobbelen et al. (2011a,b 2012) the latent period is given by p=1/γ, where γ is the probability per time unit that a lesion in the latent stage becomes an infectious lesion. The transmission rate as function of fungicide dose is given by

$$\gamma_S(D) = \gamma_0 \left(1 - \beta_{mS}\left(1 - e^{-k_S D}\right)\right)$$

for the sensitive strain and

$$\gamma_R(D) = \gamma_0 \left(1 - \beta_{mR}\left(1 - e^{-k_R D}\right)\right)$$

Where $1/\gamma_0$ is the latent period when no fungicide is used. We have assumed that the shape parameters, k_S and k_R, and the maximum reduction parameters, θm_S and θm_R, have the same values as for the effect of the fungicide on transmission rate. But this assumption can easily be changed if needed.

Mixtures: For the mixtures we get

$$\beta_S(D_1, D_2) = \beta_0 \left(1 - \beta_{mS1}\left(1 - e^{-k_{S1} D_1}\right)\right)\left(1 - \beta_{mS2}\left(1 - e^{-k_{S2} D_2}\right)\right)$$

$$\beta_R(D_1, D_2) = \beta_0 \left(1 - \beta_{mR1}\left(1 - e^{-k_{R1} D_1}\right)\right)\left(1 - \beta_{mR2}\left(1 - e^{-k_{R2} D_2}\right)\right)$$

And when the insensitive strain is absolutely resistant to fungicide F_1 we have

$$\beta_R(D_1, D_2) = \beta_0 \left(1 - \beta_{mR2}\left(1 - e^{-k_{R2} D_2}\right)\right)$$

Since we assume that the mixing partner is a multi-site fungicide they do not have an effect on other pathogen life-cycle parameters than the infection efficiency.

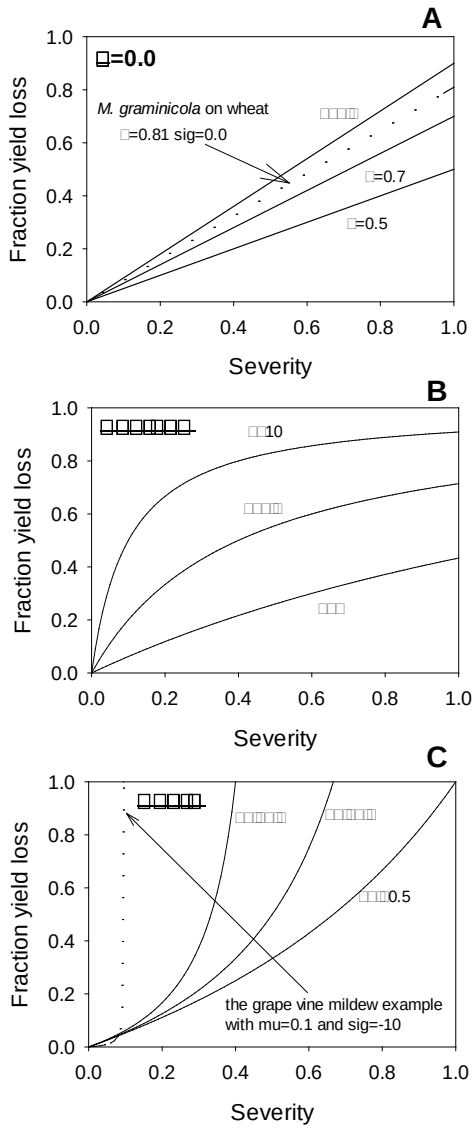


Figure 1. Disease severity versus yield loss curves as used in this chapter. The two key examples of grape powdery mildew and septoria blotch are drawn in the figures A and C.

The yield loss function, g(S,A)

The only remaining quantity to define is the yield loss function, $g(S,A)$, describing the effect of disease severity on the fraction yield lost to disease. A range of yield loss functions has been published for different plant pathogens (Madden et al. 2007). Any relation describing the effect of disease severity on yield loss can be used in our calculations of costs. Here we will use a simple and flexible disease yield relation that covers linear disease yield loss as well as convex and concave yield loss functions.

$$g(S,A) = \frac{\mu X}{1 + \sigma X}$$

X is the severity as it develops under the fungicide application program,

$$X = (1-\theta\ )S\ e^{-(r - r_S(D))\Delta} + \theta\ \ S\ e^{-(r - r_R(D))\Delta}$$

For σ=0 this function describes a linear disease yield loss relation (Figure 1.A). For σ>0 the relation is concave with the slope of the yield loss relation decreasing with increasing disease severity (Figure 1 B), and for σ<0 it is convex with the slope of the yield loss curve increasing with increasing disease severity (Figure 1 C).
As can be seen from the figure, there will be parameter values μ and σ for which $g(S)>1$ for a value $S>S_{max}$. In such cases yield is entirely lost and $g(S)=1$. In Appendix 1 we explain the adjustments to the cost calculation needed in these cases.

RESULTS

Applications: Septoria tritici blotch on wheat and grape powdery mildew

In this section we apply the model to two contrasting cases, septoria tritici blotch (STB) on wheat and powdery mildew on grape. STB is caused by the fungal pathogen *Zymoseptoria tritici*, and is one of the main disease on wheat in Europe, North Africa, New Zealand and NW USA. Grape powdery mildew (PM) is caused by the fungal pathogen *Erysiphe necator*, infecting crops wherever grape vines (both table and wine) are grown. One of the key differences between these two cases is the disease-yield loss relationship. The main cause of yield loss due to STB is that the pathogen reduces the green leaf area available for photosynthesis, and thereby reduces the rate of build-up of grain biomass. Both grain numbers and grain size are reduced. This results in a linear relation between disease severity and yield loss, as shown in figure 1A. PM also causes reduced berry numbers and weight, but its main effect is that grapes covered by more than 5-10% mildew are rejected by the wine making industry. This results in a gradual increase of yield loss at low disease severity, due to the loss of yield mass, with a sudden very steep increase in yield loss when disease severity exceeds 5-10% due to rejection by the industry (figure 1C).

We consider two groups of fungicides with different modes of action, to which very contrasting levels of resistance develop. We will consider the QoI fungicides as typical examples of fungicides to which (virtually) absolute resistance develops in the plant pathogens under consideration. In contrast we also consider the azole fungicides to which both pathogens developed partial resistance. Actually resistance to the azoles is developing through a sequence of strains increasing in resistance to the azole fungicide, but we will consider the simplified situation with two strains one of which is partially resistant to the azole under consideration. A summary of all model parameters for both patho-systems is given in Table 1 (at the end of the chapter). The dose response curves for all four pathogen fungicide combinations are shown in figure 2.

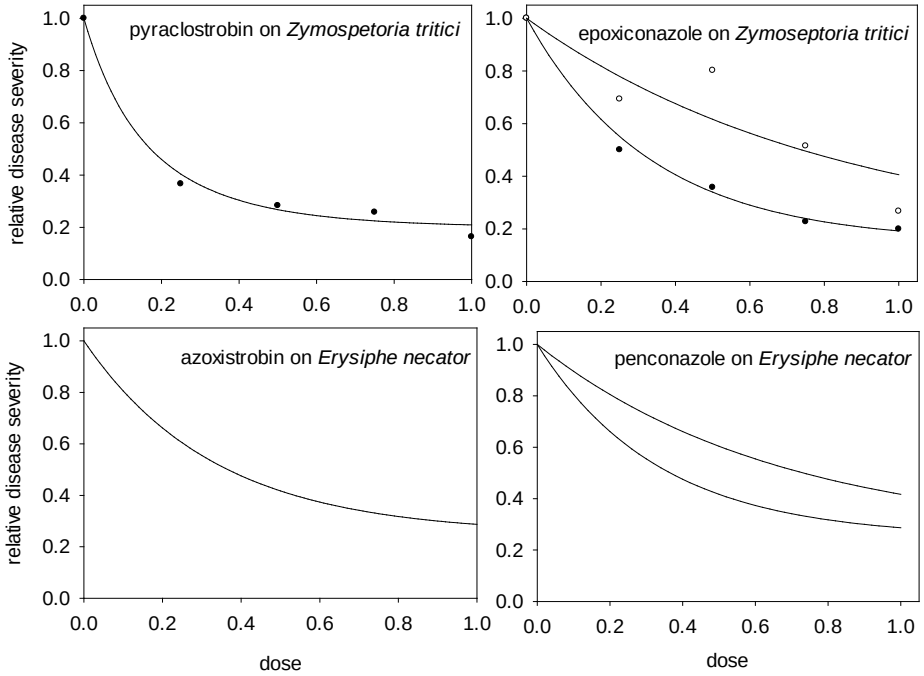


Figure 2. The dose response curves for the pathogen fungicide combinations used in this chapter. The dose response curves for septoria blotch (*Zymoseptoria tritici*) are parameterised on extensive data sets, as shown as dots in the graphs. The curves for grape powdery mildew (*Erysiphe necator*) are constructed on basis of several published sources on fungicide efficacy.

QoI case, absolute resistance

Figure 3A and 3B show the long term average cost of disease (fungicide application costs + costs due to yield losses) as function of dose for a range of frequencies of the resistant strain. Typically, starting from the untreated situation (dose is 0), mean cost decreases with increasing dose due to the increased control of the pathogen. At higher dosages the dose response curve approaches an asymptote. This means that increasing dose provides less additional control than at low dosages. The increased cost of the fungicide with increasing dose then causes the mean cost to increase with increasing dose.

The dose that minimises the mean cost varies with the frequency of the resistant strain. In the case of STB the optimal dose is smaller in situations where the frequency of the resistant strain is larger. This implies that it is not justified from a cost-benefit perspective to increase fungicide dose when resistance is developing in the pathogen population.

The situation for PM is different. As long as the frequency of the resistant strain is below 0.2 the optimum dose increases when resistance develops in the pathogen population (Fig 2B). Only when the frequency of resistance is larger than 0.2 (and remember that grapes are rejected by industry when the severity on the grapes is larger than 10%) the economic optimum dose decreases with resistance frequency. NOTE that the optimal dosages are very low as compared to what growers normally apply. As we have discussed the actual dose applied may very well be not aimed at minimising the long term mean cost but will include a

higher dose as a method of risk avoidance. We will however see that the trends shown here also apply to the case where growers are risk averse, though the applied dosages are considerably higher.

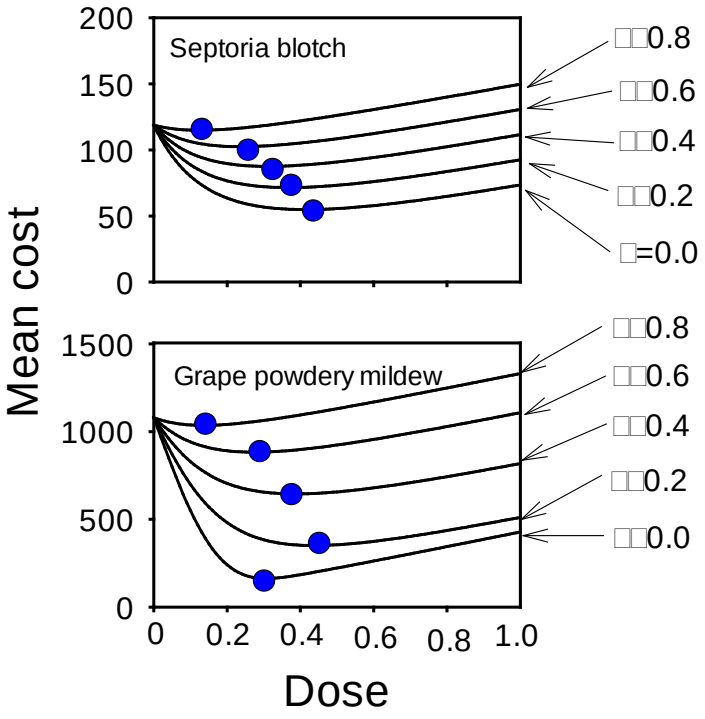


Figure 3. Long term mean cost to disease (cost of fungicide applications plus the cost due to disease induced yield loss) as function of the fungicide dose applied, for several values of the frequency of resistance in the pathogen population. The frequencies are the number on the right hand side of the graphs. Dots represent the dose at which mean cost is minimised.

At first sight it may appear strange that when resistance increases in the STB blotch case, the optimal fungicide dose decreases. One may be inclined to think that when resistance increases one should apply more fungicide to 'keep the problem under control'. However, our calculations clearly show that this is not the economically optimal way of reacting to the developing problem. For STB in wheat treated with a fungicide to which absolute resistance develops the economically optimal reaction is to apply less fungicide simply because the costs of application do not outweigh the reduction in costs due to disease induced yield loss. As the resistance to QoI is (virtually) absolute, the efficacy of the fungicide is zero. Hence, it is not surprising that fungicide use is uneconomic.

The reason that the optimal dose first increases and then decreases for PM becomes clear from figure 4A. The figure shows the fraction of the years that the yield is totally lost because the disease severity increases above 10%, and the grapes are rejected by the wine making industry, at full dose rate of the fungicide in the application program. In this figure we see that when applying full dosages of the fungicides in each application, the number of years the yield is rejected for wine making is very small for resistance frequencies below 0.2. Clearly applying an appropriate dose can prevent any problems arising. That it needs a higher dose to keep the disease severity below 0.2 when there is resistance in the population, is obvious.

Above a resistance frequency of 0.2 the number of years where the yield is rejected for wine making increases sharply with the frequency of resistance in the population. Even applying a full dose it is not possible to keep the problem at bay in all years. In such a case applying an even lower fungicide dose becomes an economically better option.

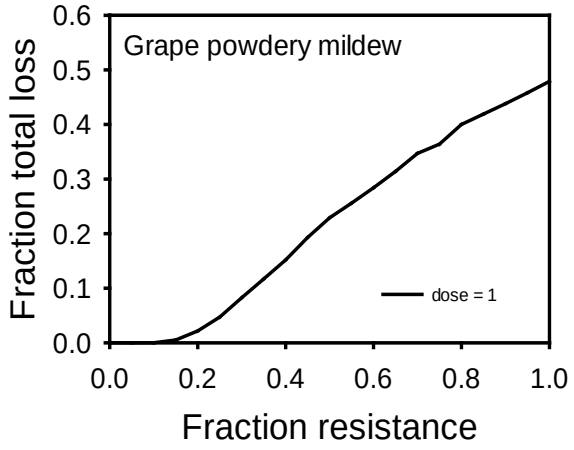


Figure 4. The fraction of years where all grape yield is lost because the severity on the grape bunches increases above 10%, when the full dose of the fungicide is applied.

We now turn to the realistic case of a risk averse grower. Figure 5 shows the optimal dose as function of the frequency of resistance for three levels of risk aversion. The 50% largest epidemics means that a grower aims to protect her crop for the epidemics in the 50% of the growing seasons that the highest epidemics develop. '10% highest epidemics' means that the grower protects her crops against the 10% largest epidemics that can develop during a growing season.

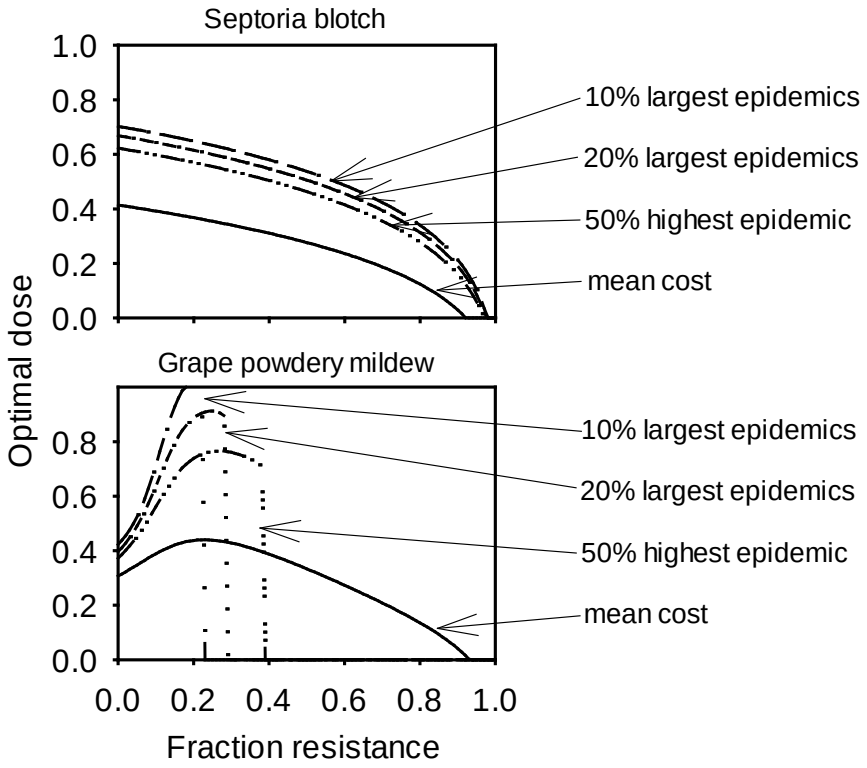


Figure 5. The optimal dose as function of the frequency of resistance for three levels of risk aversion. The 50% largest epidemics means that a grower aims to protect her crop for the epidemics in the 50% of the growing seasons that the highest epidemics develop. '10% highest epidemics' means that the grower protects her crops against the 10% largest epidemics that can develop during a growing season.

First we see that in both the STB and the PM case the optimal dosages are much larger for growers that are risk averse. The optimal dose for STB blotch on wheat is about 1.5 to 2 times as large as the optimal dose in the long term mean cost case. For PM this is even more extreme with risk averse growers applying 2 to 4 times the optimal dose in the long term mean cost case.

Strikingly, in the PM case the optimal dose jumps to a zero dose above a threshold frequency of resistance. This implies that when the resistance has increased in the pathogen population above this threshold value the use of the fungicide under consideration is no longer justified from a cost benefit point of view. This switch point is for all levels of risk aversion between a frequency of resistance of 0.2 to 0.4. When the frequency of resistance has increased above the switch point, the grower should no longer apply the fungicide and start applying other control methods including other fungicides instead. This is simply because the number of years that the yield is totally lost, i.e. not accepted by the wine making industry, is increasing.

The azole case, partial resistance

Figures 6, shows the long term average cost as function of dose, reveal that there is a very different effect of the level of resistance in the pathogen population on the optimal dose for azole fungicides compared to the QoIs. In both patho-systems the optimal dose increases with the frequency of the resistance in the pathogen population. This difference between the partial resistance case and the absolute resistance case is further shown in figures 6. Even at high levels of resistance it turns out to be economically justified to use

a high dose of the azole fungicide to compensate for the high levels of the resistant strain which is more difficult to control. This is in sharp contrast to both patho-systems and an application program of QoI fungicides.

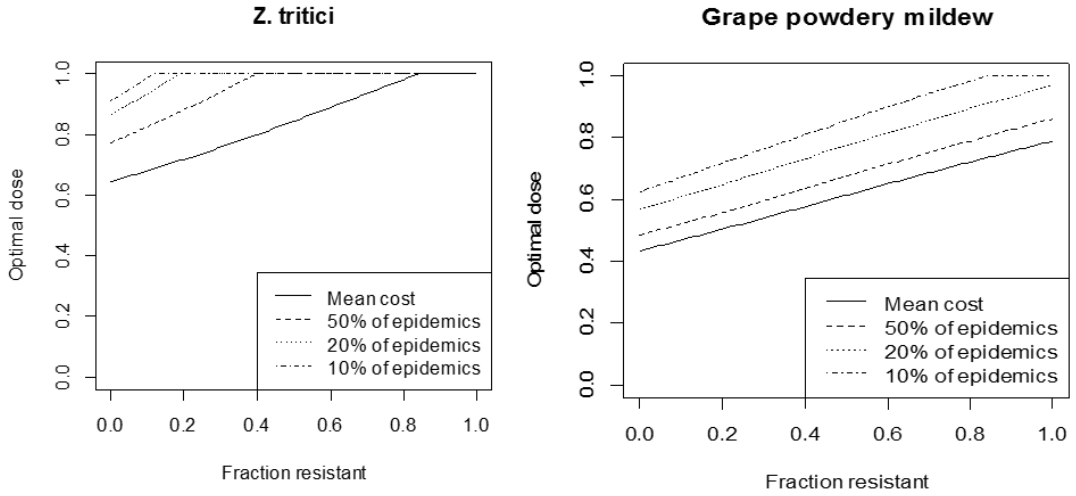


Figure 6. The optimal dose as a function of the frequency of resistance for three levels of risk aversion. The 50% largest epidemics means that a grower aims to protect their crop for the epidemics in the 50% of the growing seasons that the highest epidemics develop. '10% highest epidemics' means that the grower protects her crops against the 10% largest epidemics that can develop during a growing season.

In the PM and QoI case the optimal dose decreased at higher levels of resistance in the pathogen population because in many years it is impossible to reduce the severity below the critical 10% level when there is resistance. Why is this not the case for the azole application program? The optimal dose increases with increasing frequency of resistance, even for very high frequency, say θ>0.5, in the azole case. The solution is that the insensitive strain can still be adequately controlled by high fungicide dosages (figure 2). This leads to the situation that even when resistance is at high levels in the pathogen population it is possible in many years to keep the severity below 10% by applying high dosages. Clearly the losses in the occasional year where high fungicide dosages are not able to reduce the severity below 10% and resistance levels are high, do not change the trend that increasing frequency of resistance require higher dosages to be used.

Turning to the case of risk averse growers we consider figure 6. As in the absolute resistance case an increase level of risk averseness increases the optimal fungicide dose. In the STB patho-system the optimal dose for risk averse growers is 1 for a considerable stretch of the frequency of the resistant strain in the pathogen population. In the PM case optimal dosages increase with increasing frequency of resistance. At no point does the dose drop to zero as in the QoI case. Again this is due to the high level of control that a full dose of the azole fungicide still provides when resistance frequency is high, because the insensitive strain can be controlled by high dosages.

Cost benefit analysis for mixtures

We now turn to the use of fungicide mixtures. Here we only study the PM case as it gives a sufficient overview over the possible cost benefit effects of mixing. As the at-risk fungicides we use the QoI and the

azole as in the previous two sections. As the mixing partner we use parameters derived for the case of spiroxamine. Note that we use this parameterisation for our model exploration only because we had access to the parameter values. Spiroxamine is not considered a mixing partner in current application programs, and we are not advocating to change this viewpoint.

Figure 7 shows the effect of the dose of the at-risk fungicide (y-axis) and the mixing partner (x-axis) on the cost of disease (contour lines). When there is no resistance in the pathogen population the optimal dose of the at-risk fungicide in the mixture is approximately 0.3, which is only slightly smaller than the optimal dose under solo use. The optimal dose of the mixing partner is 0.18. Note that these calculations are for the economically mean optimum. Under risk aversion, as we have seen in the previous sections, the optimal dosages are much higher. The trends in dose with changes in resistance frequency or any other parameters is however the same for the mean economic gain as for the risk averseness calculations. Therefore, we only show here the mean economic gain results.

When resistance develops the optimum dose of the at-risk fungicide decreases gradually to zero with increasing frequency of resistance in the pathogen population. The dose of the mixing partner increases with increasing frequency of resistance. When the pathogen population is entirely resistant, θ=1, the optimum dose of the at-risk fungicide is zero, as it has no effect on the pathogen anymore but adds to the cost of the application program. The optimal dose of the mixing partner has increased to 0.4 as it is the only fungicide now available to control the disease.

For the azole case the picture is somewhat different. Figure 8 shows that when there is no resistance in the pathogen population, θ=0, the economically optimum is not to use a mixture but to use the azole as a solo product. The optimal dose for the at-risk azole is then, of course, exactly the same as in the solo use calculation in the previous paragraphs. That adding a mixing partner is not economically beneficial becomes clear is we compare the costs and efficacies of the azole and spiroxamine. Spiroxamine is more expensive that the azole and its efficacy is slightly less. This makes that adding the mixing partner only increases costs and does not add to control.

When resistance is developing, and the efficacy of the azole becomes compromised, it becomes beneficial to add the mixing partner. When the entire pathogen population consist of the partially resistant strains the optimal dose of the mixing partner has increased from zero to 0.2. Again this dose is low because we have calculated the economic mean gain. Under the risk averse scenarios, the dosages are higher but the trends remain the same.

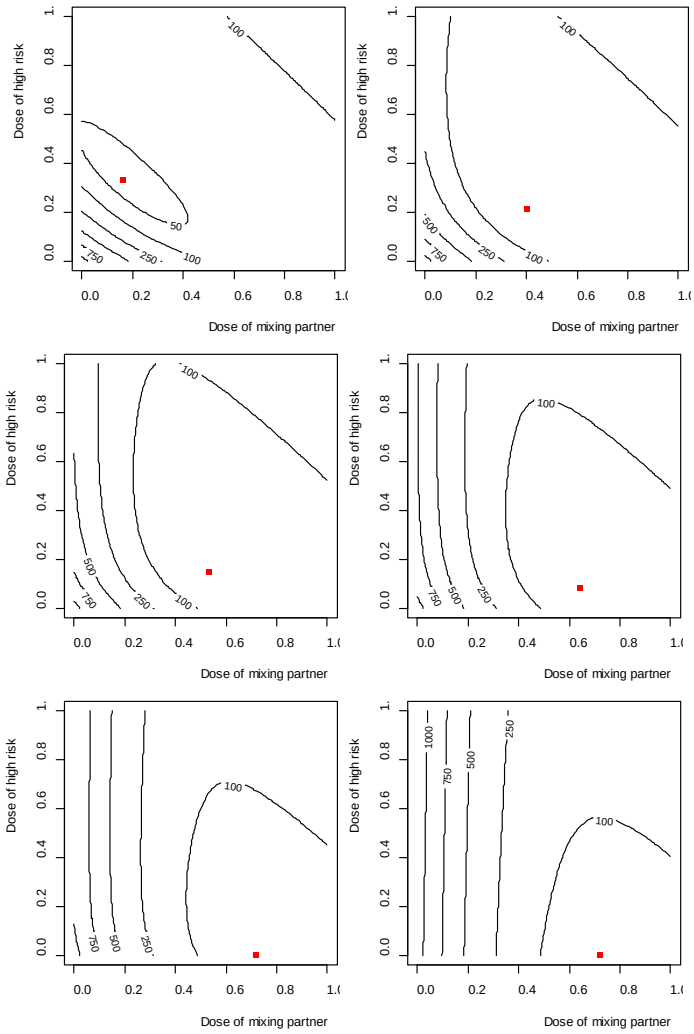


Figure 7. The effect of mixing in the QoI case. Y-axis is the dose of the at-risk fungicide, the QoI. X-axis is the dose of the mixing partner. The contour lines are lines of equal cost due to disease (fungicide application costs plus disease induced yield reduction). The red dot marks the optimal combination of at-risk fungicide and mixing partner.

Generalisations

In this section we look for generalisations of the results from our case studies. We have done a very wide parameter exploration to investigate a range of topics. Here we only show some results that answers key questions about generalisations, all results are available on request. In each section we will first introduce the specific topic to be explored and then show and discuss the results.

We have seen that when absolute resistance to the fungicide develops there are cases where the optimal dose increases, and there are cases where the optimal dose decreases, with increasing frequency of resistance. What determines whether the optimal dose increases or decreases with the

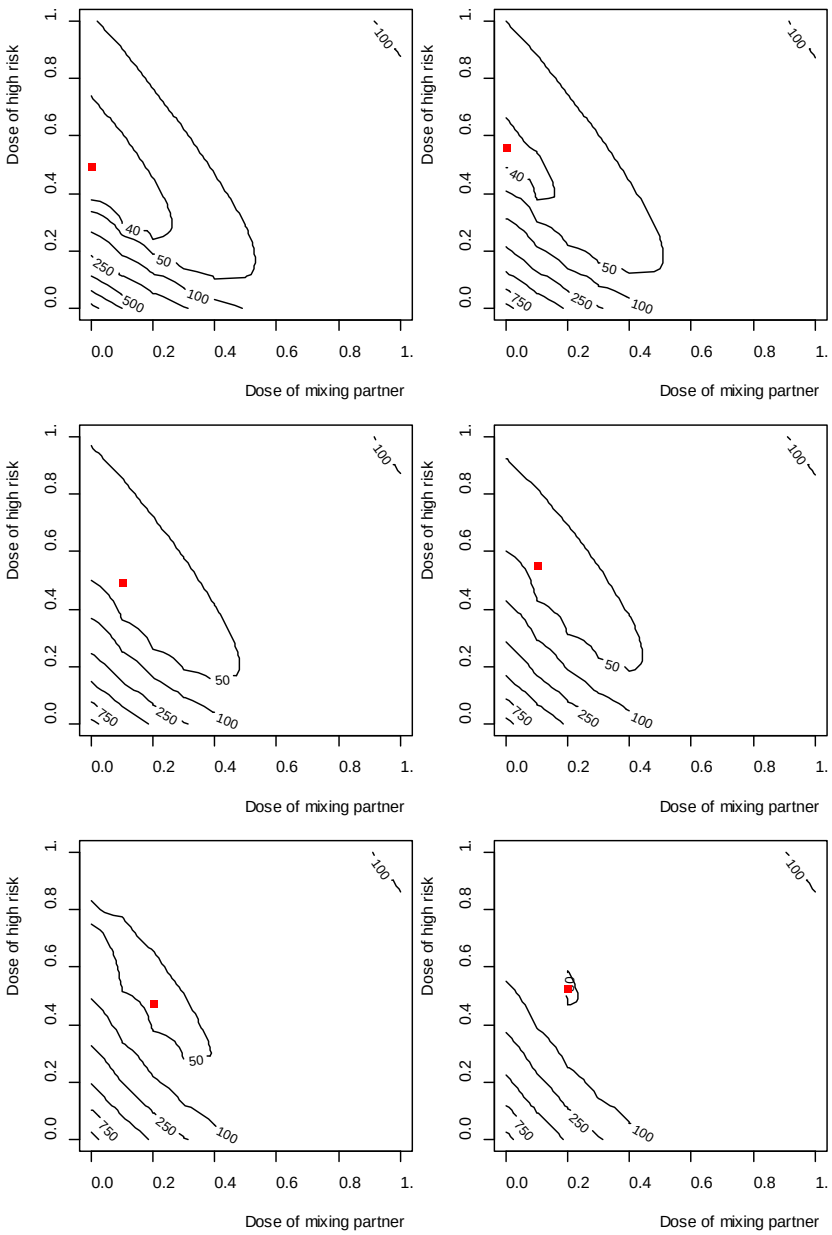


Figure 8. The effect of mixing in the azole case. Y-axis is the dose of the at-risk fungicide, the QoI. X-axis is the dose of the mixing partner. The contour lines are lines of equal cost due to disease (fungicide application costs plus disease induced yield reduction). The red dot marks the optimal combination of at-risk fungicide and mixing partner.

We have found that for all linear and for all concave disease-yield loss relations the optimum dose decreases with increasing fraction of resistance in the pathogen population. This holds both for the long term mean cost as for the case or risk averse growers. This conclusion holds for changes in any other of the parameters in the model.

Only when the disease-yield loss relation is convex we find some cases where the optimum dose increases with increasing frequency of resistance. We have found (figure 9) that there are two quantities that determine whether increasing fraction of resistance increases the optimal fungicide dose.

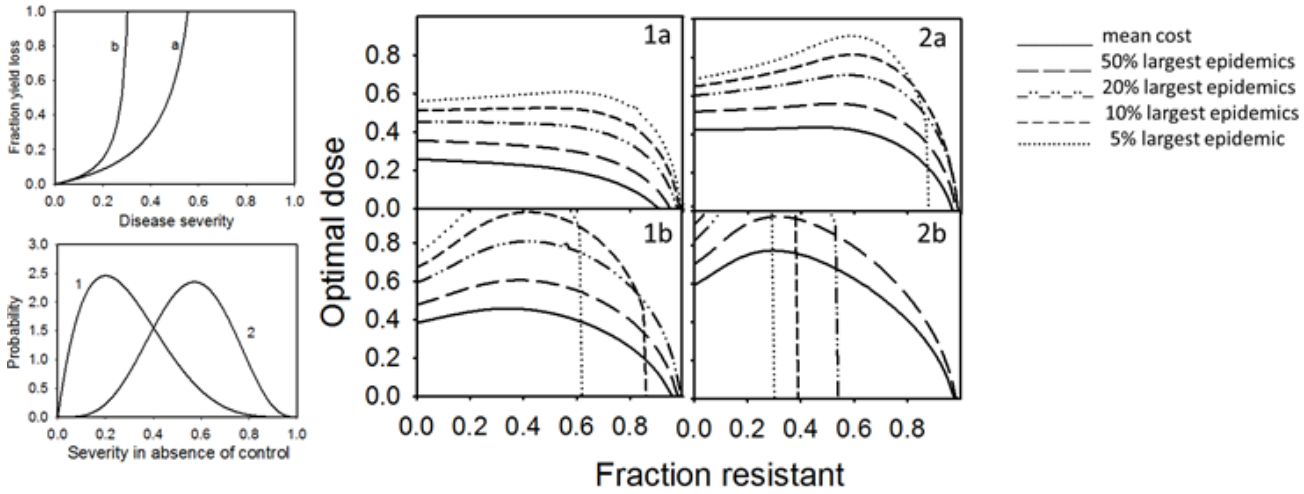


Figure 9. Left hand panels are the disease-yield relationships (top panel) used and the disease severity probability distributions (bottom panel) used in the calculations for the graphs on the right hand side. The right hand side are plots of the optimal dose as a function of the frequency resistance in the pathogen population for a range of risk aversion levels. The number and letter in the right hand upper corner of each subpanel refer to the cases shown in the left hand panel.

The first is the combination of (i) the steepness of the disease-yield relation and (ii) the untreated severity probability density, *P*(*S*). The determining quantity is the fraction of years in which the untreated disease severity is larger than the severity at which all yield is lost. The larger this fraction the more likely it is that the optimum dose increases with increasing frequency of the resistance in the pathogen population. The second determining parameter is the level of risk averseness of the grower. The more risk averse a grower is the more likely it is that the optimum dose increases with increasing frequency of resistance in the pathogen population. Note however again that the disease-yield loss relation needs to be convex and be very steep. There are no cases where a linear or a concave disease-yield loss relation leads to the optimal dose increasing when resistance develops.

For the linear severity-yield loss relation, such as for the *Z. tritici* case, the optimal dose decreases with the frequency of resistance in the pathogen population when absolute resistance develops, such as in the QoI case, and increases for the azole case where partial resistance develops. The key question thus is what level of resistance causes the optimal dose to increase or decrease with the frequency of resistance. There are two ways partial resistance can be expressed in the dose response curve. Firstly, the shape parameter β, determining the curvature of the dose response curve, is zero for absolute resistance and larger than zero for partial resistance. Secondly, the asymptote, RD, determining the maximum effect of the fungicide on the life-cycle parameter, is zero for absolute resistance and larger than zero for partial resistance.

Figure 10A shows the effect of the curvature parameter on the optimal dose versus frequency of resistance relationship. Clearly values of β very close to zero still result in the optimal dose decreasing for increasing

frequency of resistance. However, already for values of β larger than 0.1 the trend is reversed and the optimal dose increases with increasing frequency of resistance in the pathogen population. When β approaches the same value of the susceptible strain there is no difference between the susceptible and the resistant strain resulting in the same dose being optimal irrespective of the frequency of resistance. Figure 8B shows the effect of partial resistance expressed through the asymptote. Again for values of the asymptote very close to zero (absolute resistance) the optimal dose decreases with the frequency or resistance. A small change of the asymptote, RD>0.1, however reverses the trend and the optimal dose increases with the frequency of resistance. Comparing figure 10A and 10B makes clear that the curvature parameter has a much more drastic effect on the steepness of the increased dose with increasing frequency of resistance than the asymptote has.

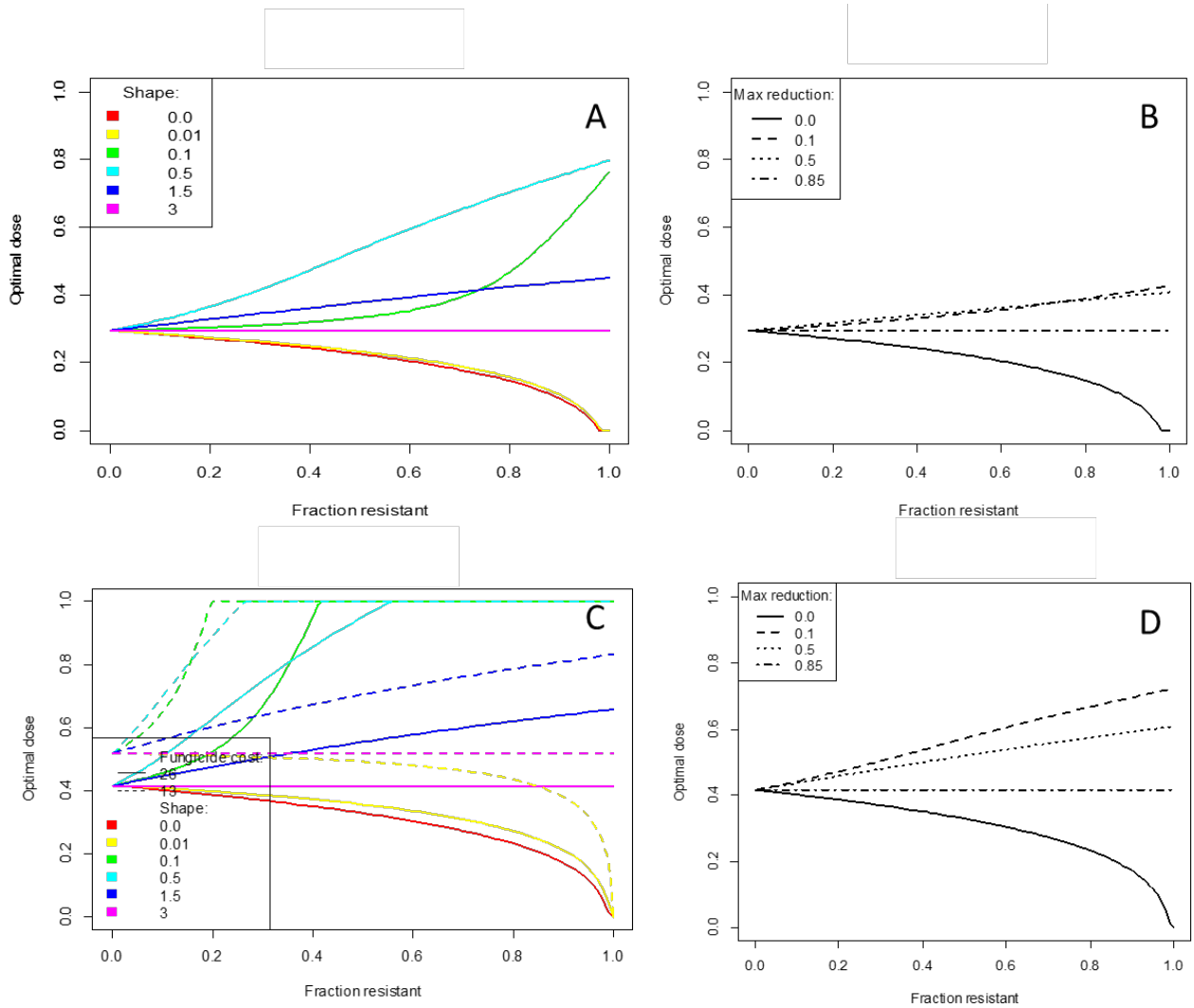


Figure 10. The effect of the shape of the dose response curve on the optimal fungicide dose. Top row, 10A and 10B, are for the mean economic gain; bottom row, 10C and 10D, are for the risk averse grower who protects his crops against the 10% largest epidemics. Left column, 10A and 10C, is for variations in the shape parameter, k, of the dose response curves. Right hand column, 10B and 10D, are for variations in the dose response curve asymptote.

This pattern is maintained when we consider risk averseness, figures 10C and 10D. Partial resistance causes the optimal dose to increase with the frequency of resistance in the pathogen population, and the effect of the curvature parameter is much larger than that of the asymptote.

We have tried a range of the other parameters in the model but the qualitative trends as described here do not change, through there are of course quantitative differences. Surprisingly the cost of the fungicide has no effect on the qualitative patterns described, only for a more costly fungicide the optimal dosages are lower, but none of the trends are changed.

Changes in the level of partial resistance in the grape powdery mildew case have similar effect as in the *Z. tritici* case (Figure 11). There is one remarkable addition to this however. We have found that in the case of risk averse growers and a fungicide to which absolute resistance develops the optimal dose can jump to zero when a critical frequency of resistance in the pathogen population is passed. This jump behaviour disappears for fungicides to which only partial resistance develops, figure 11B.

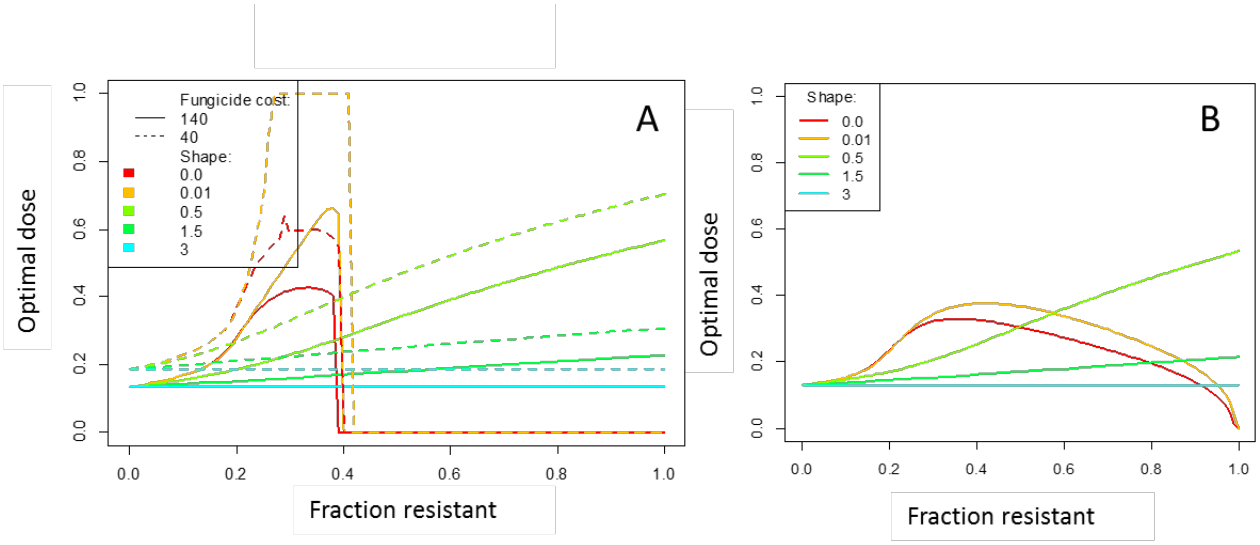


Figure 11. The effect of the shape of the dose response curve on the optimal fungicide dose. 11A for the mean economic cost, 11B for risk averse growers who protect their crop against the 10% largest epidemics.

Discussion and further developments

We have shown how to quantify the optimal dose when fungicide resistance is developing in the pathogen population. The model and analytical method are quite general and can be applied to a wide variety of pathosystems and fungicides. The numerical scenarios studied covered both two specific examples, grape powdery mildew and septoria blotch on wheat, and two contrasting classes of fungicides, the QoIs and the azoles. We furthermore did an exploratory analysis for a wide range of parameter values, to be able to generalise our results. There are several key conclusions from the study:

1. The optimal dose will for risk averse growers always be considerably higher than for growers aiming only at the long term mean economic gain. However, all trends in the optimal fungicide

dose with changes in the frequency of resistance in the pathogen population as well as trends with any model parameter are qualitatively the same for risk averse and not-risk averse growers. We can therefore study the qualitative consequences of changes in resistance and model parameters by only studying the trends in the long term mean economic gain.

2. When absolute resistance is developing to the fungicide we found that:
   - When the disease-yield loss relation is linear or concave, the optimal fungicide application dose decreases with increasing frequency of resistance in the pathogen population. This conclusion is irrespective of the value of any of the other parameters in the model.
   - When the disease-yield loss relation is convex there are cases where the optimal fungicide treatment dose increases with the frequency of resistance and there are cases that the optimal dose decreases with frequency of resistance.
   - The balance between the steepness of the disease-yield loss relation and the fraction of the years developing a high disease severity in untreated crops determines whether optimum dose increases or decreases with the frequency of resistance in the pathogen population. For practical use this will need to be analysed on a case-by-case basis as other parameters such as the risk averseness of growers affect the conclusion.

3. For fungicide to which partial resistance develops we found that:
   - The optimal dose increases with increasing frequency of the resistant strain in the pathogen population. This is irrespective of the shape of the disease induced yield loss curve. This conclusion holds for long term mean economic gain as well as for risk averseness.
   - When the partial resistance is expressed through the curvature parameter of the dose response curve, k, the optimal dose increases more steeply with frequency of resistance then when it is expressed through the dose response curve asymptote.

4. The optimal dose combination in a mixture depends on the cost of the mixing partner relative to the cost of the at-risk fungicide, (ii) the efficacy of the mixing partner relative to the efficacy of the at-risk fungicide and (iii) on the frequency of resistance in the pathogen population.
   - When the cost of the mixing partner is higher than that of the at-risk fungicide, and their efficacies are comparable, the optimal economic solution is not to use a mixture when no resistance is present in the pathogen population.
   - When in this situation resistance develops it can become economically sensible to use mixtures. Whether a mixture is the economically optimal solution is again determined by costs and efficacy of the mixing components.
   - When the mixing partner is less expensive than the at-risk fungicide and their efficacies are comparable, mixtures are economically sensible in all cases.
   - The frequency of resistance in the pathogen population determines the optimal dose combination in the mixture.

The model developed in this project has, for the first time, enabled a systematic study of the costs and benefits of fungicide application programs. Both solo use and mixture use can be analysed. Previous work on fungicide resistance models (e.g. Hobbelen et al. 2011ab; 2012; van den Berg et al. 2016; van den Bosch et al. 2014ab) studied the effect of dose and mixture using a definition of effective disease control as a threshold green/healthy leaf area loss that could be tolerated. When the resistance has developed to such an extent that this criterion for effective control is no longer met, the fungicide has lost its usefulness for disease control.

This definition however ignores that ultimately the financial costs and benefits of a fungicide treatment program determines whether it will be used in practice. With the developments in this project we have established a way to quantify these costs and benefits, and developed a systematic method to analyse these costs and benefits of different application programs.

Further development of the model in conjunction with appropriate field experimentation will make it possible to apply the analysis to actual fungicide application programs for multiple diseases threatening crop yield. The further developments needed to this end are:

1. Develop and apply an efficient method to measure the dose response curves of the pathogens to the fungicide applied in the programs to be analysed.
2. Including multiple diseases in the model and enabling combined analysis of the costs and disease induced yield losses for multiple diseases. This is still a considerable development that needs to be done but it would open the way to a full analysis of practice cases.
3. Further development of the evolutionary model and establishing its relation to the economics. This will need the development of a quantity to measure the success of a fungicide application programme during the entire time frame where resistance in emerging and spreading in the pathogen population. This quantity will measure the economic gain of the to be analysed application program from the moment the application program is introduced to the moment the costs of application exceed the benefits in terms of reduced yield loss.

We hope to be able to further develop and apply the modelling work in future research.

APPENDIX 1:

For the disease-yield loss relationship, in the case of a convex shape, there will be parameter values μ and σ for which $g(S)>1$ for a value $S>S_{max}$. In such cases yield is entirely lost and $g(S)=1$. Here we explain the adjustments to the cost calculation needed in these cases. The expected costs are calculated from:

$$E(C(A)) = \int_0^{S_{max}} P(S)C(S,A)dS \; + \; \int_{S_{max}}^{1} (MC_F + \; P_Y \, Y \,) \; P(S) \, dS$$

And to the expected costs of the 1 in N years largest epidemics two cases have to be considered.
If $S_N \geq S_{max}$ then

$$E(C|S \geq S_N) = \; P_Y \, Y$$

If $S_N < S_{max}$ then

$$E(C|S \geq S_N) = \int_{S_N}^{S_{max}} \frac{P(S)}{(1/N)}\, C(S,A)\, dS \; + \; \int_{S_{max}}^{1} \frac{P(S)}{(1/N)}\, (M\, C_F + \, P_Y\, Y)\; dS$$

and S_{max} is found from g(S_{max})=1. So

$$g(S_{max}) = \frac{\mu\,(1-\theta_n) S_{max}\; e^{-(r\,-\,r_S(A))\Delta} + \;\theta_{\,n} S_{max}\; e^{-(r\,-\,r_R(A))\Delta}}{1 + \;\sigma\,((1-\theta_n) S_{max}\; e^{-(r\,-\,r_S(A))\Delta} + \;\theta_{\,n} S_{max}\; e^{-(r\,-\,r_R(A))\Delta)}} = 1$$

$$S_{max} = \frac{1}{\mu - \sigma}\, \frac{1}{\left((1-\theta_n)\; e^{-(r\,-\,r_S(A))\Delta} + \;\theta_{\,n}\; e^{-(r\,-\,r_R(A))\Delta}\right)}$$

Table 1a. Pathogen life-cycle and economic parameter used in the model for grape powdery mildew and septoria tritici blotch on wheat.

		Grape powdery mildew	Wheat *Z. tritici*
C_{DA}	Cost of a dose of fungicide/ ha	Azoxystrobin $140	Pyraclostrobin £25
Cs	Cost of applying fungicide	Not used	Not used
P	Value of product/ tonne	$250-$300 per tonne	£100 per tonne
Y	Yield in weight per unit area	7 tonne per ha	9.15 tonne per ha
σ *and* μ	*Yield loss parameters*	μ=0.1; σ=-10	μ=0.81; σ=0.0
a	Parameter beta distribution	1.1	0.84
b	Parameter beta distribution	3.7	4.4
p	Latent period in absence of fungicide	10 days	16.2 days
i	Infectious period	10 days	27.8 days
		azoxystrobin	pyraclostrobin
β_{mSA}	Max fractional effect of fungicide	0.75	0.9
k_{SA}	Dose response curve curvature parameter	3	3
Δ	Fungicide time window	5	5

Table 1b. Dose response curve parameters used in the model for grape powdery mildew and septoria tritici blotch on wheat.

		Grape powdery mildew		*Septoria blotch*	

		sensitive	*insensitive*	*sensitive*	*insensitive*
QoI	*β*	*0.75*	*0*	*0.9*	*0*
	k	*3*	*0*	*3*	*0*
	Δ	*5*	*-*	*5*	*-*
Azole	*β*	*0.7*	*0.7*	*0.8*	*0.8*
	k	*3*	*1.5*	*3*	*1.2*
	Δ	*15*	*15*	*15*	*15*
Spiroxamine	*β*	*0.65*	*0.65*	*-*	*-*
	k	*3*	*3*	*-*	*-*
	Δ	*15*	*15*	*-*	*-*

Appendix 1: Communications

Understanding fungicide resistance in diseases of grapes using phenotyping and genotyping techniques. Lincoln Harper. Fungicide resistance group, Department of Environment and Agriculture, Curtin University 11th of November 2013. Australian agrochemical resistance meeting (AARM), Perth, WA.

Fungicide resistance in pathogen of grapevines: lessons to be learned. Lincoln Harper. Fungicide resistance group, Department of Environment and Agriculture, Curtin University. 3rd of November 2016. Australian agrochemical resistance meeting (AARM), Adelaide, SA.

Lessons through the grapevine – fungicide resistance in grapevine pathogens. Lincoln Harper. Fungicide resistance group, Department of Environment and Agriculture, Curtin University. 23rd of November 2016. Crop protection forum 2016, Wagga wagga, NSW.

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Genotyping of *Erysiphe necator* and *Plasmopara viticola* isolates from grapevines

Author: Mr Lincoln Harper, Curtin University

Introduction

Powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) are two economically important fungal diseases in Australian viticulture. Management of powdery mildew and downy mildew is dependent on the application of fungicides. Field failure of fungicides against these two diseases has been observed in Australia. Reduced sensitivity or resistance of *E. necator* isolates has been reported to Demethylation Inhibitor (DMI) fungicides (Savocchia et al. 2004) and to Quinone outside Inhibitor (QoI) fungicides (Wicks et al. 2013). Reduced sensitivity or resistance of *P. viticola* isolates to metalaxyl has also been reported (Wicks et al. 2005). To investigate fungicide resistance, a number of *E. necator* isolates from across various Australian wine regions were collected, single-spored and phenotyped at South Australian Research and Development Institute (SARDI). A small number of non-single spored *P. viticola* samples were collected for preliminary genotypic analysis. To provide molecular evidence to support the *E. necator* phenotyping results and to acquire molecular data on *P. viticola* resistance, known target genes were genotyped for these two species. *E. necator* isolates were genotyped for the cyp51 gene (DMI target) and the cytB G143A region (QoI). *P. viticola* isolates were genotyped for *ces*A3 G1105s region (CAA target) and the cytB G143A region (QoI target).

Methods

Fungal DNA was extracted from infected leaf material using a CTAB extraction method (Cubero et al. 1999). Flanking and sequencing primers were designed for the *E.necator cyp*51 gene, *E. necator cyt*B hotspot region, and *P. viticola ces*A3 hotspot region using the reference sequences UNU72657, AF343441, and GQ258975 respectively. The *P. viticola cyt*B hotspot region primers as described in Grasso et al. 2006 were used in this study. Amplification was carried out using high-fidelity Phusion polymerase (New England Biolabs) according to the supplier's protocol using the primers listed in table 1. Un-purified amplified DNA was sent to Macrogen Inc. (Korea) for sequencing. Consensus sequences were aligned to the reference sequences; UNU72657 (*E. necator cyp*51), GQ258975 (*P. viticola ces*A3), and AY696297 (*P. viticola cytB*). *E. necator cyt*B sequences were aligned to a WA wildtype sequence from this study.

Table 1. Target gene primers for *E.necator* and *P. viticola*

Primer name	Species	Gene	Sequence 5’ – 3’	Primer type
1F_UnCyp51	*E. necator*	*cyp*51	TGAGGCGGGTAAATCGGCCA	Flanking/sequencing
1R_UnCyp51	*E. necator*	*cyp*51	ACGGACCTCTTCTCCTTGTAAG GCAT	sequencing
2F2_UnCyp51	*E. necator*	*cyp*51	ATGAAAACGGCTCTTACC	sequencing
2R_UnCyp51	*E. necator*	*cyp*51	TGGGAGCGTGAAGTCGCAGA	sequencing
3F_UnCyp51	*E. necator*	*cyp*51	AGCCCTTCTGATGGCTGGACA	sequencing
3R3_UnCyp51	*E. necator*	*cyp*51	TCATCTCTTTTCCCAGCC	Flanking/sequencing
UnectCytB F	*E. necator*	*cyt*B	GAGCACCAAGAACATTAG	Flanking/sequencing
UnectCytB F	*E. necator*	*cyt*B	GAGAAACCTCCTCAGATG	Flanking/sequencing
Pv cesA3 F	*P. viticola*	*ces*A3	ATGAAAGCGAAGTCGACC	Flanking/sequencing
Pv cesA3 R	*P. viticola*	*ces*A3	GCAGCATCCCCAGTTTCG	Flanking/sequencing
PvcytB 1f	*P. viticola*	*cyt*B	TATACATGCGAATGGTGCATCT	Flanking/sequencing
PvcytB 4r	*P. viticola*	*cyt*B	GCGATCACACCACCAATTTTAT	Flanking/sequencing

Results

Y136F was the only *E. necator cyp*51 mutation identified across the population tested. Of the 46 *E. necator* isolates which were tested and produced a sequence for *cyp*51, 35 (76%) had the Y136F mutation (Table 2). A number of these isolates (26%) containing the Y136F mutation exhibited a mixed mutant and wildtype chromatogram peak (an overlapping A and T) at the second nucleotide position within the 136 codon. The frequency of isolates exhibiting Y136F across SA, WA, NSW, VIC and TAS are 87%, 88%, 100%, 38%, and 33% respectively.

Of the 36 *E. necator* isolates which were tested and produced a sequence for the *cyt*B hotspot region, 33 (92%) had the G143A mutation. The majority of these isolates (85%) exhibited a mixed mutant and wildtype chromatogram peak (an overlapping G or C) at the second nucleotide position within the 143 codon. All states except WA and SA exhibited a 100% frequency for the G143A allele.

Nineteen of the 30 (63%) *E. necator* isolates that had genotyping results for both *cyp*51 and *cyt*B had the Y136F and G143A mutations.

11 non-single spored samples of *P. viticola* from WA were genotyped, with no mutations found (data not shown).

Discussion

Of the all the *E. necator* isolates tested, there was a high proportion of the Y136F (76%) and G143A (92%) mutations present in the population. Furthermore, there is a high number of isolates exhibiting the Y136F and G143A mutations simultaneously. The mixed Y136 and Y136F genotypes could indication cross-contamination of isolates and/or DNA, or multiple *cyp*51 copies present in the mixed genotype isolates. Jones et al. 2014 showed that gene duplication of the *cyp*51 gene in *E. necator* can occur, but specific isolates appear to only have multiple copies of the Y136F containing gene, so would not exhibit a mixed genotype.

The high proportion of mixed cytB genotypes could also indicate cross contamination of isolates and/or DNA samples. A more likely reason is because of *cyt*B heteroplasmy; multiple copies of the *cyt*B gene are present in the mitochondria and isolates can contain both mutant and WT *cyt*B copies (Colcol and Baudoin 2015). A qPCR based approach could be a useful method to investigate proportions of mutant and WT *cyt*B alleles within isolates (Colcol and Baudoin 2015). Once relative amounts of mutant alleles were established, these could subsequently be related to resistance factors and any fungicide failure in the field. Further work is thus required to provide more insights into the relationship between laboratory results and field efficacy.

No mutations were found from all the non-single spored WA samples of *P. viticola*; more sampling is thus required to identify molecular evidence for *P. viticola* fungicide resistance in Australia.

Table 2. *E. necator* genotyping results. Isolates exhibiting the mutation are indicated in red. n/a indicates the sample was not tested or had a failed DNA amplification result.

state	Wine region	*cyp*51 gene	*cyt*b hotspot
NSW	Hunter Valley	Y136F	mix G143A/G143
NSW	Hunter Valley	mix Y136/Y136F	n/a
NSW	Orange	mix Y136/Y136F	n/a
SA	Adelaide Hills	Y136F	mix G143A/G143
SA	Adelaide Hills	n/a	mix G143/G143A
SA	Adelaide Hills	Y136F	mix G143A/G143
SA	Adelaide Hills	Y136F	n/a
SA	Adelaide Hills	Y136F	n/a
SA	Adelaide Hills	Y136F	n/a
SA	Adelaide Hills	Y136F	n/a
SA	Adelaide Hills	Y136F	n/a
SA	Adelaide Plains	Y136	n/a
SA	Adelaide Plains	Y136	mix G143A/G143
SA	Adelaide Plains	Y136F	mix G143A/G143
SA	Adelaide Plains	Y136F	n/a
SA	Adelaide Plains	Y136F	mix G143A/G143
SA	Adelaide Plains	n/a	G143A
SA	Barossa	n/a	mix G143A/G143
SA	Barossa	n/a	G143
SA	Barossa	Y136	mix G143/G143A
SA	Barossa	Y136F	mix G143/G143A
SA	Langhorne Creek	mix Y136/Y136F	G143
SA	Langhorne Creek	mix Y136/Y136F	mix G143/G143A
SA	Langhorne Creek	mix Y136/Y136F	mix G143/G143A
SA	Padthaway	Y136	mix G143A/G143
SA	Padthaway	Y136F	mix G143/G143A
SA	Riverland	Y136F	mix G143/G143A
SA	Riverland	mix Y136/Y136F	mix G143/G143A
SA	Riverland	mix Y136/Y136F	mix G143/G143A
SA	Riverland	n/a	G143A
SA	Riverland	Y136F	mix G143/G143A
TAS	Tamar Valley	Y136	mix G143A/G143
TAS	Tamar Valley	Y136F	mix G143A/G143
TAS	Tamar Valley	Y136	mix G143A/G143
VIC	Bellarine peninsula	mix Y136/Y136F	n/a
VIC	Goulburn Valle	Y136	mix G143A/G143
VIC	Goulburn Valley	Y136	G143A
VIC	King Valley	Y136	mix G143A/G143
VIC	King Valley	Y136	mix G143A/G143
VIC	Mornington	Y136	n/a
VIC	Mornington	Y136F	n/a
VIC	Mornington	Y136F	n/a
VIC	Yarra Valley	mix Y136/Y136F	n/a
WA	Frankland river	Y136F	n/a
WA	Great Southern	Y136F	mix G143A/G143
WA	Great Southern	Y136	n/a
WA	Margaret River	n/a	G143A

WA	Margaret River	Y136F	mix G143A/G143
WA	Margaret River	Y136F	mix G143A/G143
WA	Margaret River	Y136F	G143A
WA	Margaret River	Y136F	mix G143A/G143
WA	Swan District	Y136	G143

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9.4.4. CSU: Downy mildew

DOWNY MILDEW FINAL REPORT

Method

Collection of downy mildew infected grapevine material

Diseased leaves or berries were collected from commercial vineyards in New South Wales (NSW), Victoria (VIC), Tasmania (TAS) and Western Australia (WA) in 2014 and 2015. The vineyards sampled and the *Plasmopara viticola* isolates established from each vineyard are presented in Table 1. Following collection, the diseased material was transferred to the National Wine and Grape Industry Centre (NWGIC) laboratory and established in culture on detached leaves.

Propagation of glasshouse-grown grapevines

To establish a constant supply of disease-free leaves for the mass production of conidia, grapevines were established and maintained in the glasshouse. Grapevines (*Vitis vinifera* cv. Sultana) were propagated from two-bud cuttings taken from the Charles Sturt University vineyard, Wagga Wagga, NSW. The base of each cutting was dipped in Plant Cutting Powder (Yates, Australia) and planted into 20 cm diameter pots containing general purpose soil mix. After six weeks, cuttings were fertilised fortnightly with a complete water soluble fertiliser (1:200 Moeco 30® (NPK 12%:3%:8%), Moeco Pty Ltd, Australia). During the autumn and winter months, active shoot growth was maintained by providing a 1h photoperiod, from 00.00 to 01.00h (330 μE $s^{-1}m^{-2}$ from cool white fluorescent bulbs) positioned above the plants. Grapevines were watered for 15 min/day using an automatic dripper system and were maintained free from powdery mildew by burning sulfur powder in a 220V, 2 wave verdamper (Nivola B.V., Sassenheim Holland) for 1 h/day. The predatory mite, *Phytoseiulus persimilis* (Bugs For Bugs, Mundubbera Queensland) was used to control two-spotted spider mite (*Tetranychus urticae*). Flying insects, including, white flies and aphids were controlled by sticky aphid and white fly traps (Bugs for Bugs, Mundubbera, Queensland).

Leaf disc culture for maintenance of isolates

Petri plates (10 cm diameter, 2 cm deep: Falcon®, Becton Dickinson Europe, France) containing 20 ml of 1% distilled water agar (Bitek, Difco Laboratories, Michigan), 25 μg/μL Pimaricin (Sigma Chemical Co., St Louis) and 50 mg/L streptomycin (Sigma Chemical Co., St Louis) were prepared. In the laminar-flow cabinet, fully expanded leaves taken from the fourth to sixth node from the distal shoot tip were surface-sterilised in 0.5% sodium hypochlorite and 0.1% Tween20 for 90 secs and rinsed three times, for 2 min each time, in

sterile distilled water. The leaves were blotted dry with sterile paper towel and 9 mm discs were cut from each leaf. The leaf discs were placed with the abaxial side facing upwards onto the pre-prepared water agar plates.

A number of different techniques were trialled to transfer the *P. viticola* sporangia from the infected field samples to the sterile leaf discs including; (i) rubbing the diseased sample directly onto the leaf discs, (ii) using an artist brush to collect the sporangia and transfer them to the leaf discs and (iii) preparing a spore suspension to transfer to the leaf disc. Out of these methods the preparation of a spore suspension was most successful. On arrival of the infected field material to the laboratory it was either processed on the same day or stored at -20^{o}C. The sections of leaf infected with downy mildew were cut and placed into a sterile 100 mL plastic bottle containing 10 mL of sterile distilled water and vortexed for 1 min. Spore suspensions were adjusted to between 1 x 10^5 and 1 x 10^6 spores/mL and a 10 μL droplet was placed onto the leaf disc to yield an approximate spore concentration of 1 x 10^4 spores/mL. Leaf discs were incubated overnight at room temperature and the water droplets containing the spores allowed to dry in a laminar flow the following day. The dried leaf discs were further incubated at room temperature in the dark until sporulation was observed. Leaf discs covered in *P. viticola* were stored at -20^{o}C or freeze-dried until required for further experiments.

Preparation of fungicides

Technical grade pyraclostrobin, mandipropamid and metalaxyl were stored at 4^{o}C until use in bioassays for fungicide sensitivity. Fungicides were dissolved in 1 mL of acetone and diluted to the desired concentrations. Pyraclostrobin was diluted to concentrations of 0.1, 0.05, 0.01, 0.005, 0.001, metalaxyl to 10, 1.0, 0.5, 0.1, 0.05 and mandipropamid to 10, 1.0, 0.1, 0.01, 0.001 μg/mL in sterile distilled water immediately prior to each assay. These concentrations were determined by preliminary testing of selected isolates.

Preparation, inoculation and incubation of leaf discs

Disease-free immature, bright, shiny leaves were harvested from the leaves taken from the fourth to sixth node from the distal shoot tip of glasshouse-grown *V. vinifera* cv. Sultana and surface sterilised as described above. In the biohazard cabinet, discs were cut from each leaf using a sterile 9 mm diameter cork borer. Two methods were tested for the bioassay (i) floating the leaf discs in fungicide solution or (ii) imbibing the leaf discs in fungicide solution and then placing them onto water agar. The latter method produced the most consistent results so was the chosen method for the all bioassays. The leaf discs were placed into sterile beakers with a given fungicide concentration, agitated for 1 hr (Wong and Wilcox, 2000) and then dried in the biohazard cabinet. The discs were randomised and 10 were placed into each Petri plates containing 1.5% water agar amended with 25 μg/μL pimaricin (Sigma Chemical Co., St Louis) and 50 mg/L streptomycin (Sigma Chemical Co., St Louis) and

with the abaxial surface facing upwards. Sterile distilled water was used for the control. Each disc was considered a pseudo-replicate and each plate was considered a replicate with a total of three replicates for each treatment and isolate combination.

Each of the 10 replicate discs was inoculated with a single isolate of *P. viticola* by placing a 10 μL droplet of 1 x 10^6 spore suspension onto each leaf disc. The discs were incubated for 24 hr in the dark and then dried in the biohazard hood until the inoculation droplet was no longer visible before transferring back to the incubator for a further seven days. Disease incidence was recorded by assessing for presence or absence of *P. viticola* relative to the control. The effective doses for 50% control (EC_{50}) of disease incidence were calculated by plotting the percentage of leaf discs that were infected with *P. viticola* against the fungicide concentration. Linear regression was used to determine the EC_{50} concentrations for each isolate (Microsoft Excel 2013).

Results/Discussion

Determination of EC_{50} values

The mean EC_{50} values for each *P. viticola* isolate and each fungicide are provided in Table 2.

Pyraclostrobin

In the preliminary tests to determined effective concentrations, the isolates used were very sensitive to pyraclostrobin and therefore the highest test concentration chosen was 0.1 μg/mL, which was not high enough to effectively determine whether there was reduced sensitivity. While some of the isolates had EC_{50} values below the highest level of 0.1 μg/mL, many were still viable at the highest test concentration. From the Hunter and Tamar Valley, 58% and 50% of isolates respectively were still viable at the highest test concentration, as were all five from Margaret River and one from Wagga. By contrast, all three isolates from the Yarra Valley were sensitive to pyraclostrobin, with EC_{50} <0.04 μg/mL.

Pyraclostrobin, a strobilurin in the quinone outside inhibitor class (QoI) is registered for use against grapevine downy mildew as Cabrio® (250g/L pyraclostrobin; Nufarm Australia Ltd) and has had widespread use in Australian vineyards since its introduction in 2003. QoI fungicides are used as preventatives 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 13 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 sub-tropical regions such as the Hunter Valley.

One limitation of this study is that the bioassay could not be repeated to include higher concentrations of pyraclostrobin due to time and budget constraints. Many isolates were still producing visible sporangia at the maximum tested pyraclostrobin concentration of 0.1 μg/mL and therefore future bioassays should test concentrations greater than this. Higher ranges of EC_{50} values for isolates exposed to pyraclostrobin have been reported in the USA with Baudoin *et al*. (2008) reporting an EC_{50} of 25 μg/mL and Gauthier and Amsden (2014) reporting an EC_{50} of 390 μg/mL. In contrast, Giraud *et al*. (2013) found isolates of *P. viticola* resistant to 3 μg/mL of pyraclostrobin in Luxembourgian vineyards. Baseline sensitivities to pyraclostrobin were first reported by Wong and Wilcox (2000) and for disease incidence ranged from 0.05 – 0.94 μg/mL and for disease severity 0.04 to 0.78 μg/mL. Further surveys should be conducted to ascertain the spread of reduced sensitivity in Australian viticultural regions.

Metalaxyl

The phenylamide class of fungicides include Metalaxyl-M the active ingredient in the widely used Ridomil Gold® (Syngenta Australia) and which was registered for first use against downy mildew in 2000 in Australia. Prior to this, fungicides containing formulations of metalaxyl were widely used in Australia since the 1970s (Wicks *et al*. 2005) to control downy mildew of grapevines. The mode of action of the phenylamide fungicides involves the inhibition of rRNA biosynthesis (polymerase complex 1) in the target pathogen. Resistance involves one to two major genes and potentially several minor genes. Mutations in the genome conferring resistance to phenylamides have not been mapped to date and therefore there are no molecular methods for detection of resistance (FRAC, 2014).

The sensitivity of *P. viticola* to metalaxyl in Australian vineyards was first assessed in 1987 by Wicks *et al.* (1987) by testing 15 isolates at 10 μg/mL. All isolates in this bioassay were found to be sensitive to benalaxyl. The first report of *P. viticola* isolates with reduced sensitivity to metalaxyl then occurred almost 20 years later in the 2004/05 season in the Hunter Valley region (Wicks *et al*. 2005), where a small subset of samples showed that resistant isolates could still grow at 10 μg/mL whereas the sensitive isolate had no growth at 0.1 μg/mL.

From our studies, 67% and 33% of isolates from the Hunter and the Yarra Valley showed reduced sensitivity to metalaxyl, respectively. Mean EC_{50} values for isolates tested against metalaxyl for these two regions ranged from 0.01 to 10 μg/mL. The two isolates tested from the Tamar Valley, Tasmania and the two from Margaret River, WA showed reduced sensitivity to metalaxyl as they were still able to grow at the highest test concentration of 10 μg/mL.

Mandipropamid

Revus® fungicide (active ingredient 250 g/L mandipropamid, Syngenta Australia), a mandelic acid amide fungicide, was recently introduced in July 2014 to the Australian wine industry and therefore to date has not had widespread use. However, cross-resistance between mandipropamid, dimethomorph (Acrobat, first registered for use against grapevine downy mildew in Australia in 2001) and other carboxylic acid amide (CAA) fungicides was reported in *P. viticola* in a study conducted by Gisi et al. (2007). Resistance to CAA fungicides has been reported for *P. viticola* in a number of countries overseas (FRAC, 2005) and also more specifically to mandipropamid (Yoshinao, 2011; Nanni et al., 2016). In the study by Nanni et al. (2016) EC_{50} values for sensitive and resistant isolates ranged from <1 to 300 μg/mL and 200 to >300 μg/mL. Studies by Gisi et al. (2007) suggested that isolates with EC_{50} values of 0.03 to 3.6 μg/mL were considered sensitive to mandipropamid.

In our study and to our knowledge, the isolates collected from the vineyard in the Tamar Valley were the only isolates that had previous exposure to Revus® fungicide. All isolates tested except one from Wagga were sensitive to mandipropamid with mean EC_{50} values ranging from 0.06 – 2.62 μg/mL. Therefore, the data obtained from this study can be considered as baseline sensitivity data and may be useful for tracking shifts in sensitivity as the product becomes more widely used in the industry.

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Appendix 4: Staff

Sandra Savocchia (Senior Lecturer)
Lindsay Greer (Technical Officer)
Helen Waite (Casual Technical Officer)
Jennifer Bannister (Casual Technical Assistant)

Table 1. Origin of *Plasmopara viticola* isolates used in this study.

Viticultural Region	Location	Host Cultivar	Isolate Code	Spray History[a]	Date Collected
Hunter Valley, NSW	Pokolbin, Block 1	Chardonnay	DBCH	2 x Phosphorous acid, copper and dithane in previous season	1.11.14
	Pokolbin, Block 2	Chardonnay	DBMC*	2 x Phosphorous acid, copper and dithane in previous season	13.12.14
	Pokolbin, Block 1	Verdelho	DBVE*	2 x Phosphorous acid, copper and dithane in previous season	1.11.14
	Pokolbin, Block 2	Verdelho	DBMV*	2 x Phosphorous acid, copper and dithane in previous season	13.12.14
	Pokolbin, Block 1	Semillon	DBMS*	2 x Phosphorous acid, copper and dithane in previous season	13.12.14
	Pokolbin, Block 2	Semillon	V1	2 x Phosphorous acid, copper and dithane in previous season	9.2.15
	Pokolbin, Block 1	Shiraz	V2	2 x Phosphorous acid, copper and dithane in previous season	9.2.15

Viticultural Region	Location	Host Cultivar	Isolate Code	Spray History[a]	Date Collected
	Broke, Block 1	Sangiovese	V3		9.2.15
	Branxton, Block 1	Pinot	BXPN	Dithane, copper, 2 x metalaxyl	1.11.14
	Branxton, Block 1	Semillon	BXSM*	Dithane, copper, 2 x metalaxyl	13.12.14
	Lovedale, Block 1	Pinot Gris	LPGT*	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	1.11.14
Hunter Valley, NSW	Lovedale, Block 1	Pinot Gris	LPGC*	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	1.11.14
	Lovedale, Block 2	Shiraz	LDSH*	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	1.11.14
	Lovedale, Block 1	Sangiovese	AD1	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	9.2.15
	Lovedale, Block 3	Chardonnay	EM1	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil,	9.2.15

Viticultural Region	Location	Host Cultivar	Isolate Code	Spray History[a]	Date Collected
				metalaxyl + copper, phosphorous acid	
	Lovedale, Block 1	Cabernet	MI1	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	9.2.15
	Lovedale, Block 3	Shiraz	ML1	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	9.2.15
	Block 1	Chardonnay	MVCH	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	1.11.14
	Block 1	Chardonnay	MVCH2	2 x Delan, 1 x Captan, 2 x Cabrio, 4 x Oxydul, 1 x Peratec (hydrogen pyroxide), 1 x Ridomil, metalaxyl + copper, phosphorous acid	1.11.14
Hunter Valley, NSW	Block 1	Shiraz	JYT	Dithane, copper	15.12.14
	Block 1	Verdelho	WG*	Dithane, copper	15.12.14
	Block 1	Vermentino	HH*	Copper, 1 x Phosphorous acid	15.12.14

Viticultural Region	Location	Host Cultivar	Isolate Code	Spray History[a]	Date Collected
Wagga Wagga, NSW	CSU Campus	Flame Seedless	CS1	Copper	28.1.15
	CSU Campus	Flame Seedless	CS2	Copper	28.1.15
Swan District WA	Block 1	Shiraz	HR1	Unknown	4.11.14
	Block 2	Shiraz	HR2	Unknown	7.11.14
	Block 1	Grenache	GR1	High use of Ridomil this and previous season	20.11.14
Margaret River, WA	Block 1	Chardonnay	WR1	Unknown	10.11.14
	Block 2	Chardonnay	CBWA	Unknown	10.11.14
Tamar Valley, TAS	Block 1	Pinot Noir	PBS1	Copper, Revus, Delan, Ridomil Gold	20.2.15
	Block 1	Pinot Noir	PBS2	Copper, Revus, Delan, Ridomil Gold	20.2.15
	Block 1	Pinot Noir	PBS3	Copper, Revus, Delan, Ridomil Gold	20.2.15
	Block 1	Pinot Noir	PBS4	Copper, Revus, Delan, Ridomil Gold	20.2.15
	Block 1	Pinot Noir	PBS5*	Copper, Revus, Delan, Ridomil Gold	20.2.15
Yarra Valley,	Block 1	Cabernet	YCS1	Revus, Cabrio, Ridomil Gold	1.3.15

Viticultural Region	Location	Host Cultivar	Isolate Code	Spray History[a]	Date Collected
VIC	Block 1	Cabernet	YCS2*	Revus, Cabrio, Ridomil Gold	1.3.15
	Block 1	Cabernet	YCS3	Revus, Cabrio, Ridomil Gold	1.3.15
	Block 1	Cabernet	YCS4*	Revus, Cabrio, Ridomil Gold	1.3.15
	Block 1	Cabernet	YCS5	Revus, Cabrio, Ridomil Gold	1.3.15

[a] Number and type of sprays used in the season of which the sample was collected.

*Isolates in storage at NWGIC but not tested in the fungicide sensitivity bioassay.

Table 2. Mean EC_{50} of *Plasmopara viticola* isolates exposed to fungicides.

Viticultural Region	Isolate	EC_{50} [a] (µg/mL)		
		Pyraclostrobin	Metalaxyl	Mandipropamid
Hunter Valley, NSW	DBCH	0.01	0.01	0.01
	V1	>0.10	NT	0.25
	V2	>0.10	0.54	2.03
	V3	>0.10	NT	1.19
	BXPN	0.10	10	0.50
	AD1	>0.10	>10	1.10
	EM1	>0.10	>10	0.81
	MI1	0.02	0.61	1.34
	ML1	>0.10	>10	1.46
	MVCH	0.01	10	0.84
	MVCH2	0.01	10	0.24
	JYT	0.04	1.54	1.90
Wagga Wagga, NSW	CS1	NT	NT	4.95
	CS2	>0.1	>10	1.21
Swan District, WA	HR1	>0.1	1.57	1.64
	HR2	>0.1	NT	NT
	GR1	0.1	NT	1.27
Margaret River, WA	WR1	>0.1	>10	1.21
	CBWA	>0.1	>10	1.42
Tamar Valley, TAS	PBS1	0.01	NT	0.07
	PBS2	>0.10	>10	0.08
	PBS3	>0.10	10	2.62
	PBS4	0.01	NT	0.06
Yarra Valley, VIC	YCS1	0.04	>10	0.02
	YCS3	0.01	7.78	0.01
	YCS5	0.02	0.94	0.018

[a] Concentration inhibiting development of *Plasmopara viticola* by 50%.

NT = not tested.

9.4.5. AWRI: NGS and genotyping

Project SAR 1204: Population genotyping of *Erysiphe necator, Plasmopara viticola* and *Botrytis cinerea* populations using amplicon sequencing

Anthony Borneman, AWRI

Introduction

While genotyping of known resistance loci can be performed on purified isolates of fungal pathogens using standard PCR amplification of the locus combined with Sanger sequencing (See 9.4.4), this technique can not accurately determine the proportions of alleles that are present within samples containing multiple, genetically distinct individuals.

Unlike Sanger sequencing, in which a single sequence read is obtained per PCR amplicon, next-generation sequencing methods produce millions of individual sequencing reads, with each read deriving from a single DNA molecule. It is possible to determine the proportion of unique DNA sequences within a mixed population by counting the number of sequencing reads of each distinct type. This technique is therefore ideally suited to determining the proportions of alleles within mixed populations, such as is required when attempting to measure the levels of resistant mutations in vineyard pathogen populations.

Methods

Amplicon sequencing for Erysiphe necator and Plasmopara viticola

DNA was obtained from either SARDI or Curtin University from the grapevine pathogens *E. necator* and *P. viticola* from a mixture of sources, including single-spore isolates and swabs taken directly from grapevine surfaces.

DNA was amplified using primer pairs based upon locus sequence data supplied by Curtin University, that were specific for either the *cyp51* or *cytB* locus (Table 1) and which surrounded the known resistance mutation hotspots G143A (*cytB*) and Y136F (*cyp51*). To increase the sensitivity of the detection (required for the population swabs), 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 (Stage 2 amplicon).

Following PCR, individual amplicons were pooled at approximate equimolar ratios and sequenced using Illumina chemistry (2 x 300 bp or 2 x 150 bp depending on overall amplicon length) on a Miseq sequencing machine (Ramaciotti Centre for Functional Genomics).

Raw sequencing data was the processed using an analytical pipeline which consisted of quality trimming (Trimmomatic, Bolger et al. 2014), adaptor trimming (Cutadapt, Martin 2011) and paired-end read merging (FLASH, 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 (Varscan, Koboldt et al. 2012).

The proportion of both the sensitive and resistance alleles at the relevant hot-spot base were then extracted using custom python scripts and plotted in R.

Table 1. Primers used in this study

Primer name	Species	Gene	Sequence[a]	Primer type
En_Cyp51_Y136F-F	*E. necator*	*cyp51*	TAGACCCTACGAGCCACCAG	Flanking
En_Cyp51_Y136F-R	*E. necator*	*cyp51*	AGAGCGGCAAAAGATGAGTC	Flanking
En_Cyp51_Y136F_F_Nxxx	*E. necator*	*cyp51*	CCTACACGACGCTCTTCCGATCT*xxxxxxxx*TCTTTTACATGCCCATCTCC	Stage 1 amplicon
En_Cyp51_Y136F_R_Nxxx	*E. necator*	*cyp51*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT*xxxxxxxx*TCCTTGTAAGGCATGTGAAGC	Stage 1 amplicon
En_cytB_F	*E. necator*	*cytB*	GAGCACCAAGAACATTAG	Flanking
En_cytB_R	*E. necator*	*cytB*	GAGAAACCTCCTCAGATG	Flanking
En_cytBnest_F_Nxxx	*E. necator*	*cytB*	CCTACACGACGCTCTTCCGATCT*xxxxxxxx*TTGACCTACTCAAGGTATAGCAC	Stage 1 amplicon
En_cytBnest_R_Nxxx	*E. necator*	*cytB*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT*xxxxxxxx*AGTTTGAACAATTGGAACTGTAA	Stage 1 amplicon
Pv_cytB_G143A_292F	*P. viticola*	*cytB*	TTTAGGGGTTTGTATTACGG	Flanking
Pv_cytB_G143A_628R	*P. viticola*	*cytB*	ATGGATTATTTGAACCTACCTC	Flanking
Pv_cytB_G143A_331F_Nxxx	*P. viticola*	*cytB*	CCTACACGACGCTCTTCCGATCTTAAGGCGAGAAGCTTTATGGTGTTCAGG	Stage 1 amplicon
Pv_cytB_G143A_567R_Nxxx	*P. viticola*	*cytB*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAGGCGATACAAATGGAAAGGTGAAATG	Stage 1 amplicon
Illumina Nextera dual-index F primer	NA	NA	AATGATACGGCGACCACCGAGATCTACACxxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATCT	Stage 2 amplicon
Illumina Nextera dual-index R primer	NA	NA	CAAGCAGAAGACGGCATACGAGATxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC	Stage 2 amplicon

[a] *x* refers to a variable 8bp inline barcode used to multiplex samples within a single run

Whole-genome sequencing of Botrytis cinerea for multi-locus genotyping

DNA was obtained from either SARDI or Curtin University from both purified and mixed cultures of the fungal pathogen *B*. *cinerea* that were originally sourced from grapevine infections.

Illumina-compatible genomic libraries were prepared using the Nextera XT protocol and sequenced using 2 x 250 bp chemistry on a Hiseq2500 machine (Ramaciotti Centre for Functional Genomics).

Raw sequencing data were quality trimmed and then aligned to existing genome sequence of the *B. cinerea* strain BcDW1 (Blanco-Ulate et al. 2013) using Novoalign. The whole-genome alignment files were then interrogated for specific nucleotide variation at several loci that had been investigated previously in this project by Sanger sequencing (see 9.4.3; Table 2) using Varscan (Koboldt et al. 2012). In addition to a presence/absence call for these mutations, the next-generation data enabled allele proportions to be calculated for the mixed populations.

Table 2. Gene loci investigated for *B. cinerea*

Gene	**Mutation**	**Confirmed Sanger result**
sdhB	ND[a]	Y
sdhD	ND[a]	Y
erg27	ND[a]	Y
cgs	L8P strain Bc-39 D146E strain Bc-40	Y Y
bos-1	I365S strain Bc-39 Q369P strain Bc-40 N373S strain Bc-40	Y Y Y
cyp51	ND[a]	Y
cytB	G143A	N
benA	E198A strain Bc-39	Y

[a] No mutations detected in the Curtin University Sanger sequencing

All mutations could be confirmed in the strains that were previously sequenced by Curtin University using Sanger sequencing, with the exception of *cytB* G143A, for which the next generation analysis was not able to produce data due to complications with the reference genome.

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9.4.6. Journal articles and conference

Still some sting in the tail of strobilurins

Researchers Trevor Wicks, Barbara Hall **and** Doug Wilson **want grapegrowers to understand strobilurin resistance, and the strategies they can follow for continued use of these valuable fungicides against powdery and downy mildew.**

THE STROBILURIN GROUP of fungicides – including Amistar, Cabrio and Flint ¬– are the only fungicides controlling both powdery and downy mildews on grapes.

With some providing more effective control of downy mildew than others.

Strobilurins have been used in Australia for at least 10 years, and generally have provided good control of these two major grape diseases.

Since their introduction, growers have been advised to always include other products in programs which include strobilurins, applying no more than 2-3 strobilurin sprays per season, and confining them to around flowering.

The reasons for this are:

- Research has shown the most effective time to apply strobilurins is during flowering.
- Restricting their use to a few sprays per season reduces the risk of strobilurin-resistant strains of fungi developing in Australian vineyards.

STROBILURIN RESISTANCE OVERSEAS AND IN AUSTRALIA

In overseas vineyards, resistant strains of both downy mildew and powdery mildew have developed within a few years of strobilurin use.

This resistance appears suddenly, often without warning, and normally in isolated areas of vineyard blocks.

Strobilurins remain effective in other areas, so the resistance can be described as an "on/off" situation rather than a gradual loss of performance.

Strobilurin-resistant strains of powdery mildew have now been found in Australian vineyards but no strobilurin-resistant downy mildew has been detected.

HOW DOES RESISTANCE DEVELOP?

- The use of fungicides does not induce resistance in the fungus population.
- Resistant or insensitive spores of fungi occur naturally in the environment.
- It is only after regular use of the same fungicide or fungicide group that insensitive fungi survive and multiply, causing significant loss of control by that fungicide.

It's important to look closely at some of the Australian situations where strobilurin-resistant strains have developed.

FIRST CASE - LENSWOOD RESEARCH CENTRE, SOUTH AUSTRALIA

The first confirmed Australian case of strobilurin-resistant powdery mildew was in Chardonnay vines at Lenswood Research Centre in the Adelaide Hills,

Grow straight up to here

without any hand training, hand spraying or hand weeding.

Vines grow naturally straight inside GroGuards without any hand training or pruning. No need to touch the vines until they run along the wire!

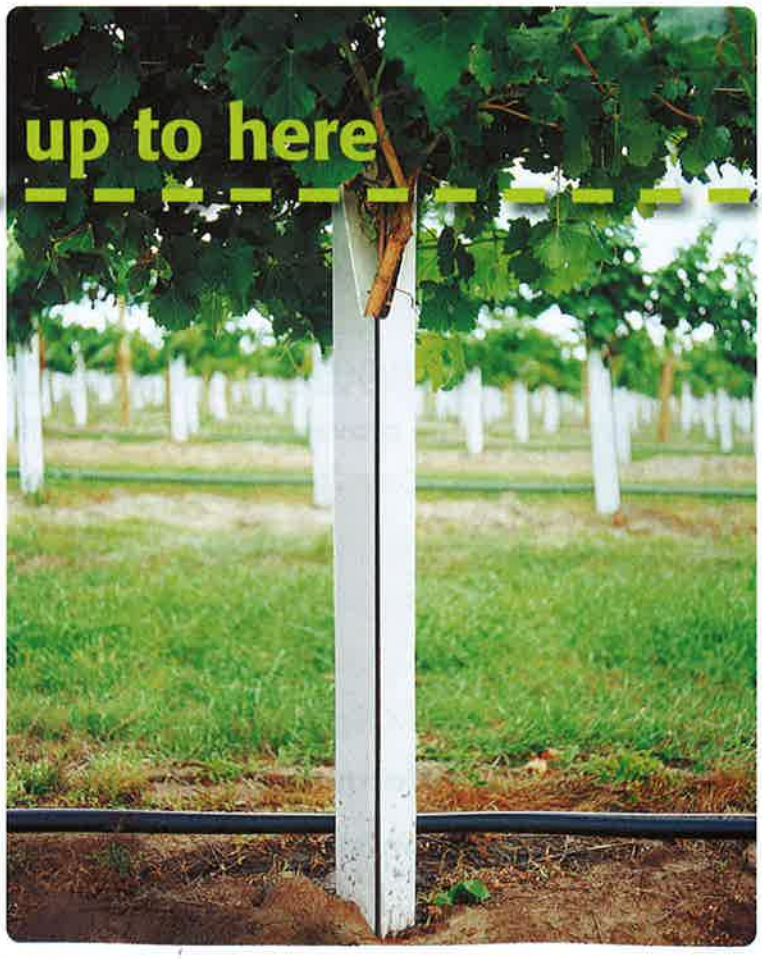
What's more, GroGuard's waterproof **Zip-Safe** seal protects vines from herbicide spray so you can control weeds from a tractor.

GroGuard's legendary strength and reliability are backed by a 3-year guarantee. You can use and re-use each GroGuard on successive plantings.

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Mandatory droplet spectrum requirements

A hot topic for discussion at many of the viticulture meetings I've attended recently has been the use of crop protection products in accordance with expanding legal requirements. As many of you would be aware, labels for newly registered products and labels for products that are already registered (but have recently been updated) are including additional information. Often this information relates specifically to droplet size and is aimed squarely at minimising off-target drift resulting from use of spray equipment.

So under the general instructions section on these labels it is increasingly common to see more detail on how these products are to be applied with a mandatory droplet size or range being specified.

An example of what might be seen on a label could be; Ground Application Only: Apply as a spray in a minimum of 400 L of water/ha using equipment delivering a MEDIUM spray quality.

Given these directions are on these labels and the label is legal document, spray operators clearly have a responsibility to show they have taken all appropriate steps to ensure they have followed label directions.

The question many people are asking at the meetings is how do I know the droplet size of my spray unit?

Calibrating spray equipment is relatively straightforward and growers will know their water volumes, speed of travel and spray pressures. Droplet size however is not so straightforward and it's not something a grower can really determine easily on-farm.

To that end you will have to rely on the information you are given by your spray equipment supplier or nozzle manufacturer. If you haven't already done so, I would strongly recommend growers contact their supplier to discuss what options they have and ensure their equipment complies with label directions.

It would also be a very good idea to document and record the information you are given to show you have taken the appropriate steps to conform to legal requirements. Keep that information with your spray records.



Whilst this may seem like more work and more formalities, it will give you piece of mind and may even advert that spray drift situation which will be good news for everyone.

Scott Mathew
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For details, please call the Syngenta technical product advice line on 1800 067 108 or visit www.syngenta.com.au

AT A GLANCE:

- Even though strobilurin-resistant strains of powdery mildew have been detected in Australian vineyards, this important group of fungicides will continue to provide useful control of powdery mildew – provided they are restricted to one or two sprays per season, and they are always mixed with another non-strobilurin fungicides registered for powdery mildew.
- Powdery mildew resistant to strobilurins can be controlled by using three or more fungicides of different activity in spray programs.
- No strobilurin-resistant strains of downy mildew have been detected in Australian vineyards. When used for downy mildew control, strobilurins should always be tank mixed with a powdery mildew fungicide from a different activity group.
- Strobilurins are most effective when applied at the start of flowering. A tank mixture of a strobilurin such as Cabrio with an alternative product such as Filan applied at this stage will control powdery mildew, downy mildew and Botrytis bunch rot.
- New fungicides such as Flute, Talendo and Vivando – with different modes of activity – increase the range of alternative products available for use in resistance management.

where strobilurin had been used since 2000.

Resistance was detected in 2010/11 in a trial where strobilurins had not been applied according to recommendations. On reflection, everything was done incorrectly:

- Strobilurins were not applied until powdery mildew was well established in the vines.
- Strobilurins were applied in four consecutive applications.
- Fungicides with different modes of activity were not always used in the spray program.

By 2010/11, neither Amistar nor Cabrio controlled powdery mildew, while sprays of different activity groups remained effective.

This was in contrast to previous trials, where programs including strobilurin around flowering provided good control of powdery mildew.

Samples of powdery mildew were collected from the various treatments, and sent overseas for testing for the marker gene indicating strobilurin resistance.

The results showed a high level of resistant genes in powdery mildew collected from the Amistar and Cabrio sprayed areas – compared with low to nil amounts in powdery mildew collected from unsprayed plots, and from vines never treated with these fungicides.

NURIOOTPA, SOUTH AUSTRALIA

A similar situation developed in Chardonnay vines at Nuriootpa in the 2011/12 season.

Poor powdery mildew control occurred when strobilurin-resistant strains developed after four consecutive applications of either Amistar or Cabrio.

In contrast, good to complete control of powdery mildew was achieved with similar applications of tebuconazole and other non-strobilurin fungicides.

Downy mildew developed at both the Lenswood and Nuriootpa sites in the 2011/12 season, but was well controlled in areas sprayed with strobilurins – indicating strobilurin fungicides still have an important place in grape spray programs.



STROBILURIN-resistant powdery mildew in Chardonnay grapes where strobilurins had not been applied according to recommendations.



STROBILURIN fungicides will continue to provide useful control of powdery mildew – provided their use is restricted to one or two sprays per season, and they are always applied as a mixture with another registered powdery mildew fungicide with a different mode of activity.

THE LESSONS LEARNED?

These two cases showed strobilurin-resistant strains of powdery mildew can develop quickly, and can result in significant crop loss if only strobilurin products are applied in a season.

To avoid crop losses due to the development of fungicide resistant strains, powdery mildew spray programs need to be carefully planned and include several activity groups.

TRIALS OF ALTERNATIVE SPRAY PROGRAMS

The strobilurin-resistant powdery mildew in Chardonnay vines at both Lenswood and Nuriootpa provided an ideal opportunity to evaluate different spray programs for controlling resistant strains.

In the 2011/12 season, a number of different fungicides were evaluated – in different combinations of product and spray timing.

Many of the spray programs included one or two applications of Cabrio at flowering – either alone, or with the first flowering-spray tank-mixed with Filan.

The reasoning behind this was Cabrio at flowering is one of the most effective sprays for controlling downy mildew; however it was uncertain how these applications would impact on powdery mildew control.

LENSWOOD TRIAL

- In untreated Chardonnay vines most leaves and bunches were severely mildewed, with severity values of 93% developing on bunches.
- In vines treated with sulphur before and after flowering – plus two applications of either Cabrio or Flint at flowering – the sulphur sprays provided good control of leaf powdery mildew, but the strobilurins did not control bunch infection.
- Better control of bunch infection was achieved when the first flowering-spray was a tank mix of Filan and Cabrio.
- Similarly effective powdery mildew control was achieved in programs where the first two sulphur sprays were replaced with either Vivando or Legend.
- Other programs including Legend, Filan, Prosper or Vivando and sulphur also provided good control of powdery mildew on leaves and bunches.

NURIOOTPA TRIAL

Tank mixtures of Filan and Cabrio as the first flowering-spray, followed by a single Cabrio application were evaluated in programs with the first two sprays being either sulphur, Vivando or Legend. All programs included sulphur as the final two sprays.

- In the unsprayed vines, powdery mildew developed extensively, with more than 70% of leaves and bunches diseased.
- All of the trialled spray programs provided complete, or almost complete, control of powdery mildew.

OTHER TRIALS

- Powdery mildew was controlled where Talendo was applied before and after the Filan/Cabrio flowering sprays, with sulphur and Rubigan included in the program.
- Another new fungicide Flute provided good control of powdery mildew when



applied twice in programs including a total of six fungicide applications, and various combinations of other fungicides such as sulphur, Prosper, and Cabrio.

CONCLUSION FROM ALL TRIALS

These trials showed strobilurin-resistant powdery mildew can be controlled by a spray program including three or more fungicides, each with a different mode of activity.

The trials also showed including a strobilurin fungicide at flowering to control downy mildew does not compromise the control of powdery mildew in resistant situations – provided the strobilurin is applied with an unrelated fungicide.

Strobilurin fungicides will continue to provide useful control of powdery mildew – provided their use is restricted to one or two sprays per season, and they are always applied as a mixture with another registered powdery mildew fungicide with a different mode of activity.

According to recent visiting Cornell University professor Dr Wayne Wilcox, it is common practice for many US grapegrowers to tank mix any strobilurin application with an effective powdery mildew fungicide from a different activity group – to prevent resistant strains from building up and causing economic losses.

A number of powdery mildew fungicides are suitable mixing partners for the strobilurin flowering-sprays.

Filan, for example – the product we used in many of our trials – has excellent activity against powdery mildew as well as Botrytis bunch rot. As a result, three major diseases – powdery mildew, downy mildew and Botrytis bunch rot – are controlled with a tank mix of two products applied at the one time of flowering.

Another is Flute, a new fungicide shown to provide excellent control of powdery mildew when applied in two sprays around flowering, and reported overseas to control strobilurin-resistant strains of powdery mildew.

FUTURE RESEARCH

The development of strobilurin-resistant strains of downy mildew in Australian vineyards may be the next challenge.

Further work needs to be done in this area – particularly to determine the appropriate mixing partners and spray timings when using strobilurins for downy mildew control.

A new project funded by GWRDC is investigating resistance in Australian vineyards.

A collaboration between scientists from SARDI, Charles Sturt University, Curtin University, DAFWA, AWRI, University of Adelaide and industry partners, the project aims to provide comprehensive information on the fungicide sensitivity of the three key pathogens affecting Australian vineyards – powdery mildew, downy mildew and botrytis – and their distribution.

This information will contribute to improved fungicide-resistance management strategies through collaboration with CropLife, agrichemical companies and the industry.

Maintaining the effectiveness of all fungicide groups will contribute significantly to reducing the economic and environmental impact of disease in Australian viticulture for the future. GW

Trevor Wicks, formerly South Australian Research and Development Institute (SARDI). **Barbara Hall**, Senior Research Scientist – Horticulture Pathology, SARDI. **Doug Wilson**, Research & Development Projects Coordinator, Nufarm Australia Ltd. (03) 9282 1427. 0427 806 386. doug.wilson@au.nufarm.com

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Powdery mildew in grapevines has the potential to devastate the vine's health and structure, as seen on the right versus treated vines on the left.



While strobilurin-resistant strains of downy mildew have not been observed in Australian vineyards, researchers believe work needs to be done to determine optimal spray timings.

By TREVOR WICKS, BARBARA HALL, Senior Research Scientist – Horticulture Pathology, SARDI and DOUG WILSON, Research & Development Projects Coordinator, Nufarm Australia

THE strobilurin group of fungicides – including Amistar, Cabrio and Flint – are unique. They are the only fungicides that control both powdery and downy mildews on grapes, with some providing more effective control of downy mildew than others.

Strobilurins have been used in Australia for at least 10 years, and have generally provided very good control of these two major grape diseases.

Since their introduction, growers have been advised to always include other products in programs that include strobilurins, applying no more than two to three strobilurin sprays per season, and confining these sprays to around flowering.

Research has shown that the most effective time to apply strobilurins is during the flowering period, while restricting their use to a few sprays per season reduces the risk of strobilurin-resistant strains of fungi developing in Australian vineyards.

In overseas vineyards, resistant strains of both downy mildew and powdery mildew have developed within a few years of strobilurin use.

This resistance appears suddenly, often without warning, and normally in isolated areas of vineyard blocks. Strobilurins remain effective in other areas, so the resistance can be described as an "on/off" situation rather than a gradual loss of performance.

Strobilurin-resistant strains of powdery mildew have now been found in Australian vineyards, but no strobilurin-resistant downy mildew has been detected on grapes in Australia to date.

Scientists have determined that the use of fungicides does not induce resistance in the fungus population, but rather resistant or insensitive spores of fungi occur naturally in the environment.

It is only after regular use of the same fungicide or fungicide group that the insensitive fungi survive and multiply, causing significant loss of control by that fungicide.

On a local level, strobilurin resistance has been observed in Lenswood and Nurioopta research vineyards.

These two cases showed that strobilurin-resistant strains of powdery mildew can develop quickly, and can result in significant crop loss if only strobilurin products are applied in a season.

FAST FACTS

- **Strobilurins still have vital role to play**
- **No downy mildew resistance observed**
- **Trials unveil importance of variation**

To avoid crop losses due to the development of fungicide resistant strains, powdery mildew spray programs need to be carefully planned and include several activity groups.

The trials also showed that including a strobilurin fungicide at flowering to control downy mildew does not compromise the control of powdery mildew in resistant situations – provided the strobilurin is applied with an unrelated fungicide.

Strobilurin fungicides will continue to provide useful control of powdery mildew – provided their use is restricted to one or two sprays per season, and they are always applied as a mixture with another registered powdery mildew fungicide with a different mode of activity.

According to recent visiting Cornell University professor

MB MIRCO BROTHERS MACHINERY



keeps resistance at bay

Wayne Wilcox, it is common practice for many US grape growers to tank-mix any strobilurin application with an effective powdery mildew fungicide from a different activity group to prevent resistant strains from building up and causing economic losses.

A number of powdery mildew fungicides are suitable mixing partners for the strobilurin flowering-sprays. Filan, for example – the product used in many of these trials – has excellent activity against powdery mildew as well as Botrytis bunch rot.

As a result, three major diseases – powdery mildew, downy mildew and Botrytis bunch rot – are controlled with a tank mix of two products applied at the one time of flowering.

Another is Flute – a new fungicide shown to provide excellent control of powdery mildew when applied in two sprays around flowering, and reported overseas to control strobilurin-resistant strains of powdery mildew.

The development of strobilurin-resistant strains of downy mildew in Australian vineyards may be the next challenge. Further work needs to be done in this area – particularly to determine the appropriate mixing partners and spray timings when using strobilurins for downy mildew control.

A new project funded by the Grape and Wine Research and Development Corporation has commenced to investigate resistance in Australian vineyards.

A collaboration among scientists from the South Australian Research and Development Institute, Charles Sturt University, Curtin University, the Department of Agriculture and Food Western Australia, the Australian Wine Research Institute, University of Adelaide and industry partners, the project aims to provide comprehensive information on the fungicide sensitivity of the three key pathogens affecting Australian vineyards – powdery mildew, downy mildew and botrytis – and their distribution in powdery mildew collected from unsprayed plots, and from vines that had never been treated with these fungicides.



▲ Strobilurin-resistant powdery mildew has been observed on grapes in both Lenswood and Nuriootpa vineyards.

Strobilurin over-use creates Aust's first resistant vines

THE first confirmed Australian case of strobilurin-resistant powdery mildew was in chardonnay vines at Lenswood Research Centre in the Adelaide Hills, where strobilurin fungicides had been applied to vines since 2000.

Resistance was detected in the 2010-11 season in a trial where strobilurins had not been applied according to recommendations. On reflection, everything was done incorrectly – strobilurins were not applied until powdery mildew was well established in the vines, fungicides with different modes of activity were not always used in the spray program and strobilurins were applied in four consecutive applications.

By 2010-11, neither Amistar nor Cabrio controlled powdery mildew, while sprays of different activity groups remained effective.

This was in contrast to previous trials, where programs that included strobilurin around flowering provided good control of powdery mildew.

Samples of powdery mildew were collected from the various treatments, and sent overseas for testing for the marker gene that indicates strobilurin resistance.

The results showed a high level of resistant genes in powdery mildew collected from the Amistar and Cabrio sprayed areas – compared with low amounts or none in powdery mildew collected from unsprayed plots, and from vines that had never been treated with these fungicides.

Key observations

- In untreated Chardonnay vines, most leaves and bunches were severely mildewed, with severity values of 93 per cent developing on bunches.
- In vines treated with sulphur before and after flowering – plus two applications of either Cabrio or Flint at flowering – the sulphur sprays provided good control of leaf powdery mildew, but the strobilurins did not control bunch infection.
- Better control of bunch infection was achieved when the first flowering-spray was a tank mix of Filan and Cabrio.
- Similarly effective powdery mildew control was achieved in programs where the first two sulphur sprays were replaced with either Vivando or Legend.
- Other programs that included Legend, Filan, Prosper or Vivando and sulphur also provided good control of powdery mildew on leaves and bunches.



Wine & Viticulture Journal
Tuesday 1/10/2013
Page: 60
Section: General News
Region: National, AU
Circulation: 3000
Type: Magazines Trade
Size: 879.81 sq.cms.

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CUT STRAIGHT TO YOUR NEWS

Understanding fungicide resistance in Australian vineyards

By The Fungicide Resistance Project Team#*
*Corresponding author: Barbara Hall, SARDI. Email: Barbara.Hall@sa.gov.au

A project involving research partner organisations from across Australia is studying three key pathogens affecting the nation's vineyards.

A collaborative project funded by GWRDC is under way involving industry partners and researchers from SARDI, Curtin University, National Wine and Grape Industry Centre, Department of Agriculture & Food WA, AWRI, and The University of Adelaide. Three key pathogens affecting Australian vineyards will be targeted: *Botrytis cinerea*, *Erysiphe necator* and *Plasmopara viticola*.

Although there are currently a number of fungicide groups available for these pathogens, the investment required to discover and develop new molecules is in the order of $300-400 million. It is, therefore, important to manage the existing chemistries to ensure that they continue to provide effective control.

This project will provide comprehensive information on the fungicide sensitivity of these pathogens, the incidence and distribution of resistant populations, and improve knowledge about the mechanisms of fungicide resistance in Australia to the main fungicides used. The information will contribute to improved fungicide resistance management strategies through collaboration with CropLife, agrochemical companies and industry.

Maintaining the effective use of all fungicides will contribute significantly to the objective of reducing the economic and environmental impact of disease in Australian vineyards for the future.

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Wine & Viticulture Journal
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Fungicide resistance to the fungicide group metalaxyl has been reported in the pathogens that cause *Plasmopara viticola* (downy mildew, pictured).

Project researchers have developed a method for the rapid analysis of *Botrytis* growth in the presence of the major fungicides used in the viticultural industry.

HOW GROWERS CAN HELP

If you suspect resistance, or are having difficulty in controlling botrytis bunch rot, powdery mildew or downy mildew that cannot be attributed to other causes, we would like to hear from you. We are aiming to sample from properties throughout Australia, and would like to receive representative samples from as wide a geographical range as possible.

As these samples will be used for research, there will be no charge for this testing. Results will only be available when all testing is complete and data analysed. We cannot guarantee a rapid turnaround time for results.

If you would like to help, please contact the appropriate laboratory listed at the end of this article to arrange sample collection. The sample needs to be sent fresh and arrive in good condition, as *Erysiphe necator* (powdery mildew) and *Plasmopara viticola* (downy mildew) are both obligate parasites, which means they will only grow on living tissue and cannot be artificially cultured like *Botrytis*.

WHAT IS FUNGICIDE RESISTANCE?

Fungicide resistance occurs when a plant disease-causing organism is able to survive exposure to doses of a fungicide that would normally control it. Resistance is usually due to one or more genetic mutations which enable the fungus to overcome the activity of a fungicide. Each fungal population may contain a very small number of individuals that naturally have the mutation(s) and can survive the application of a particular fungicide. If the same fungicide or fungicides from the same activity group are used repeatedly and exclusively, the susceptible individuals are killed, yet those with the mutation survive, multiply and become dominant.

Fungicide resistant populations add to the cost of managing a vineyard due to reduced fungicide efficacy and failure of spray programs to manage disease. While industry is advised to follow recommended anti-resistance strategies, there is confusion over which compounds are best to use as mixtures or in alternation. More information on managing fungicide resistance can be found at CropLife Australia (http://www.croplifeaustralia.org.au) ▶

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Ref: 215959718

Wine & Viticulture Journal
Tuesday 1/10/2013
Page: 60
Section: General News
Region: National, AU
Circulation: 3000
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Size: 879.81 sq.cms.

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The most recent report of fungicide resistance was of *Erysiphe necator* (powdery mildew, pictured) to the QoI (Strobilurin) group of fungicides. As this group of fungicides is also used to control downy mildew, it is important to determine if there is also resistance in *Plasmopara viticola* populations in Australia.

FUNGICIDE RESISTANCE IN AUSTRALIA

Botrytis bunch rot, powdery mildew and downy mildew are the three most economically important diseases in Australian vineyards. Fungicide resistance in the pathogens causing these diseases have been reported within the grape industry, for *Botrytis cinerea* to the fungicide groups dicarboximide (e.g. Rovral®) and anilinopyrimidine (e.g. Scala®), *Plasmopara viticola* (downy mildew) to metalaxyl and *Erysiphe necator* (powdery mildew) to the demethylation inhibiting (DMI) group of fungicides. The most recent report of fungicide resistance was of *Erysiphe necator* to the QoI (Strobilurin) group of fungicides. As this group of fungicides is also used to control downy mildew, it is vital to determine if resistance also occurs in *Plasmopara viticola* populations in Australia.

WHAT WILL THIS PROJECT ACHIEVE?

It is important to establish the occurrence and geographical distribution of pathogens resistant to fungicides to enable adaptation of spray programs to maintain optimal efficacy. The viticulture industry needs access to tests for rapid and accurate detection of fungicide resistance. Apart from those used for *Botrytis cinerea*, tests are currently not available for routine testing at a population level, due to the time and resource demands of conventional phenotyping and genotyping methods. Conventional phenotyping methods are those using the spores and growing the fungi on artificial media or leaf material, where as genotyping methods use molecular techniques on the DNA. The genetic basis of resistance is known in some cases and not in others, so this project will combine both phenotypic and genotypic tests to monitor fungicide resistance development in the three key pathogens. The aims of this project are to:

- determine the occurrence and distribution of populations of *E. necator*, *P. viticola* and *B. cinerea* resistant to 'at risk' fungicides within Australia
- validate high throughput genotypic testing using established DNA markers and next generation DNA sequencing techniques to develop rapid and accurate tests to detect fungicide resistance in pathogen populations
- develop and evaluate effective and sustainable resistance management strategies for the 'at risk' fungicides
- disseminate the information to industry.

To ensure an industry focus in meeting these aims, the project team is working in conjunction with an industry advisory group represented by Colin Bell (AHA Viticulture), David Braybrook (Vitisolutions), Warren Birchmore (Accolade Wines), Di Davidson (Davidson Viticulture), Suzanne McLoughlin (Treasury Wine Estates), Scott Paton (NuFarm), Liz Riley (Vitibit Pty Ltd), Leonard Russell (Watershed Wines, WA), David Sanderson (Wine Tasmania) and Shane Trainer (Bayer).

RESULTS SO FAR

More than 30 samples of *Botrytis cinerea* and *Erysiphe necator* have been collected. Unfortunately, many of the samples of powdery and downy mildew collected late season were old and could not be grown on fresh leaves, so could not be phenotypically tested. However, enough samples were recovered to establish methods for both phenotypic and genotypic testing for these two pathogens.

Researchers working within this GWRDC initiative have developed a high-throughput method for the rapid analysis of *Botrytis* growth in the presence of major fungicides used in the viticultural industry. This method will allow researchers to detect shifts in fungicide sensitivity in *Botrytis* populations before fungicide failure occurs, so alternative chemistry can be put in place on time. Additionally, researchers have found a correlation between resistance and genetic mutations in *Botrytis* isolates coming from tablegrape samples. Similarly, genetic analysis of powdery mildew samples collected from the wine regions of Western Australia has been shown to carry two mutations responsible for decreased sensitivity to DMI fungicides and resistance to QoI fungicides, respectively. This finding will be useful for the detection of genetic resistance in vineyards.

SAMPLE COLLECTION

Samples collected from the main viticultural regions of Australia, with collaboration from industry and agrochemical representatives, will be sent to and processed by one of three research organisations. Powdery mildew samples will be processed by the South Australian Research and Development Institute (SA), downy mildew by the National Wine and Grape Industry Centre (Charles Sturt University, NSW) and botrytis bunch rot by the Australian Centre for Necrotrophic Fungal Pathogens (Curtin University, WA). Please contact the appropriate people for further information and instructions on sample collection and handling:

- **Botrytis**
  Fran Lopez:
  Telephone (08) 9266 3061, or email fran.lopezruiz@curtin.edu.au
- **Powdery mildew**
  Suzanne McKay:
  Telephone (08) 8303 9759, or email suzanne.mckay@sa.gov.au
- **Downy mildew**
  Sandra Savocchia: Telephone (02) 6933 4341, or email ssavocchia@csu.edu.au

REFERENCES

Fungicide Resistance Action Committee, http://www.frac.info

AWRI Dog Book, http://www.awri.com.au

#The project team includes Barbara Hall, Suzanne McKay, Mark Sosnowski (SARDI); Fran Lopez, Lincoln Harper (Curtin University); Sandra Savocchia, Helen Waite (National Wine and Grape Industry Centre); Markus Herderich, Anthony Borneman (AWRI); Eileen Scott (University of Adelaide); Andrew Taylor (Department of Agriculture & Food WA); Doug Wilson (NuFarm); Hugh Armstrong (Bayer Crop Science); Scott Matthew (Syngenta); Trevor Wicks (private consultant); Keith Hayes (GWRDC).

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Persistent Identifier: urn:nbn:de:0294-sp-2014-Reinh-1

Wicks TJ *et al.*, Development of grape powdery mildew strains resistanct to QoI fungicides in Australia. In: Dehne HW; Deising HB; Fraaije B; Gisi U; Hermann D; Mehl A; Oerke EC; Russell PE; Stammler G; Kuck KH; Lyr H (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VII, pp. 253-256. © 2014 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-13-6

Development of grape powdery mildew strains resistant to QoI fungicides in Australia

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INTRODUCTION

Powdery mildew caused by *Erysiphe necator* is the most widespread and destructive fungal disease of grapes in Australia. Depending on the weather and the disease pressure most growers control the disease with 6-8 fungicide applications per season.

A wide range of different fungicide activity groups are available to Australian growers (Table 1) with integrated programs of sulphur and DMI fungicides being used most commonly (Wicks et *al.* 1997).

Table 1 Fungicide activity groups registered in Australia for the control of powdery mildew on grapevines (AWRI 2013)

Group	Active Ingredient	Activity group
Inorganic	Wettable Sulphur	M2
DMI'S	triadimenol, tetraconazole, myclobutanil, fenarimol, penconazole	3
Aza-naphthalenes	quinoxyfen, proquinazid	13
SDHI	boscalid	7
Aryl-phenyl-ketone	metrafenone	U8
Morpholine	spiroxamine	5
QoI	azoxystrobin, pyraclostrobin, trifloxystrobin	11

Since the introduction of QoI fungicides more than 10 years ago, this group, particularly pyraclostrobin and trifloxystrobin, are widely used in Australia. These are applied around flowering in spray programs that include fungicide applications with different activity at other timings (Wicks & Hitch 2002). Since the release of the QoI fungicides in Australia coincided with overseas reports on the development of fungal strains resistant to this activity

Persistent Identifier: urn:nbn:de:0294-sp-2014-Reinh-1

group (Wilcox et *al.* 2003) the recommendations were to limit their use to no more than three applications per growing season.

Until recently, this strategy has been successful, as the QoI fungicides have provided excellent control of powdery mildew and downy mildew on grapes in Australia.

However in the 2010/11 season, programs that included QoI fungicides failed to control grape powdery mildew at an experimental site in the Adelaide Hills of South Australia.

This paper reports on the situation where QoI resistance developed and the survey for the resistance mutation in powdery mildew isolates collected from Australian vineyards.

MATERIALS AND METHODS

The fungicides Amistar (250 g/L azoxystrobin), Cabrio (250 g/L pyraclostrobin) and a mixture containing tebuconazole (200g/L) and azoxystrobin (120 g/L) were applied at 100 ml, 40 ml and 130 ml/100 L respectively to mature chardonnay vines at the Lenswood Research Centre situated 30 km west of Adelaide, South Australia. Fungicides were applied on 4 occasions between 80% cap fall (December 10) and pre bunch closure (December 29) using a motorised back pack sprayer that utilised spray volumes of up to 1800 l/ha. Plots consisted of 4 vines with treatments including an unsprayed control arranged in a randomised block. The incidence and severity of powdery mildew was assessed on February 25 on 25 mature leaves and bunches selected at random from the middle two vines of each plot. Disease severity was rated on a 0-10 scale which reflected the area of upper leaf surface or bunch with obvious infection. At the time of assessment, sterile cotton bud swabs were used to collect samples of powdery mildew from leaves and bunches of sprayed and unsprayed vines by wiping the swabs over the surface of infected tissue. A further sample of powdery mildew was collected from grape vines never exposed to fungicides.

The DNA of all samples was extracted using standard procedures and the presence and frequency of the G143A mutation in the cytochrome *b* gene which confers QoI resistance (Gisi et *al.* 2002) analysed by pyrosequencing.

In the 2011/12 season powdery mildew samples were collected from widely dispersed vineyards in Western Australia, South Australia and Victoria which had been either unsprayed, sprayed with fungicides other than QoI chemistry or sprayed with QoI one or more times in the previous 5 years. Samples were mainly collected from vineyards where the fungicide programs had provided poor control. The samples were collected with sterile cotton bud swabs and analysed as previously described.

RESULTS

Powdery mildew developed extensively in the unsprayed vines, severely infecting all leaves and bunches (Table 2). A high amount of disease also developed on leaves and bunches of vines sprayed with QoI fungicides and although the incidence was similar to that of the

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unsprayed vines the severity on both leaves and bunches was reduced by 25% or more in most cases. Where tebuconazole was included in a treatment powdery mildew control was markedly improved.

Table 2 Powdery mildew incidence, severity and presence of the G 143A mutation on leaves and bunches of Chardonnay vines treated with and without fungicides.

Treatment	Leaves		Bunches		%G143A
	Incidence	Severity	Incidence	Severity	
Untreated	100	84	100	91	12
Azoxystrobin	97	41	100	66	98
Pyraclostrobin	99	40	100	55	99
Azoxystrobin + tebuconazole	32	5	58	11	17
Unsprayed	-	-	-	-	0

The G143A mutation was detected in all samples of powdery mildew except those that were not exposed to fungicides (Table 2). The highest level of mutation of 98% or more were found in the vines where QoI fungicides were applied, whereas amounts of 17% or less were found in the unsprayed control treatments or those where tebuconazole was applied.

In the 83 powdery mildew samples collected in 2011/12, the mutation was found in 95% of the samples (Table 3). Levels of mutation greater than 95% were found in most of the samples from Victoria and South Australia and were mainly sampled from vineyards with a history of QoI application. Of the four samples where no mutation was detected, two were obtained from unsprayed areas and the remainder from vines where boscalid and spiroxamine had been applied.

Table 3 Incidence of the G143A mutation in powdery mildew isolates collected in 2011/12 from vineyards in Western Australia, South Australia and Victoria.

	Number of samples with mutated allele G143A				
	0	1-25	25-50	50-95	>95
Victoria	0	0	3	8	26
South Australia	4	2	2	1	28
Western Australia	0	2	0	1	6
Total	4	4	5	10	60
Percent	4.8	4.8	5	12	72

DISCUSSION

These results are the first confirmed case of QoI resistance of *Erysiphe necator* in Australia. They were detected in an experimental vineyard where the QoI fungicides had been applied for more than five years and in the season of detection, QoI fungicides were applied

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consecutively on four occasions. However in commercial vineyards, losses following the application of QoI fungicides have not been widely reported, most likely as a result of growers adhering to the recommendation of applying no more than three applications per season. Nevertheless the survey of powdery mildew undertaken in the 2011/12 season showed that the G143A mutation is widespread in Australian grape growing areas. This suggests that further solo use of QoI fungicides in vineyards could result in increased selection of resistant strains and result in control failures.

In Australia the QoI fungicides still provide excellent control of *Plasmopara viticola* particularly when applied during flowering. As a result of the development of powdery mildew resistant strains and the detection of the G143A mutation in widely dispersed vineyards throughout Australia, the QoI fungicides are now recommended to be tank mixed with a powdery mildew fungicide of a different mode of activity.

Further monitoring is underway in Australia to determine the extent of resistance in vineyards to the fungicides commonly used to manage powdery mildew, downy mildew and Botrytis bunch rot. This work will provide comprehensive information on the fungicide sensitivity and distribution of these three key pathogens affecting Australian vineyards. The results will contribute to improved fungicide resistance management recommendations and assist with maintaining the effective use of all the fungicide groups in Australian viticulture for the future.

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Testing fungicide resistance in grapevine powdery mildew: how to work with biotrophs

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Key words: powdery mildew, *Erysiphe necator*, fungicide resistance, techniques.

Grapevine powdery mildew caused by *Erysiphe necator*, is the most economically important fungal disease of grapevine in Australia. While reduced sensitivity to SBI fungicides was confirmed in Australian vineyards in 2004 [1], resistance to QoI fungicides was only recently detected in 2010 [2].

In the 2011/12 and 2012/13 seasons, 88 powdery mildew samples were collected from widely dispersed vineyards in Western Australia, South Australia and Victoria where fungicide programs that included QoI fungicides had provided poor control. The samples were collected with sterile cotton bud swabs and extracted using standard procedures and the presence and frequency of the G143A mutation in the cytochrome b gene which confers QoI resistance [3] analysed by pyrosequencing by BASF in Germany. Only four of the samples had no G143A mutated allele present, with most having >96% (Table 1).

A project is underway to investigate the incidence and distribution of fungicide resistant populations of *Erysiphe necator* in Australia. Both phenotypic and genotypic testing will be undertaken on the same samples to compare results. However there are difficulties with phenotypic testing of biotrophs such as *E. necator* that can only be grown on living plant tissue. Many methodologies for fungicide sensitivity testing of *E. necator* are reported in the literature however very few discuss the pros and cons of techniques.

This paper reports on the development of an effective phenotypic test for powdery mildew using published methods and highlights problems that have arisen. The aim is to assist others who wish to travel this challenging path.

Table 1 Incidence of the G143A mutation in powdery mildew isolates collected in 2011/12 and 2012/13 from vineyards in Western Australia, South Australia and Victoria.

	Number of samples with mutated allele G143A in each frequency range				
	0	**1-25**	**25-50**	**50-95**	**>95**
Victoria	0	0	3	8	26
South Australia	4	2	3	2	31
Western Australia	0	2	0	1	6
Total	**4**	**4**	**6**	**11**	**63**

Isolation and maintenance of cultures of *E. necator*

To successfully grow and test *E. necator* on grapevine tissue, green leaf material that will remain healthy for at least 2 weeks is required. To assess which variety was most suitable for culture maintenance using detached leaves and for using leaf discs in fungicide sensitivity tests, three *Vitis vinifera* varieties, Cabernet Sauvignon [4] Chardonnay [5, 6] and Sultana were tested. Sultana, although being very conducive for growth of powdery mildew, was unsuitable because the leaves deteriorated within two weeks, both when detached or cut in discs. Chardonnay and Cabernet Sauvignon were both suitable, however Cabernet Sauvignon was used for all future experiments as it was readily available at the time of testing. Cabernet Sauvignon plants were grown from rooted cuttings and maintained in pots in a controlled growth room at 25^{o}C for a 16/8 hr day/night light regime. Strict hygiene and restricted entry into growing areas are used to reduce the likelihood of natural mildew infection.

Mildew infected leaves and bunches were supplied by growers from the major grape-growing regions around Australia. Plant material ranged in age from growth stage EL 19 to EL 36 with both recently sporulating and old colonies present. Powdery mildew was successfully subcultured from both leaves and bunches, however much greater success was achieved with younger plant material. Fewer sporulating colonies were observed with older plant material and the viability of powdery mildew spores was lower in bunches with old infections resulting in difficulties obtaining a viable culture. Also, as most sporulation was inside bunches, it was difficult to reach in older tight bunches (>EL31) without getting contamination with grape juice or other fungi. High levels of saprophytic fungi, including *Paecillomyces, Penicillium* and *Aspergillus* were present and these grew easily on either the detached leaves or on the tap water agar (TWA) media on which the leaves were placed. This resulted in the need for repeated subculturing by transfer of single spores to obtain clean cultures.

Single spore isolates are often obtained by picking a single powdery mildew conidium from a colony using an eyelash fastened to a holder [7] or using a single camel hair bristle [8]. We found the use of a single sable hair bristle still attached to an artist's paintbrush, to be superior to the

use of an eyelash as the conidia attached more easily to the sable bristle.

Fungicide testing: method development

To determine the most suitable media and inoculation method to use with the leaf disc method of fungicide sensitivity tests, three media and three inoculation methods were compared in a factorial experiment.

Three media were tested using 60 mm petri dishes: 1. 1.5% tap water agar (TWA, Bacto™ agar, BD, USA) amended with pimaricin (2.5 µL/mL, Sigma Aldrich); 2. TWA with one piece of sterile filter paper moistened with 1 mL sterile water on the surface [5]; and 3. two layers of sterile paper towel covered with two layers of Miracloth (EMD Millipore USA) moistened with 2 mL sterile water [9]. Leaves, cv. Cabernet Sauvignon, were removed from the third and forth node from the growing tip, surface sterilised in 1.0% sodium hypochlorite solution (White King® bleach) for 3 mins, washed 3-4 times in sterile distilled water (SDW), then blotted dry between 2 layers of sterile paper towel. Ten mm diam discs were cut using a sterile cork borer and placed on the surface of each of the various media. Three inoculation methods were tested: 1. tapping an infected leaf at the top of a spore settling tower (plastic tube, 270 mm diam × 270 mm high) to dislodge conidia [5]; 2. a single conidium, transferred using a single bristle attached to a sable hair paintbrush [6] and 3. one 10 µL droplet of spore suspension (2 x 10^5 spores/mL). No inoculation was used as the control. Two replicate Petri dishes, each containing three leaf discs, were used for each treatment combination i.e. media × inoculation method. Dishes were incubated at 22ºC, 12/12 hr day/night under fluorescent light for 14 days then the number of discs with powdery mildew colonies recorded (Table 3).

Table 3. The percentage of leaf discs with powdery mildew colonies following inoculation with three different methods on three different media, after 14 days incubation.

	Droplet of spore suspension[4]	Single conidium	Spore settling tower	No inoculation
Tap water agar[1]	0	63	67	0
Tap water agar and filter paper[2]	33	67	100	0
Paper towel and Miracloth[3]	0	66	33	0

(1) 1.5% amended with 2.5 µL/mL pimaricin (2.5% aqueous suspension, Sigma Aldrich)
(2) sterile filter paper moistened with sterile water placed on amended TWA
(3) two layers of sterile paper towel were covered with two layers of sterile Miracloth (EMD Millipore, USA)
(4) 10 µL droplet of spore suspension, 2 × 10^5 spores/mL

Inoculation with single conidium resulted in similar colony growth on all media. The greatest percentage of infection was achieved using the spore settling tower with the TWA + filter paper media and the least number of colonised discs were following the use of the droplet inoculation on TWA alone and on the paper towel and Miracloth media. Although there was similar colonization of discs using the single conidium and the spore settling tower methods, the single conidium technique was time consuming, and a longer time was required for growth of each colony prior to assessment which frequently resulted in deterioration of the leaf discs. Either TWA alone or TWA plus paper towel are considered suitable, however, TWA alone is simple and more time efficient. Discs deteriorated quicker using the TWA plus paper towel compared to using TWA alone. The spore settling tower is simple and effective but requires a greater amount of initial inoculum that either the single conidium or droplets of spore suspension. Although using a droplet of spore suspension enables quantification of inoculum, we found it to give inconsistent results and considerably higher levels of contamination were observed using this method. If there is any contamination present among the powdery culture, it is often not visible and it is time consuming to check every leaf under the microscope prior to subculturing or inoculation, particularly when there are many isolates to maintain.

For future fungicide tests using concurrent multiple disc inoculations, it was concluded that the simplest and most effective method was using TWA alone as the medium and a spore settling tower to inoculate.

Fungicide sensitivity testing

Testing isolates of powdery mildew obtained from all the main grape growing regions of Australia has now commenced using the following technique. Commercial grade fungicides, namely, Cabrio® (pyraclostrobin 250g/L ai, Nufarm Australia Ltd), Topas® EC (penconazole (100g/L ai, Syngenta Crop Protection) and Mycloss™ Xtra (myclobutanil 200g/L ai, Dow Agro Sciences), mixed in sterile double distilled water (SDDW), are diluted to 10, 1.0, 0.1, 0.01 and 0.001 µg/mL with SDDW used as the control.

Leaves cv Cabernet Sauvignon were collected and surface sterilised as previously described. Following cutting, leaf discs were placed immediately into a Petri dish containing SDDW until needed. Discs were removed from SDDW, randomised and placed abaxial surface upwards in a 140 mm diam Petri dish lined with sterile filter paper containing 5 mL of fungicide at a given concentration. The time of exposure to the fungicides was taken from published methods, and has not been re-evaluated as part of this research. After a 30 mins for Cabrio® [10] and Topas® EC [7], and 60 mins for Mycloss™ [6], discs were removed and blotted dry between two layers of sterile paper towel. Discs were placed adaxial surface upwards in 60 mm Petri dishes containing 1.5% TWA amended with 2.5 µL/mL of

pimaricin (2.5% aqueous suspension, Sigma Aldrich). Three discs per dish and 3 dishes per fungicide and isolate combination were used.

For inoculation, petri dishes containing leaf discs with the lids removed were placed at the bottom of a plastic spore settling tower. Conidia were dislodged by tapping leaves with freshly sporulating powdery mildew colonies at the top of the tower and allowed to settle for 1 min. One sporulating leaf was used as an inoculum source per replicate of each isolate, with each replicate inoculated separately. The tower was cleaned with 80% ethanol in between isolates and allowed to dry for 5 mins.

After 10 days incubation at 22^{o}C, 12/12 hr day/night under fluorescent light, each leaf disc was assessed for the percentage of leaf area colonised by powdery mildew. Analysis of the data (results not presented) will include mean % inhibition of growth of the nine discs per fungicide concentration for each isolate with generation of a dose-response curve. The EC_{50} for each isolate will be calculated by Probit analysis using Genstat 15th edn (VSN International, UK).

Discussion

Several of the published methods for fungicide sensitivity assays were trialled and although we found that methodologies repeatable some were more difficult and time consuming than others, for example, paper towel and Miracloth media, single conidium inoculation. With over 80 samples currently being tested phenotypically, and the amount of time and resources needed to maintain cultures on detached leaves, a repeatable, time effective and user friendly method of fungicide sensitivity testing was vital. We found that inoculation using a spore settling tower and maintaining the leaf discs for the fungicide test on TWA alone is currently giving consistent results.

Many lessons have been learned in the development phase of this project. These include:

1. Sample plant material with young sporulating powdery mildew colonies to maximise the chance of successful isolation and culturing.
2. If the powdery mildew colony on the plant material is old, culturing from this material will likely result in high contamination by saprophytes. Here, it is necessary to subculture from the first culture as soon as possible to eliminate contaminants.
3. Check the colonies microscopically before subculturing or testing, especially the first couple of subcultures directly from plant material, as some contaminants are often cryptic and difficult to see.
4. Use a natural bristle brush for obtaining single spore isolates as eyelashes can be painful to remove and the spores adhered better to the sable brush.
5. Inoculation using a spore suspension was rapid and easy but it also consistently had the highest levels of contamination.
6. Ensure the leaf tissue is as healthy as possible at the start of each test.

Acknowledgements

This research was funded by the Grape and Wine Research and Development Corporation. The project team includes scientists from the South Australian Research and Development Institute, Australian Wine Research Institute and University of Adelaide in South Australia; Curtin University and the Department of Agriculture, Food and Wine in Western Australia; the National Wine and Grape Industry Centre in New South Wales; NuFarm, Bayer, Syngenta; and Industry partners from throughout Australia.

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Speaker Abstracts #AARM2015

Session 1 Recent Field Developments

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Fungicide resistance in Australia: from traditional monitoring to rapid identification of resistance in the field

Fungicides are a critical component of food security worldwide contributing about $8 extra productivity for every $1 spent in Australia. However this fungicidal crop protection is threatened by ever-increasing regulatory stringency and the development of resistance. Along with cultural practices, the main control measures for most fungal diseases are the application of effective fungicides and the use of cultivars with genetic resistance. Unfortunately for Australian growers, the majority of cultivars commercially available for each major crop are either susceptible or moderately susceptible to fungal infection. Therefore growers have largely relied on the application of fungicides for disease control. However, disease management has been compromised by the emergence of fungicide resistance in the last few years in different fungal species. In fungi, resistance often emerges as a result of single or multiple mechanisms. Five general mechanisms have been described to confer resistance to fungicides been target site modifications the most important and widespread. Poor anti-resistance strategies (i.e. continuous use successful fungicides and lack of mixtures) and disease management will lead to the development of resistance and failures in disease control.
Here we summarise the current status of fungicide resistance in major Australian crops to some of the most important groups of fungicides worldwide, and evaluate the best approaches to unravel the molecular mechanisms involved in the resistance found. The implications of these findings for the development and deployment of fungicide resistance monitoring strategies in Australia are discussed.

Resistance of *Erysiphe necator* to QoIs and DMIs

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INTRODUCTION

Powdery mildew, caused by the obligate biotrophic fungus *Erysiphe necator*, is the most economically important disease of grapevines in Australia. Disease management is dependent on the timely application of fungicides, which include the Quinone outside Inhibitors (QoIs) and Demethylation Inhibitors (DMIs). Resistance to the QoIs in grapevine powdery mildew is conferred by the G143A mutation, a single point mutation in the cytb gene (1). The Y136F mutation, in the cyp51 gene, is associated with the development of resistance to the DMIs, however, other mechanisms are also thought to contribute (1). Whilst resistance to QoIs (2) and DMIs (3) has been found within Australia, the overall extent of resistance is not clear. Here, we aim to determine the extent of resistance to the QoI pyraclostrobin and the DMI penconazole in populations of grapevine powdery mildew obtained from Australian vineyards.

MATERIALS AND METHODS

Forty seven isolates were obtained from powdery mildew infected leaves or bunches in 2013/14. Cultures were maintained as single spore isolates on surface sterilised detached grapevine leaves, cv. Cabernet sauvignon, on water agar. Isolates were tested in leaf-disc assays for sensitivity to pyraclostrobin (Cabrio®) and penconazole (Topas®), using concentrations from 0.001 to 10 µg/mL active ingredient. The EC_{50} µg/mL, the dose that reduces growth by 50%, was calculated by Probit analysis (Genstat 16th ed). Detection of the G143A and Y136F mutations was carried out by extraction of genomic DNA followed by Sanger sequencing of the cytb and cyp51 genes.

RESULTS AND DISCUSSION

Pyraclostrobin: The EC_{50} values were between 0.0005 to 14.4 µg/mL with the overall mean 1.6 +/- 3.0 (sd) (n=47). The G143A mutation was detected in all isolates with an EC_{50} value > 0.11 µg/mL (Fig 1). This confirms the link between reduced sensitivity to pyraclostrobin and the presence of the mutation. The bimodal distribution pattern of the wild type versus mutant isolates shows a definite sensitivity shift towards resistance (Fig 1). This pattern is consistent with major single gene resistance described for the QoIs (1). The G143A mutation was detected in 68% of isolates genotyped to date (n=37). Testing is continuing, but even with the limited numbers there is good correlation between exposure to strobilurins and presence of the mutant (data not shown).

Penconazole: The EC_{50} values ranged from 0.0004 to 1.3 µg/mL with the overall mean 0.2 +/-0.2 (sd) (n=45). The lack of obvious pattern of the wild type versus mutant genotypes (Fig 2) may be due to the small number genotyped to date (n=20), with the Y136F mutation detected in 55% of samples. Unlike the strobilurins, the correlation between exposure and presence of mutant is not as well defined (data not shown). A relationship may be clearer following further genotyping, however although the Y136F mutation is associated with DMI resistance, it is possible that there are other mutations as well as an accumulation of other resistance mechanisms (1).

Overall, these results have implications for resistance management strategies in the viticultural industry. This high proportion of mutants detected is of major concern, indicating that resistant populations are common and without effective management the potential for loss of field efficacy is significant.

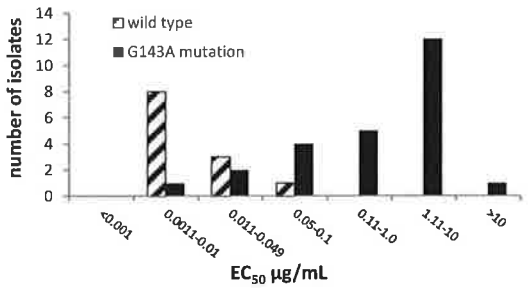


Figure 1. The distribution of sensitivities and genotype frequency of isolates of *Erysiphe necator* tested with Cabrio®.

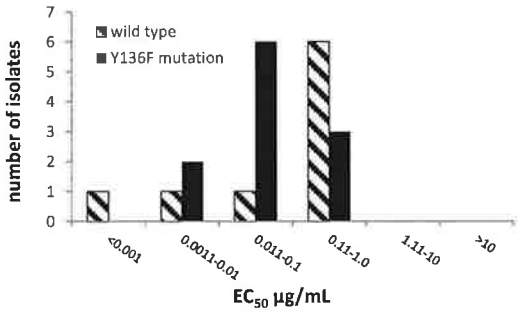


Figure 2. The distribution of sensitivities and genotype frequency of isolates of *Erysiphe necator* tested with Topas®.

ACKNOWLEDGEMENTS

Thankyou to AGWA for funding, viticulturists and growers for samples and Syngenta for the fungicides.

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Australia & NZ Grapegrower & Winemaker
March, 2015
Page: 19
Section: General News
Region: National, AU
Circulation: 3456
Type: Magazines Trade
Size: 3,585.00 sq.cms.

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CUT STRAIGHT TO YOUR NEWS

International research on downy and powdery mildew

Eileen Scott and **Tijana Petrovic**, from the University of Adelaide's School of Agriculture, Food and Wine; together with **Barbara Hall**, from the South Australian Research and Development Institute; and **Bob Emmett**, RW Emmett Pty Ltd, attended an international workshop on grapevine downy and powdery mildew held in Spain in mid-2014. They report on the highlights from the lectures, papers and posters presented at the workshop.

DOWNY and powdery mildew threaten vineyards around the world and substantial research effort goes into understanding the diseases and improving management. The international workshop on grapevine downy and powdery mildew, held every three-to-four years, provides a forum for scientists from universities, research institutes and industry to exchange new findings about the biology and management of these two diseases.

About 75 researchers, including six from Australia, attended the seventh workshop in Vitoria-Gasteiz, Spain, in July 2014. Across five days, two invited lectures, 32 papers and 19 posters were presented, covering topics in epidemiology, plant-pathogen interactions, resistance and grape breeding, disease forecasting, detection and control. Participants also visited the Basque Government research institute Neiker-Tecnalia and vineyards and wineries in Rioja Alavesa and Gipuzkoa. This article provides a summary of the workshop.

The program, invited lectures and several other presentations are available at http://gdpm2014.com/scientific-program.

INVITED LECTURES

Dr Bernardo Latorre (Pontifica Universidad Catolica de Chile) provided an overview of viticulture in Chile, where Cabernet Sauvignon and Sauvignon Blanc are the main varieties, grown mostly with irrigation. Powdery mildew, the most significant disease, is managed by cultural practices and conventional fungicide spray programs, although alternatives and biological control agents are being used in drier regions. Outbreaks of downy mildew have been sporadic and relatively minor since an epidemic in 1997, several years after the disease first appeared.

Dr Emilio Gil (Universitat Polytecnica de Catalunya, Spain) reviewed spray application practices and technologies in major viticultural regions of the world, including Australia. He summarised methods for calculating spray dose (ground area, leaf wall, row length, tree row volume, canopy row volume) and noted that canopy characteristics and spray equipment vary considerably. Live sensors are being used to determine canopy height, width and volume to allow variable rate application and minimise spray drift. He also mentioned the TOPPS (train operators to promote practices for sustainability) program (http://topps-drift.org), a drift analysis online tool developed in Europe to promote responsible use of plant protection products.

PATHOGEN AND DISEASE ASSESSMENT

Australian research on improving diagrammatic keys for assessing powdery mildew was presented by Bob Emmett and Eileen Scott. Keys with various increments at the lower end of the disease severity scale were developed and evaluated. While there was no evidence that keys improved assessment of severity, they do have potential for use in training assessors.

Tijana Petrovic outlined the development of quantitative PCR (qPCR) and spectroscopic assays for the objective measurement of powdery mildew.

A multiplex end-point PCR assay has been developed by Spanish researchers to detect spores of *Plasmopara viticola* (downy mildew) and *Erysiphe necator* (powdery mildew) simultaneously in environmental samples captured on glass microscope slides above the grapevine canopy. Another Spanish presenter reported trapping and microscopic observation of spores of *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea* on sticky glass slides in the warm, wet region of Galicia, north-west Spain. Spores of *B. cinerea* were most common, particularly during flowering and ripening whereas spores of *E. necator* were abundant between flowering and berry set. Genetic diversity of *B. cinerea* and *E. necator* was low, whereas several races of *P. viticola* were detected.

A portable fluorescence sensor, the Multiplex 330, has been used to detect downy mildew on leaves in the vineyard. The instrument detects stilbenes synthesised by the vine in response to infection and can distinguish diseased vines from healthy controls one day after inoculation. Hyperspectral imaging is also being studied as a means of automated early detection of downy mildew, before symptoms appear.

EPIDEMIOLOGY AND MODELLING/FORECASTING

A model has been developed by researchers at INRA Bordeaux, France to simulate a powdery mildew epidemic at a plot scale. The model allows investigation of the effect of vine growth and susceptibility, crop phenology and disease control measures on the development and spread of powdery mildew. The model showed that disease could be reduced by having mixtures of resistant and susceptible varieties in each row and that one fungicide application at early flowering delayed onset of the epidemic and reduced the leaf area diseased at the end of the season. This group also used modelling to confirm that bunch closure is a critical stage for control of powdery mildew and that vines should be sprayed from just before flowering to bunch closure.

Statistical analysis of data from experimental plots of five North American varieties in which downy mildew, powdery mildew and botrytis bunch rot were managed or not across four years showed that the following variables best described disease risk: phenological stage of the vine; amount of inoculum; disease severity at harvest in the previous year; and weather conditions at certain times. Also, the practice of covering vines with soil in winter for frost protection can, if the vines were affected by downy mildew, increase the risk of disease in the following spring.

The Mildium expert decision system for managing downy and powdery mildew at the plot scale has been evaluated across four▸

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Australia & NZ Grapegrower & Winemaker
March, 2015
Page: 19
Section: General News
Region: National, AU
Circulation: 3456
Type: Magazines Trade
Size: 3,585.00 sq.cms.

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CUT STRAIGHT TO YOUR NEWS



In the December column we focused on Botrytis disease management. Since then, many areas have received weather conditions conducive to berry splitting and Botrytis development, so it may be timely to do a harvest assessment and, where appropriate, focus on what can be done from here.

For crops still to be picked by hand, fruit can be selectively harvested and infected fruit avoided. Pickers must be able to recognise Botrytis bunch rot and know which bunches should be dropped to the ground.

Botrytis cinerea is by no means the only organism that causes bunches to rot. Make positive identifications in collaboration with your winery or agronomist; utilising their knowledge can be useful, especially with any forward planning.

If resistant strains of Botrytis have previously been identified or are suspected in your area, laboratory testing should be undertaken. Again your winery or adviser can help you with this.

Looking forward to next season, detailed records are most likely your biggest asset when reviewing your disease management practices and planning, so make sure you keep your records up-to-date.

Some things to remember are:

- *Botrytis cinerea* survives over winter in two distinct forms. Strands of the living fungus known as mycelia can survive in cane tissue. Also, resilient resting structures (3-6 mm in length, hard and black) known as sclerotia can survive attached to the vine or on decaying matter in ground litter. The fungus may also be harboured by other vegetation in and around the vineyard.
- Remove prunings or cultivate them into the soil and manage the vineyard floor for increased microbial activity to accelerate the breakdown of inoculum and the amount carried over from season to season.
- Consider alternatives to broadleaf cover crops that might host the disease on the vineyard floor. Some plant species also harbour Light Brown Apple Moth (LBAM), the larvae of which can damage bunches and transmit the disease. As an example of positive cover crops, some native grass species are proven to increase the populations of beneficial species such as lacewings, which are predatory to LBAM.



Vine Talk is compiled by Dave Antrobus, Syngenta Solutions Development Lead
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0429 133 436

years in various regions of France. Use of the model has allowed disease to be controlled with fewer fungicide treatments. The web-based forecasting tool, VitiMeteo Monitoring (see www.vitimeteo.de), which is now in regular use in central Europe (Germany, Belgium, Switzerland, Italy and the Czech Republic), combines inputs from weather forecasts, advisors and local trained disease assessors. Ten well-trained assessors reporting every 10 days is considered ideal. Primary infections of downy and powdery mildew in each 'wine village' are assessed on a 0-4 scale, from none-to-severe. In the Changins area of Switzerland, the downy mildew-specific component of the expert system, VitiMeteo-Plasmopara, has, over 11 years, correctly forecast primary infection events in seven years, was early in three years and late only once. Growers using VitiMeteo-Plasmopara reduced fungicide applications for downy mildew from 8-12 to 0-4. Meanwhile in Bulgaria, a forecasting model called RIMpro-Plasmopara has been adapted from RIMpro-Scab, a decision support system for apple scab, and is now being validated.

Powdery mildew in California is predicted using data from in-field weather stations which feed into the University of California Davis Powdery Mildew risk assessment model. As these weather stations are expensive, the value of virtual weather networks to interpolate data from conventional weather stations and determine risk of powdery mildew is being investigated. Discrepancies between the virtual and actual weather data at 10 vineyard sites in Lodi, California showed that site-specific microclimate information was not captured and the virtual model underestimated risk and recommended fewer sprays than those actually required.

PLANT-PATHOGEN INTERACTIONS AND BREEDING FOR RESISTANCE

Most presentations concerned downy mildew, only a few included powdery mildew.

Early stages of downy mildew at the cellular level were described by Peter Nick (Karlsruhe Institute of Technology, Germany). *P. viticola* zoospores swam to stomata on leaves within 10 minutes of inoculation of susceptible *Vitis vinifera* varieties but not on wild Chinese and Japanese *Vitis* genotypes nor on German *V. vinifera* subspecies *sylvestris*. An Asiatic genotype exuded small aldehydes (nonanal and decanal) that suppressed infection, a defence mechanism that may have been lost in breeding *V. vinifera*. Stomatal guard cells appeared to be involved in the response to infection and this knowledge could be exploited to induce resistance. Accumulation of phenolic compounds around the stomata of genotypes resistant to downy mildew was also reported. Another German group (Muller et al., DLR Rheinpfalz-Abteilung Phytomedezin) showed that downy mildew sporulation increased with susceptibility of the grapevine variety and that transcription of WRKY factors (regulators of plant immunity) and expression of pathogenesis related proteins were greatest in the resistant genotype, suggesting that pathogen effector proteins play an important role. Research by the Kassemeyer group (State Institute for Viticulture and Enology, Frieburg, Germany) showed a role for necrosis- and ethylene-inducing peptide-1 (NEP1)-like proteins (NLPs) in inducing plant defences early in infection. This group also reported activation of downy mildew resistance by pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). In collaboration with researchers from nine European Union countries, they are searching for natural non-toxic compounds that induce PAMP-triggered immunity (VineMan.org); one plant extract is promising but details were not provided. The plant enzymes glucanase and chitinase, along with callose synthesis and a signalling element EDS1, appeared to be involved in resistance of the hybrid variety Solaris to *P. viticola*.

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Australia & NZ Grapegrower & Winemaker
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DELEGATES: Australians at the Europa Palacio de Congresos, Vitoria-Gasteiz, Spain (from left) Trevor Wicks, Barbara Hall, Eileen Scott, Bob Emmett, Tijana Petrovic (absent, Peter Magarey).

The progression of infection on grapevine genotypes resistant and susceptible to powdery mildew was visualised using low temperature scanning electron microscopy (LTSEM) and restriction of hyphal growth on the former led the authors to conclude that plant immunity was triggered during appressorium development, penetration and haustorium formation. These findings support previous observations by Australian researchers using light microscopy and molecular biology techniques. LTSEM was also used by Spanish researchers to show that P. viticola infected and sporulated on Tempranillo leaves more readily than on Cabernet Sauvignon.

To assist breeding resistant cultivars, rapid techniques to assess downy mildew resistance were also presented. High throughput phenotyping of progeny from a cross between *V. vinifera* and a *Vitis* hybrid is being used to generate data for marker-assisted selection for downy mildew resistance. Researchers at the Fondazione Edmund Mach in San Michele all'Adige, Italy reported good agreement when sporulation on leaves was assessed visually and using the software program, Image J. Researchers at INRA, France also reported the use of an automatic particle analyser to count sporangia and zoospores of *P. viticola* as a rapid means of estimating infection potential.

Researchers from INRA, Villenave d'Ornon, France reported that the downy mildew pathogen has overcome the partial resistance of the variety Regent in three regions of France within five years of planting. There appears to be no fitness penalty and the pathogen sporulated as well on Regent as on susceptible varieties.

To counter the ability of *P. viticola* and *E. necator* to overcome major (single) gene resistance, European researchers are pyramiding resistance genes to breed varieties with durable disease resistance. Breeding for resistance to downy and powdery mildews has been underway at INRA, Colmar, France, since 2000 and Sabine Wiedemann-Merdinoglu and colleagues expect to register the first varieties with durable resistance in 2016. Groups at the Fondazione Edmund Mach in Italy and the Instituto Murciano de Investigacion y Desarrollo Agraria y Alimentario in Spain are also working towards durable downy and powdery mildew resistance.

DISEASE MANAGEMENT

The Optidose® method for pesticide dose adjustment to control powdery and downy mildew has been tested for three years in France. The aim is to encourage adoption of a system that takes into account canopy structure and spray efficacy rather than simply hectares of vineyard. The Optidose® rate, calculated based on susceptibility of the patch, canopy characteristics, phenology and inoculum pressure was, in most cases, as effective as the standard treatment but with fewer sprays. The next stage is the Optimised Treatment Plan® (PTO®), in which information about dose, risk, crop biomass, spray equipment and plant measurements is synthesised to explain the efficacy of treatments and adjust fungicide rate to crop requirements. Dose adjustments have allowed the amount of active ingredient sprayed on vineyards to be decreased by 30 per cent. Low drift nozzles are also being evaluated to reduce environmental impacts by reducing spray drift. Cone nozzles, air induction cone nozzles and air induction flat fan nozzles were equally effective (>99 per cent) in control of powdery mildew.

Several presentations dealt with fungicide resistance. Strobilurin resistance in Israel was first detected in 2006/07 and tank mixes of fungicides differing in mode of action from bloom to the early stages of bunch closure have improved control. Strobilurins failed to control powdery mildew in Switzerland in 2011 and resistance quickly became widespread. The powdery mildew fungus in Italy remained sensitive to quinoxyfen from 2009-2013 and results for conidial germination assays correlated well with those from plant assays. Barbara Hall and colleagues summarised the challenges of screening for fungicide resistance in *E. necator*, which grows only on living grapevine tissue. Treating leaf discs with fungicide, placing them on tap water agar and inoculating via a spore settling tower provided the most reproducible results.

The fungicide meptyldinocap reduced maturation of chasmothecia (cleistothecia) and viability of ascospores. It was also claimed to reduce ascospore viability when applied to mature chasmothecia although the effect was not significant.

Two novel fungicides were introduced. Pyriofenone (a benzoylpyridine compound), produced by Belchim Crop Protection France, has a vapour action, is translocated in plant▸

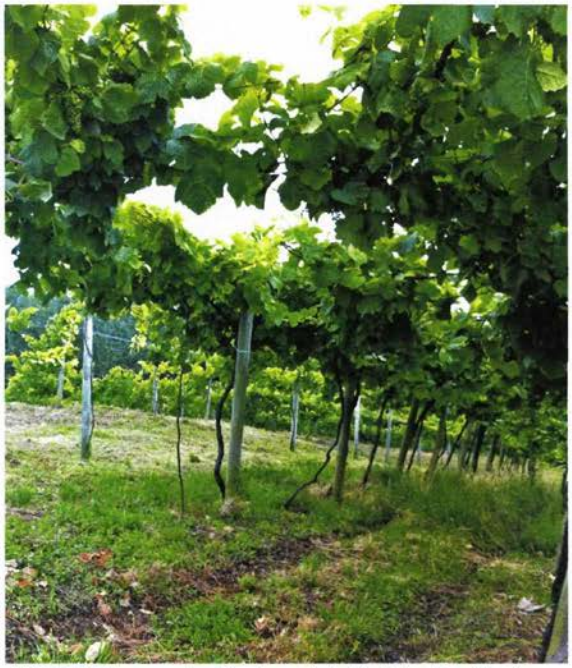
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FIELD TRIP: Vines on a traditional pergola trellis at Gipuzkoa, Atlantic coast, Spain.

tissue and is rain-fast. It was claimed to have preventative and curative efficacy against powdery mildew. Ametoctradin (triazolopyrimidylamine), produced by BASF, controls downy mildew by inhibiting zoospore formation, release, motility and germination. To reduce selection for resistance it is being used in combination with chemicals having different modes of action.

Inter-row planting of plants susceptible to powdery mildew was proposed as a means of propagating biological control agents such as the fungal parasite *Ampelomyces quisqualis* and the fungus-feeding mite *Orthotydeus lambi*. These organisms could attack powdery mildew on the vines. Also on biological control, Longxian Ran (Agricultural University of Hebei, China) reported that inoculation of grapevine leaf discs with *Streptomyces* and *Pseudomonas* bacteria inhibited development of downy mildew.

Crude methanolic extracts of dried, powdered grapevine canes inhibited germination of spores of *E. necator* and zoospore motility and sporulation of *P. viticola* in laboratory assays. Chitosan, a component of crab shell, controlled downy mildew in the vineyard. Comparison of biodynamic, organic and conventional inputs showed that programs involving sulfur generally gave the best control of powdery mildew, although alternative materials had some benefit. Ben Pike and colleagues (University of Adelaide) also found that biodynamic programs resulted in the smallest vines and least yield.

FIELD VISITS

Neiker-Tecnalia

Workshop participants visited Neiker-Tecnalia, the Basque Government agricultural research and development institute, in Vitoria. The institute employs 182 staff and has another site near Bilbao and a 72-ha farm. R&D activities cover animal husbandry, crops including cereals, potato, vines, forestry, soil health, the environment and natural resources. They also offer analytical services and conduct technology transfer (http://www.tecnalia.es/en/proyectos-item/neiker-tecnalia).

A representative from the International Organisation of Vine and Wine (OIV) outlined the organisation's charter and activities, which relate to climate change, plant protection, reduction of pesticides in viticulture using models such as Agrometeo and Optidose, and the process for clonal selection of vines (resolution VITI 1/1991). See www.oiv.int

Rioja Alavesa

La Rioja, which comprises Rioja Alavesa, Basque and Navarre regions, has 64,000 ha of vineyards, mainly dry-grown Tempranillo (50,500 ha). Other red varieties are Garnacha (Grenache) and Mazuelo (Carignan); white varieties are minor. Carbonic maceration is common. Wines are classified according to the Denomination of Origin Calificada (DOCa) Rioja. At Rioja Alta winery (ww.riojalta.com), teledetection is used to assess canopy vigour and diseases are predicted using weather-based models. Vines are not monitored by people. During a tour of the winery, we learned that mid-infrared spectral scanning (WineScan FT 120 Foss Electric) is used to assess contamination by botrytis, and consignments containing gluconic acid and glycerol reflecting 5-10% contamination are rejected.

Gipuzkoa

Gipuzkoa is on the Atlantic coast, where the terrain is steeply sloping and the climate is wet (1000-2000 mm rain per year) and windy. Vines, mainly white varieties, are grown on a traditional pergola trellis and trimmed regularly (Figure 2). Leaves are removed from the fruit zone to promote air movement and drying of bunches. Downy mildew is the most common disease and is managed by regular spraying from equipment small enough to navigate the steep terrain and overhanging trellis, and re-supplied from a tank at the end of the row.

SUMMARY

Australian researchers benefitted from taking part in the workshop. Existing collaboration with researchers in Europe was strengthened and new links were established with Chinese researchers. New knowledge is being extended to the Australian grape and wine industry via meetings, university courses and the final report to the Australian Grape and Wine Authority.

The outcome of most of the work presented was to reduce vineyard inputs, either by reducing susceptibility of the vines to the diseases or by more effective management. The main focus for many papers was developing techniques to understand and measure the pathogens to assist with breeding disease resistant varieties. While not immediately applicable by growers, disease resistant varieties are seen as the future of disease management in the industry. In addition, many techniques developed during this work will have wider applicability in disease detection and management.

Models are being widely used to reduce spray applications, manage dose and drift and improve disease forecasting. Many are available online. These models and techniques to improve early detection of the diseases may need to be evaluated for Australian conditions before use, but could be beneficial to growers and researchers.

ACKNOWLEDGEMENTS

Barbara Hall, Tijana Petrovic and Eileen Scott received travel support (GWT 1328, 1322 and 1325) from Australia's grape growers and winemakers through their investment body the Australian Grape and Wine Authority. Hall also received support from SARDI while Petrovic and Scott were supported by the University of Adelaide. GW

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Fungicide resistance in Australian vineyards

Suzanne McKay and Barbara Hall

Fungicide resistance in Australian vineyards

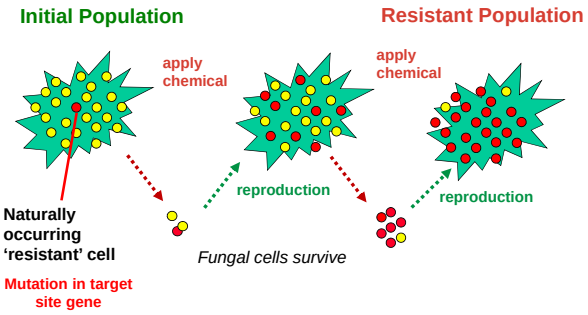
- **What is resistance?**
- **Pre-project status**
- **Project background**
- **Current status**
- **Future**

What is resistance?

Loss of acceptable control when fungicide is used according to recommendations

- Caused by increased frequency **(selection)** of resistant individuals in the fungal population
- Resistant fungal cells need to **survive** then **reproduce**

Practical Resistance is a step-wise process



from W Wilcox, Cornell USA

Development of resistance depends on:

- Mode of Action of fungicide
  - o *single site e.g. QoI*
  - o *multi site e.g. suphur*
- Genetics of fungus- *target site(s)*
- Fungus life cycle
  - *-generation time/reproduction*
- Fungicide applications
  - *-number*
  - *-timing*

***CRITICAL**

➢ **Resistance is difficult to predict**

Types of resistance

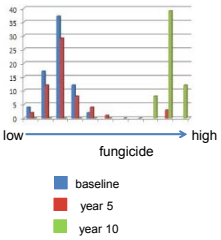
Qualitative

➢ Sudden loss of field control

"All or nothing"

– single gene mutations

e.g. QoI, metalaxyl



Types of resistance

Qualitative

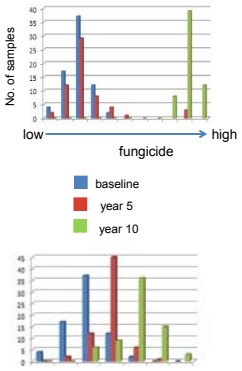
- Sudden loss of field control
  - "All or nothing"
  - – single gene mutations
    e.g. QoI, metalaxyl

Quantitative

- Gradual loss of control
  - "Shades of grey"

"Partial effectiveness"

- – several mutations, interacting or many genes e.g. DMI



Resistance detected in Australia

Powdery mildew	Botrytis	Downy mildew
QoI (11)	Benzimidazoles (1)	metalaxyl (4)
DMI (3)	iprodione (2)	Carboxylic acid amides (40)
metrafenone (U8)	Anilinopyrimidines (9)	
boscalid – SDHI (7)	QoI (11)	QoI (11)
azanaphthalenes (13) spiroxamine (5) cyflufenamid (U6)	fenhexamid (17)	phosphonates (33)
+3 Multi-site groups	boscalid – SDHI (7)	+5 Multi-site groups
	cyprodonil (9)	
	+4 Multi-site groups	

QoI resistance - powdery









- 1st used commercially in 1996 (grapes in US 1997)
  - Resistance detected soon after
  - Multiple failures in New York 2002
    - 15-20 applications since registration
    - few problems if tank-mixed w/sulfur, even with >20 applications
- Registered in Aust ~2000: >20 applications?
- Resistance confirmed in Australia in 2010
  - Vic, WA and SA

How widespread is QoI resistance in Australia?

DMI resistance - powdery



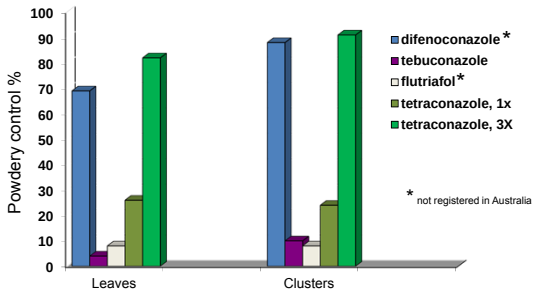




- Triademefon approved in US 1979, myclobutanil and fenarimol 1989
  - – Reduced sensitivity to triademefon detected in 1986
  - – Variable sensitivity to various DMIs reported
- Australia
  - – Reduced sensitivity to triademenol and fenarimol 2004
  - – Maybe widespread in Australia but controlled by changing product or increasing rates
- Activity spectra of DMIs varies

What is the sensitivity status of commonly used DMIs in Australia?

DMIs are not the same!



W. Wilcox, Cornell Uni, New York

Effect of QoI dose

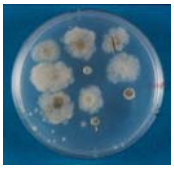
Treatment a.i. / ha	% cluster with powdery
None	98
pyraclostrobin 55g	97
pyraclostrobin 76g	94
pyraclostrobin 95g	95

(7 sprays at 2 week intervals)

Increase dose: control not achieved

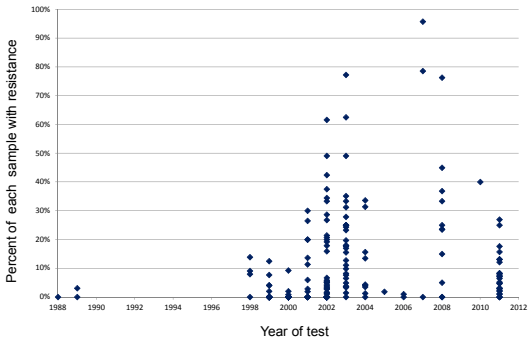
W. Wilcox, Cornell, USA

Iprodione resistance - Botrytis

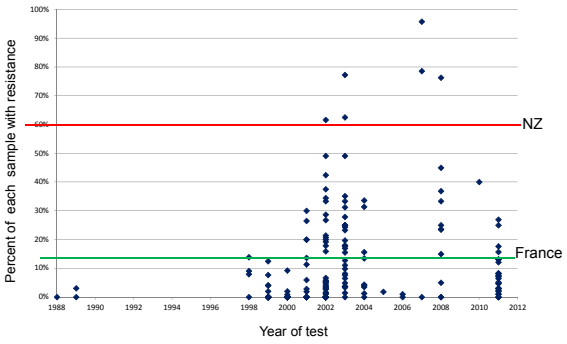


- Resistance known since late 1970's
- Widespread throughout world
- Several classes of resistance levels
  Competitive ability vs virulence
  - Highly resistant strain not as "fit"
  - Sensitive strain "fit" but not as virulent
- Resistance levels decreased in SA over last few years
- Only fungicide available close to harvest

Iprodione resistance in South Australia



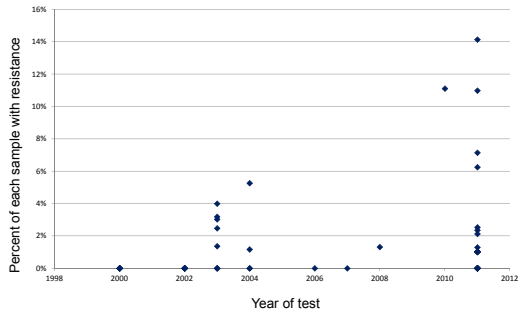
Iprodione resistance in South Australia



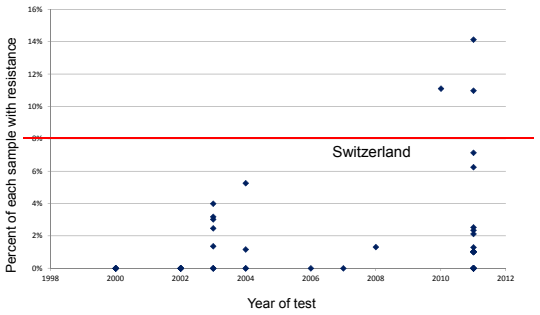
Anilinopyrimidine resistance - Botrytis

- Low proportion of less sensitive strains in wild populations
- Resistance "reversible"?
- Switzerland:
  - 4 sprays/year for 8 years - resistance 8-17% = loss of field control
  - 1 spray/year - no increase in resistance= field control
- First report in Australia in 2002
- Resistance levels increased in SA in last 10 years

Anilinopyrimidine resistance in SA



Anilinopyrimidine resistance in SA



Metalaxyl resistance - Downy

- Metalaxyl 1st used in Europe 1977
- Resistance detected in France 1983
- No reports of resistance on grapevines in US…yet
- Resistance detected in Australia
  - Hunter Valley 2004
    - High disease + suspected resistance following late eradicant applications
    - No reports from vineyards with a reasonable protectant programme
  - Yarra Valley 2010

QoI resistance - Downy

- Resistance first noted in
  - Europe 2001, now common
  - USA 2005, in warm, wet areas- North Carolina, Texas, now widespread
- Resistance in northern regions, e.g. New York, erratic
  - Somewhat less disease pressure (cooler)
  - Limited use since 2003
- Australia?

Research project 2013-2016

What is the incidence and severity of fungicide resistance in Australian vineyards?



Powdery mildew (SARDI)



Botrytis bunch rot (Curtin University, WA)



Downy mildew (Charles Sturt University, NSW and SARDI)

SARDI

Wine Australia for Australian Wine

Project team and industry collaborators

SARDI · Curtin University · The Australian Wine Research Institute · Charles Sturt University · Department of Agriculture and Food · The University of Adelaide · Nufarm · Bayer · syngenta

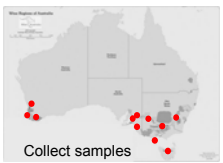
Industry reference group

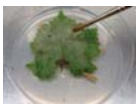
Accolade Wines · Treasury Wine Estates · VITIBIT PTY LTD · Wine Tasmania · Watershed · AHA viticulture

- Vitisolutions

SARDI

Approach





Powdery/Downy



Botrytis

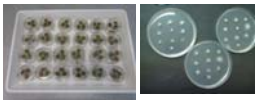
Sensitive? ↕ Resistant?

GENOTYPE
Detect mutation: 'resistance' gene(s)



\+

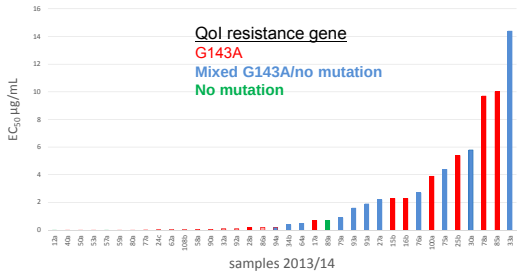
PHENOTYPE
Laboratory test for sensitivity EC_{50}



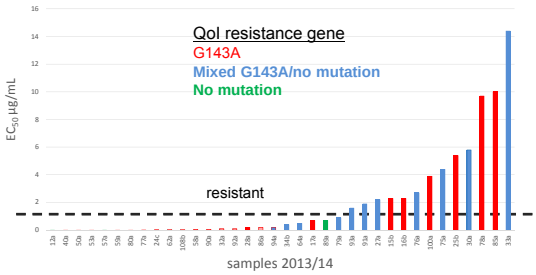
Industry partners selected groups for testing

Powdery mildew	Botrytis	Downy mildew
QoI (11) ✓	Benzimidazoles (1)	metalaxyl (4) ✓
DMI (3) ✓	iprodione (2) ✓	Carboxylic acid amides (40) ✓
metrafenone (U8)	anilinopyrimidines (9) ✓	
boscalid – SDHI (7)	QoI (11) ✓	QoI (11) ✓
azanaphthalenes (13) spiroxamine (5) cyflufenamid (U6)	fenhexamid (17) ✓	phosphonates (33)
+3 Multi-site groups	boscalid – SDHI (7)	+5 Multi-site groups
	cyprodonil (9)	
	+4 Multi-site groups	

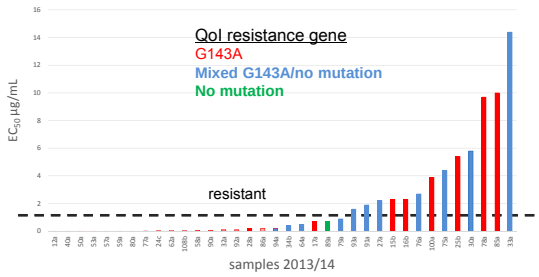
Powdery and QoI Cabrio®



Powdery and QoI Cabrio®



Powdery and QoI Cabrio®



phenotype 'resistant' 36%
resistance gene present in 86%

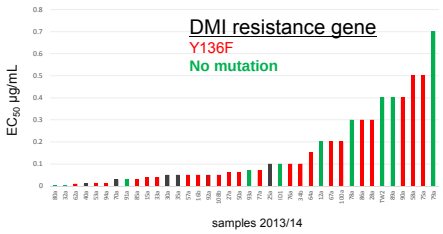
Summary QoI and powdery

	No. of sites tested	No. reduced sensitivity	No. with resistance gene
2013/14	36	13 (36%) [1]	31 (86%)
2014-16	58	37 (63%) [2]	Not tested yet

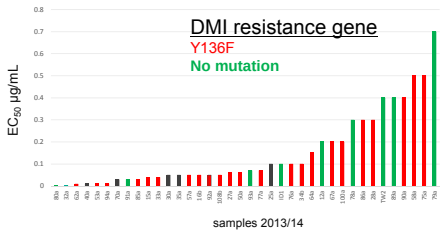


[1] EC_{50} >1.0 µg/mL
[2] Discriminatory dose , growth at ≥ 1.0

Powdery mildew: DMI Topas® (penconazole)

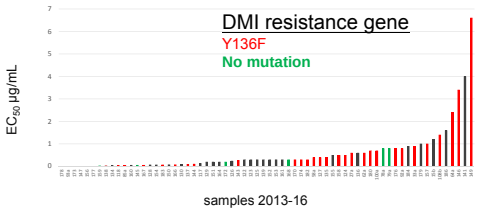


Powdery mildew: DMI Topas® (penconazole)

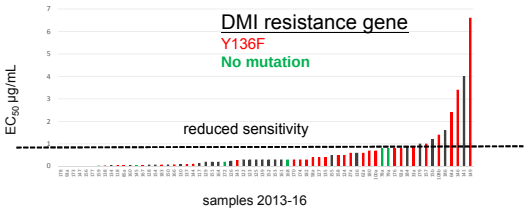


All phenotype sensitive
DMI resistance gene present 68%

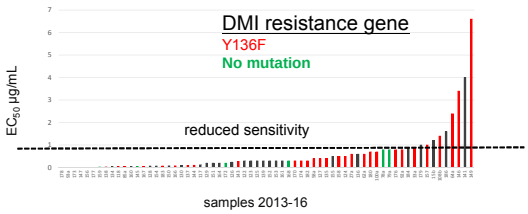
Powdery mildew: DMI Mycloss® (myclobutanil)



Powdery mildew: DMI Mycloss® (myclobutanil)

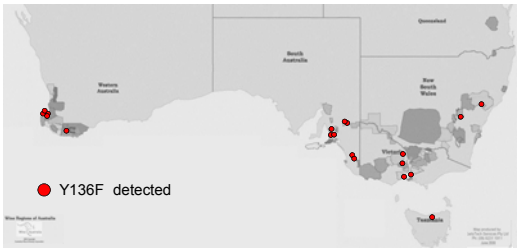


Powdery mildew: DMI Mycloss® (myclobutanil)



~ 9 samples reduced sensitivity
DMI resistance gene present in 86%

Powdery: DMI resistance gene distribution



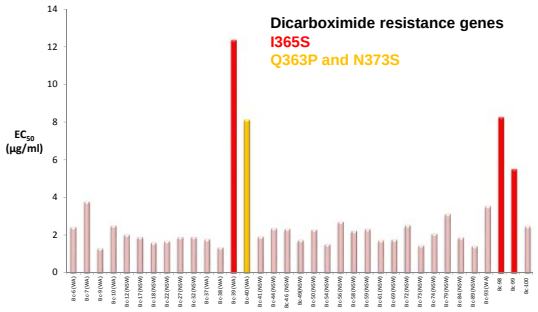
34

Summary: Powdery and DMIs

	No. of sites tested	No. reduced sensitivity	% samples with resistance gene
Topas	38	0	68%
Mycloss	66	9	84%
Domark	21	0	82%

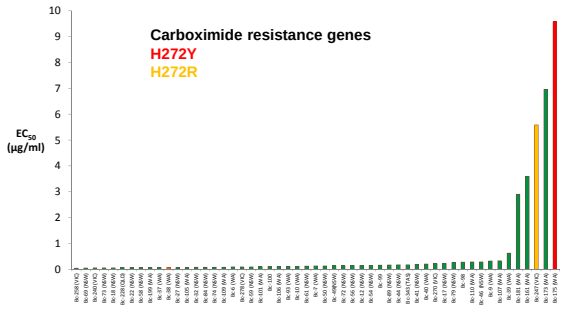


Botrytis: Dicarboximides (gp 2), iprodione



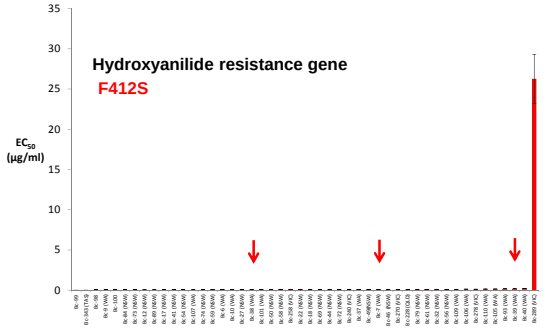
L. Harper & F. Lopez, Curtin Uni, WA

Botrytis: SDHIs (gp 7), boscalid



L. Harper & F. Lopez, Curtin Uni, WA

Botrytis: Hydroxyanilides (gp 17), fenhexamid



L. Harper & F. Lopez, Curtin Uni, WA

Botrytis

Number (%) of sites with reduced sensitivity (n=72)

Carboximides, 7 (boscalid - Filan®)	Hydroxyanilides, 17 (fenhexamid - Teldor®)	Dicarboximides, 2 (iprodione - Rovral®)	Anilinopyrimidines, 9 (pyrimethanil-Scala®)
15 (21%)	**5 (7%)**	20 (28%)	27 (38%)

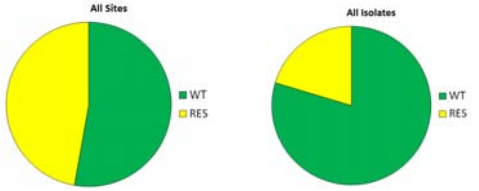
0	1 MOA	2 MOA	3 MOA	4 MOA
38 (46%)	15 (21%)	8 (11%)	**8 (11%)**	**2 (3%)**

Boscalid: EC_{50} = >3 µg/mL and/or MIC = >10 µg/mL
Fenhexamid: EC_{50} = >20 µg/mL and/or MIC = >5 µg/mL
Iprodione: EC_{50} = >5 µg/mL and/or MIC = >2 µg/mL
Pyrimethanil: EC_{50} = >0.8 µg/mL and/or MIC = >5 µg/mL

L. Harper and F. Lopez, Curtin Uni, WA

Botrytis - summary

- 54% of all sampling sites tested have some degree of resistance
- 20% of all isolates tested have some degree of resistance
- Boscalid and fenhexamid resistance confirmed
- 8 sites resistant to 3 MOA
- 2 sites resistant to 4 MOA



L. Harper and F. Lopez, Curtin Uni, WA

Downy mildew

- 61 samples received
- 12/18 (67%) phenotypically resistant to metalaxyl
- *WA and Tasmania now have metalaxyl resistant populations (as well as NSW and Victoria)*
- mandipropomid – 1 isolate resistant??
- Genotype
  - QoI resistance gene detected 3/23 samples
  - 4 sites tested so far– all negative for CAA resistance gene

S. Savvochia, CSU, NSW
A. Borneman, AWRI, SA

Current status of fungicide resistance

Botrytis	Downy mildew	Powdery mildew
benzimidazoles	metalaxyl (4)	QoI (11)
iprodione (2)	Carboxylic acid amides (CAA) (40) ??	DMI (3)
anilinopyrimidines (12)	QoI (11)	metrafenone (U8)
QoI (11)	phosphonates (33)	boscalid – SDHI (7)
fenhexamid (17)		fenhexamid (17)
boscalid – SDHI (7)		azanaphthalenes (13)
cyprodonil (9)		spiroxamine (5)
		cyflufenamid (U6)

Future research

- Understanding how our results relate to field failure
  - What % of resistance results in field failure
  - Effect of mixed genotypes
  - Relate presence of resistance gene to each fungicide
    - e.g. Y136F present-which DMI is best to use?
- Development of a rapid & accurate method to detect resistance in a vineyard
  - How many samples to collect
  - Early detection of hot spots
  - Understand spread



Be alert!
and slightly alarmed…….

<u>Always</u> follow resistance management guidelines



Management



Rotate or mix different modes of action

Use label rates

Limit total number of applications per fungicide group

Understand fungicide activity, modes of action & resistance groups

Start a fungicide program with a multi-site MOA to reduce populations – **DO NOT** use single site MOA to control well-established infections

<u>TIMING</u> is critical

from UC Davis, USA

Thanks to:
- Project team and collaborators
- Everyone who sent samples
- Wine Australia


Photo: M. Ayres

FUNGICIDE RESISTANCE IN AUSTRALIAN VITICULTURE



Dr Barbara Hall[1], Dr Suzanne McKay[1], Dr Fran Lopez[2], Lincoln Harper[2], Dr Sandra Savocchia[3]
[1]South Australian Research and Development Institute, Adelaide, SA, Australia
[2]Centre for Crop and Disease Management, Curtin University, WA, Australia
[3]National Wine and Grape Industry Centre, Wagga Wagga, NSW, Australia
E: barbara.hall@sa.gov.au

Dr Barbara Hall is a plant pathologist with over 35 years' experience in diseases of horticultural crops, and currently works as a Senior Research Scientist within the Horticulture Pathology Group at SARDI. Barbara manages the horticultural diagnostic service and the Quarantine Unit, and coordinates and supervises research projects. Her areas of expertise include: management and control of fungal diseases, resistance detection in fungicides, diagnosis of plant diseases and extending information on disease management to growers.

Abstract
Powdery mildew, botrytis bunch rot and downy mildew are the three most economically important diseases in Australian vineyards. To determine the incidence and severity of fungicide resistance in Australia, samples of all three diseases were collected from vineyards in the main viticultural regions of Australia and tested against a range of commonly used fungicides. Leaf disc assays were used to phenotypically test isolates of *Erysiphe necator* (powdery mildew) for sensitivity to pyraclostrobin, penconazole, myclobutanil and tetraconazole, and *Plasmopara viticola* (downy mildew) to metalaxyl M, mandipropamid and pyraclostrobin. *Botrytis cinerea* was tested by mycelial growth assay for sensitivity to fenhexamid, iprodione, pyrimethanil and boscalid. Representative samples were genotyped for the presence of known mutations conferring resistance. Results showed that QoI resistance in powdery mildew was found in most viticultural regions. While phenotypic resistance of *E. necator* to the demethylation inhibitors was not widespread, the Y136F allele was present in approximately 60% of the isolates. Populations of *P. viticola* resistant to metalaxyl have been confirmed in all states except South Australia, and the allele conferring resistance to QoI has been detected. *B. cinerea* populations resistant to fenhexamid, iprodione, boscalid and pyrimethanil were detected. While 54% of sites had no resistance detected, two sites had populations resistant to all four fungicides. The results of the testing so far have confirmed the presence of resistant populations of these three pathogens to many fungicides throughout Australia. However, more work is needed to confirm how these results relate to the potential for field failure.



Fungicide resistance in Australian Viticulture





Barbara Hall, SARDI

Fungicide resistance

- What is resistance?
- Testing for resistance
- Current status
- Information from Germany
- Current management

What is resistance?

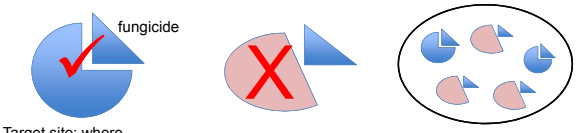
Loss of acceptable control when fungicide is used according to recommendations

Caused by increased frequency **(selection)** of resistant individuals in the fungal population

No mutation
GGG GGT TTC

Mutation
GCG GGT TTC

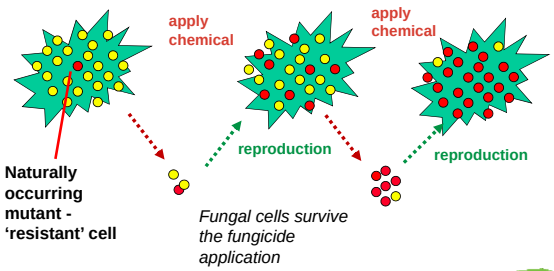
Mixed (mutation/no mutation)



Target site: where fungicide binds

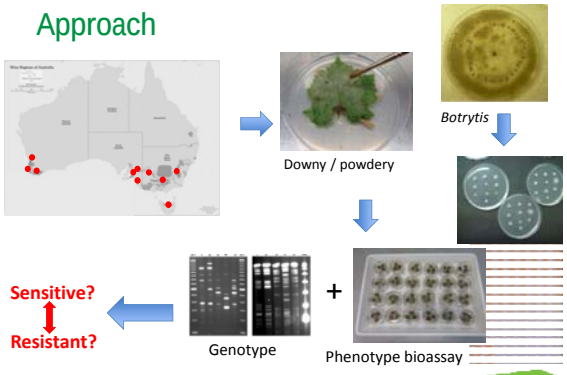
Partial efficacy

Resistant fungal cells need to **survive** then **reproduce**



from W Wilcox, Cornell USA

Approach



Result summary 2013-16 – *Erysiphe*

	No. sites tested	Reduced sensitivity	Mutant present
Cabrio® pyraclostrobin[1,2]	94	53%	86%
Topas® penconazole[1]	38	0	68%
Mycloss® myclobutanil[1]	66	14%	84%
Domark® tetraconazole[1]	21	0	82%

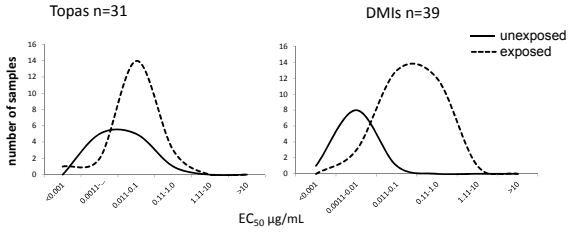
[1] EC_{50} >1.0 µg/mL
[2] Discriminatory dose , >20% growth at 1.0 µg/mL

S McKay SARDI

SARDI

Powdery: DMI sensitivity profile

Exposure within the previous 2 years



S McKay, SARDI

SARDI

Result summary 2013-16 - *Botrytis*

Percent of sites with reduced sensitivity (n=72)

fenhexamid (Teldor®)	boscalid (Filan®)	iprodione (Rovral®)	pyrimethanil (Scala®)
7%	21%	28%	38%

0	1 MOA	2 MOA	3 MOA	4 MOA
54%	21%	11%	11%	3%

L. Harper and F. Lopez, Curtin Uni, WA

Boscalid: EC_{50} = >3 µg/mL and/or MIC = >10 µg/mL
Fenhexamid: EC_{50} = >20 µg/mL and/or MIC = >5 µg/mL
Iprodione: EC_{50} = >5 µg/mL and/or MIC = >2 µg/mL
Pyrimethanil: EC_{50} = >0.8 µg/mL and/or MIC = >5 µg/mL

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Result summary 2013-16 - *Botrytis*

Resistance detected in:

- 46% of all sampling sites
- 20% of all isolates



L. Harper and F. Lopez, Curtin Uni, WA

SARDI

Result summary - *Plasmopara*

42 samples received, only 18 tested phenotypically:

- 12 (67%) resistant to metalaxyl
- Mandipropomid – 1 isolate reduced sensitivity
- QoI tests not completed

23 samples so far genotyped:

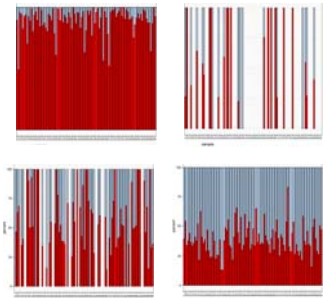
- QoI resistance gene detected in 3 samples - one 100%

S Savocchia, CSU

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Population studies

Resistance gene frequency % - 4 vineyards



- How many samples are needed?
- Distribution of resistance in a block?

Results

- 5 (40% variation)
- 30+ (10% variation)

Issues

- Consistency of sample collection

SARDI

18th International Reinhardsbrunn Symposium

Modern Fungicides and Antifungal Compounds

- Increasing numbers of papers on resistance: ~50% in 2011 & 2014, >60% in 2016.
- Resistance monitoring in Europe
  - Spore traps & phenotyping: private companies
  - Chemical companies only do genotyping
- QoI & SDHI allele frequency high (50-75%) but stable
- Sulphur affects uptake of DMIs - weather dependent relationship

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Management



Rotate or mix different modes of action

Use label rates

Limit total number of applications per fungicide group

Understand fungicide activity, modes of action & resistance groups

Start a fungicide program with a multi-site MOA to reduce populations – **DO NOT** use single site MOA to control well-established infections

TIMING is critical

from UC Davis, USA

SARDI

Resistance is here to stay and we need to manage it

Always follow resistance management guidelines



SARDI

Growers, agronomists, viticulturists in the wine industry

Project team and industry collaborators

SARDI, Curtin University, The Australian Wine Research Institute, Charles Sturt University, The University of Adelaide, Department of Agriculture and Food, syngenta, Nufarm, Bayer

Wine Australia for Australian Wine

Industry reference group

Accolade Wines, Treasury Wine Estates, VITIBIT PTY LTD, Wine Tasmania, Watershed, Vitisolutions, AHA viticulture

SARDI



Research finds some 'red warning flags' in the vineyard

FUNGICIDE RESISTANCE in Australian vineyards is not at crisis levels but, as Barbara Hall puts it, there are 'red warning flags all over the place'.

Hall, a pathologist and Senior Research Scientist with the South Australian Research and Development Institute (SARDI), is leading a collaborative national project designed to determine the incidence and severity of resistant fungal populations of the big three diseases (powdery mildew, downy mildew and botrytis), better understand how resistance develops, and work out the best response.

The researchers have largely completed the first bit and are making progress with the others but have quickly discovered just how complex a task it is, and why very few researchers have ventured into the area.

First things first.

"What we can say at this point is that fungicide resistance is an issue, but growers are still getting control if they've got a good spray program," Hall said. "But if we don't start looking after the fungicides we've got, there could be serious issues.

"By looking after fungicides I mean understanding them, knowing and following resistance management strategies, mixing and matching, and minimising their use."

The trap with fungicides is that a grower can be technically doing the right thing in terms of how often they spray, but it might be the wrong thing for their vineyard at a given point in time.

The ultimate goal for Hall and her colleagues is to be able to understand each of the 15 or so individual fungus/fungicide combinations to the extent that they can develop a testing service so growers can know where they sit and how to respond. Or, in the first instance, discover whether it is a resistance issue, or just an inappropriate spray program and/or poor spray technique.

"With any fungus/fungicide combination, if you can keep the resistance below that level you are going to keep achieving efficacy. Once resistance goes above that level you're going to start seeing loss of field control."

While this research work continues, the best approach is to stay vigilant and to understand the basics. The rule of thumb is that the more often you use an individual fungicide, the more likely it is to build resistance.

"Look what happened with Cabrio," Hall said. "Everyone embraced it as the most fantastic fungicide because it controlled powdery and downy and had an effect on botrytis. The industry used it widely and resistance grew. Scientists found in America that resistance can start with just 20 applications, so if you spray three times a year that can happen in less than eight years."

Resistance usually – but not always – develops when mutant genes that already exist at a low level in a fungal population are able to survive and thrive after exposure to a fungicide. Whether or not that happens depends on how many mutant genes there are, how strong they are relative to the other genes, and whether the conditions are right.

What makes the science so complicated is that mutant genes can be present at even quite high levels without a vineyard actually having a resistance problem.

"With any fungus/fungicide combination, if you can keep the resistance below that level you are going to keep achieving efficacy. Once resistance goes above that level you're going to start seeing loss of field control.

There are relatively simple molecular tests to assess whether mutants are present (in Europe they are part of a compulsory annual test for all chemical companies) but they don't tell you whether this is leading to failure in the field.

The path Hall's team plans to take will use more complicated phenological and genetic testing in vineyards and in the laboratory. They started with the DMI and QoI fungicide groups, which the sector told them were the priorities, and will now start to look at the newer SDHI group.

The project, funded by Wine Australia, is a collaboration between SARDI – a division of Primary Industries and Regions SA (PIRSA), the Australian Wine Research Institute, Curtin University, the University of Adelaide, the National Wine and Grape Industry Centre and the WA Department of Agriculture and Food.

