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# Fungicide Resistance in Australian Viticulture

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## ABSTRACT

Fungicide resistance has previously been reported within Australian vineyards: in *Erysiphe necator* (powdery mildew) to the demethylation inhibiting (DMI) and QoI (strobilurin) group of fungicides, in *Botrytis cinerea* to the dicarboximide and anilinopyrimidine fungicide groups and in *Plasmopara viticola* (downy mildew) to metalaxyl. To determine the incidence and severity of fungicide resistance, 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. The pathogens were tested phenotypically for resistance using leaf disc assays (*E. necator* and *P. viticola*) or mycelial growth assays (*B. cinerea*). Representative samples were genotyped for the presence of known mutations conferring resistance. Phenotypic resistance of *E. necator* to QoI was found in 42% of 72 isolates, with the G143A allele present in 87% of 46 tested isolates. Phenotypic resistance of *E. necator* to DMIs was not observed, however the Y136F allele was present in ~77% of the isolates. *B. cinerea* resistant populations to fenhexamid, iprodione, boscalid and pyrimethanil were detected in 7, 20, 21, and 27% respectively of the 72 sites tested. While 54% of sites had no resistance detected, two sites had populations resistant to all four fungicides. A number of mutations were found in the target genes; H272R or H272Y in the sdhB gene (boscalid target), I365S or Q369P/N373S in the bos-1 gene (iprodione target) and F412S in the erg27 gene (fenhexamid target). Populations of *P. viticola* resistant to metalaxyl now exist in Western Australia and Tasmania as well as Victoria and New South Wales. The results of the testing 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 laboratory results relate to field performance of fungicides in spray programmes.

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## INTRODUCTION

The three most economically important diseases in Australian viticulture are powdery mildew caused by *Erysiphe necator*, downy mildew caused by *Plasmopara viticola*, and Botrytis bunch rot caused by *Botrytis cinerea*. Performance issues of fungicides has occurred in all three diseases, attributed to resistance or reduced sensitivity reported in *E. necator* to Demethylation Inhibitor (DMI) fungicides (Savocchia et *al.* 2004) and to Quinone outside Inhibitor (QoI) fungicides (Wicks et *al.* 2013); in *P. viticola* to metalaxyl (Wicks et *al.*. 2005); and *B. cinerea* to anilinopyrimidines (Sergeeva et *al.* 2002) and dicarboximides (Hall et *al.* 2001). This paper reports results of a survey conducted throughout the main viticulture areas of Australia over two seasons (2013/4 and 2014/5) to determine the extent of resistance to selected fungicides in these three pathogens.

## *ERYSIPHE NECATOR*

### Materials and methods

Seventy two isolates of *E. necator* were collected for evaluation against selected commercial grade QoI and DMI 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). Fungicides were mixed in sterile double distilled water (SDDW) and diluted to 5-6 different concentrations between 0.001 and 16 µg/mL, with SDDW used as the control.

Young, glossy leaves cv. Cabernet Sauvignon were collected from plants grown in a controlled environment room and surface sterilised in 0.5 % bleach (White King®) for 3 mins, washed 3-4 times in SDDW. Ten mm diam discs were cut using a sterile cork borer from leaves cv. Cabernet Sauvignon, collected and surface sterilised as previously described. Discs were 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. After soaking for 30 mins for Cabrio® and Topas® EC and 60 mins for Mycloss™ (Wong and Wilcox 2002) 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 tap water agar 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. The discs were left overnight and inoculated with *E. necator* the following morning. Each disc was inoculated in the centre with ~300 *E. necator* spores by touching the end of a sterile cotton tip on to the surface of a 14-day-old sporulating colony of *E. necator* to collect spores on the cotton tip and depositing these spores on the centre of a disc by touching once with the cotton tip. After 14 days incubation at 22°C, 12/12 hr day/night under fluorescent light, each leaf disc was assessed for the percentage of leaf area colonised by powdery mildew. The $EC_{50}$ for each isolate was calculated by Probit analysis using Genstat 15th edn (VSN International, UK).

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. 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.

**Results and discussion**

A range of sensitivities to pyraclostrobin were observed, with $EC_{50}$ values ranging from 0.0005-14.4. Testing confirmed that resistance to QoI fungicides was widespread, with an EC50 value of >1.0 ug/mL in 42% of the 72 isolates. The G143A allele was present in 87% of the 46 isolates tested so far, with sequencing showing that many isolates were a mixture of wild type and mutant (Fig. 1).

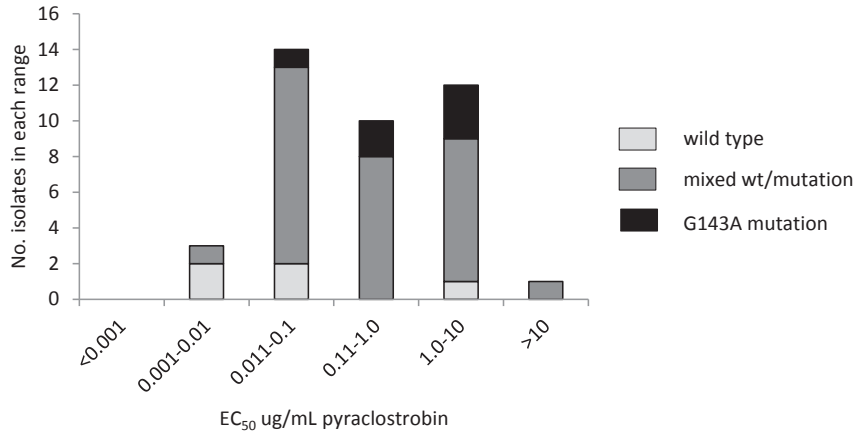


Figure 1 Frequency and mutation distribution of the QoI fungicide pyraclostrobin $EC_{50}$ values for isolates of *Erysiphe* necator.

No phenotypic resistance to penconazole or myclobutanil was detected, the $EC_{50}$ values ranged from 0.0004 to 0.7. However preliminary testing with an additional DMI fungicide (tetraconazole ai) has shown some isolates have reduced sensitivity to this fungicide.

While all isolates tested were sensitive to the two DMI fungicides in leaf tests, the CYP51 mutation (Y136F allele) was detected in 23 out of 33 isolates tested with penconazole and 32 of the 39 isolates tested with mycobutanil. While testing is not yet complete, initial studies on limited isolates with the Y136F allele present have shown no relationship between the sensitivities of the isolates to the three DMI fungicides (Table 1).

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Table 1 Penconazole, myclobutanil and tetraconazole $EC_{50}$ values for isolates of *Erysiphe necator* with the Y136F allele present.

| **Fungicide** | **$EC_{50}$ ug/mL** | | | | |
|---|---|---|---|---|---|
| **penconazole** | 0.04 | 0.009 | 0.03 | 0.2 | 0.05 |
| **myclobutanil** | 0.9 | 0.6 | 0.04 | 0.7 | 1.4 |
| **tetraconazole** | 0.01 | 0.008 | 0.05 | 0.0008 | 0.003 |

## *BOTRYTIS CINEREA*

### Materials and Methods

Seven hundred and forty five single spores isolates of *B. cinerea* were established from grape material collected between 2013 and 2015 from 72 sites throughout Australia. A subset of 54 of these isolates was screened against four fungicides using a microtiter plate method to establish $EC_{50}$, MIC values 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 a range of increased concentrations of fungicides. 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. There were two biological replicates each with two technical replicates for each isolate. After 72 h incubation at room temperature in darkness the optical density (OD) was measured at 450 nm wavelength in a Synergy HT microplate reader (BioTek). 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 as 1 µg/ml fenhexamid, 3 µg/ml iprodione, 0.4 µg/ml pyrimethanil and 1 µg/ml boscalid. Isolates were subsequently tested in a mycelial growth assay using discriminatory doses of fungicide active ingredient as previously described. After 3 days incubation in the dark, fungal growth was scored as either present (resistant isolate) or absent (sensitive isolate). *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.

### Results and discussion

*B. cinerea* resistant populations to fenhexamid, iprodione, boscalid and pyrimethanil were detected in 5, 15, 20 and 27 respectively of the 72 sites tested. While 38 (54%) of sites had no resistance detected and 15 (21%) of sites had resistance to only one fungide, multiple

resistance was observed in the remaing sites, with eight sites resistant to two fungicides, eight to three fungicides and two sites with populations resistant to all four fungicides. The detection of multidrug resistance limits the fungicide choices available to growers and impacts their resistant management strategies.

Of the isolates tested, mutant target genes were found only in samples with phenotypic resistance detected: I365S and Q369P/N373S were detected in the bos-1 gene (iprodione target), H272R or H272Y in the sdhB gene (boscalid target) and F412S in the erg27 gene (fenhexamid target). Further testing is underway on more isolates and additional fungicides, including azoxystrobin, tebuconazole and fludioxonil.

## *PLASMOPARA VITICOLA*

### Materials and Methods

A leaf-disc assay used to phenotype isolates of *Plasmopara viticola* for fungicide sensitivity was similar to that described for *E. necator*, using cvs. Sultana or Tempranillo. A 10 µL suspension ($10^6$ spores/mL) was placed on to each leaf disc and incubated for 24 h in the dark at room temp (~22$^o$C) at high humidity. The surface of leaf discs were dried for 2-3 h then incubated at 23$^o$C for 12 h light/dark. The percent of leaf infection was assessed at 7 days and $EC_{50}$ determined. The fungicides tested were mandipropamid (Revus®) at 0.001 to 10 µg/mL, metalaxyl M (Ridomil®) at 0.05 to 10 µg/mL, and pyraclostrobin (Cabrio®) at 0.001 to 0.1 µg/mL.

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.

### Results and discussion

Due to the sporadic nature of the infections only 18 isolates were able to be collected and tested. When tested on leaf material, resistance to metalaxyl was detected in 12 (67%) of the samples, confirming that resistant populations to metalaxyl now occur in Western Australia and Tasmania, as well as the previously known areas in New South Wales and Victoria. Resistant sites were perhaps unsurprisingly in the regions with high disease pressure and hence high fungicide application. However there is also possible bias from the inability to obtain samples from areas with low disease pressure. Testing of pyraclostrobin on leaf material needs to be repeated, however the G143A mutation was detected in three of the 23 sites tested.

## CONCLUSION

Field failure of fungicides has many potential causes, including incorrect timing of application and inadequate coverage. However results of this research indicate resistant strains of these

pathogens to currently used fungicides are present in Australian vineyards and careful management of fungicide programs is needed to ensure that these strains do not become a significant problem. Significant gaps in knowledge have been identified during this project. These include an understanding of the differences in DMI resistance and efficacy among the various products, determining the relative fitness of resistant populations compared to wild type, and elucidating the link between phenotype results and presence of the resistance alleles. An improved understanding of the relationship between laboratory testing results and field performance will provide more effective resistance management tools and options.

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