Fungicide resistance characterised across seven chemical classes in a *Botrytis cinerea* population isolated from Australian vineyards

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Abstract

Key words

Botrytis cinerea, fungicide resistance, mutation, grape, *CytB*, *SdhB*, *Erg27*, *Bos1*, *Pos5*, *Mdl1*, *Cyp51*, *Mrr1*

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Abstract

Gray mold, caused by *Botrytis cinerea*, is an economically important disease of grapes in Australia and across grape growing regions worldwide. Control of this disease relies heavily on canopy management and the application of single site fungicides. Fungicide application can lead to the selection of fungicide resistant *B. cinerea* populations, which has an adverse effect on the chemical control of the disease. Characterising the distribution and severity of resistant *B. cinerea* populations is needed to inform resistance management strategies. In this study, 725 isolates were sampled from 75 Australian vineyards during 2013 – 2016 and were screened against seven fungicides with different MOAs. The resistance frequencies for azoxystrobin, boscalid, fenhexamid, fludioxonil, iprodione, pyrimethanil and tebuconazole were 5, 2.8, 2.1, 6.2, 11.6, 7.7 and 2.9% respectively. Nearly half of the resistant isolates (43.7%) were resistant to more than one of the fungicides tested. The frequency of vineyards with at least one isolate simultaneously resistant to 1, 2, 3, 4 or 5 fungicides was 19.5, 7.8, 6.5, 10.4 and 2.6%.

Resistance was associated with previously published genotypes in *CytB* (G143A), *SdhB* (H272R/Y), *Erg27* (F412S), *Mrr1* (D354Y), *Os1* (I365S, N373S + Q369P, I365S + D757N) and *Pos5* (P319A, L412F). Expression analysis was used to characterise fludioxonil resistant isolates exhibiting overexpression (6.3 - 9.6-fold) of the ABC transporter encoded by AtrB (MDR1 phenotype). Novel genotypes were also described in *Mrr1* (S611N, D616G) and *Cyp51* (P357S). Resistance frequencies were lower when compared to most previously published surveys of both grape and non-grape *B. cinerea* resistance. Nonetheless, continued monitoring of critical chemical groups used in Australian vineyards is recommended.

Introduction

Botrytis cinerea Pers.:Fr., anamorph *Botryotinia fuckeliana (De Bary) Whetzel*, is a broad host range necrotrophic fungal pathogen responsible for one of the most economically important diseases of grapevines worldwide. *B. cinerea* has been stated as only second to *Magnaporthe oryzae* (Rice blast disease) in terms of its scientific and economic importance (Dean et al. 2012). There is currently a lack of scientific literature on the scale of crop losses caused by *B. cinerea* (Carisse 2016). In Australian vineyards, *B. cinerea* is second only to powdery mildew in economic impact (Scholefield and Morison 2010). *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 $AUD50 M per annum (Emmett and Nair 1991; Scholefield and Morison 2010). Yield losses in Australia from *B. cinerea* and other bunch rots can be anywhere between 3 – 30 % depending on the climatic zone (Scholefield and Morison 2010). Aside from yield, *Botrytis* infection can affect grape quality, as important qualitative compounds (e.g. sugar) can be impacted (Ribereau-Gayon et al. 1980). The control of *B. cinerea* in vineyards relies heavily on the application of fungicides (Elad et al. 2016). In Australia, a wide range of both multi-site and broad spectrum single-site fungicides are registered for *B. cinerea* control. A number of single-site fungicides that are specific botryticides are also registered (Australian Pesticides and Veterinary Medicines Authority).

B. cinerea is a "high risk" pathogen for fungicide resistance development due to its short life cycle and high reproductive rate (Brent and Hollomon 1998). Resistance in *B. cinerea* has been linked to target site modifications, target site overexpression, efflux pump activation and detoxification. Resistance to single site fungicides was first reported in Germany in the 1970s after heavy use of dicarboximides and benzimidazoles (Dekker 1976). To date, populations resistant to the single site MOA anilinopyrimidines (AP), hydroxyanilides, phenylpyroles (PP), succinate dehydrogenase inhibitor (SDHI) and quinone outside inhibitor (QoI) classes, have been found in grapevines in most grape growing countries (Avenot et al. 2018; Baroffio et al. 2003; Beresford et al. 2017; Campia et al. 2017;

De Miccolis Angelini et al. 2014; Latorre and Torres 2012; Leroch et al. 2011; Leroux et al. 1999; Mosbach et al. 2017; Sang et al. 2018; Walker et al. 2013). Resistance to these fungicides has been linked to target site modifications. The multiple drug resistance (MDR) phenotype is characterised by reduced sensitivity to fungicides and test compounds with diverse MOAs (e.g. cycloheximide, cyprodinil, fenhexamid, and tolnaftate). MDR has been found in *B. cinerea* populations from grapevines, soft fruit crops and vegetables (Fernández-Ortuño et al. 2014; Leroch et al. 2013; Li et al. 2014b; Ren et al. 2016; Rupp et al. 2017). Two mechanisms that cause MDR in *B. cinerea* have been characterised; overexpression of the ABC transporter *atrB* (giving the MDR1 phenotype) and overexpression of the Major Facilitator Superfamily (MFS) transporter *mfsM2* (MDR2) (Kretschmer et al. 2009).

In Australia, *B. cinerea* grapevine isolates resistant to benzimidazoles, dicarboximides and anilinopyrimidines have been previously described (Nair et al. 1997; Sergeeva et al. 2002a). Similarly, a preliminary report showed *B. cinerea* isolates from vegetable crops have shown high levels of resistance to SDHI, QoI and phenylpyrrole classes (Hailstones 2011).

Current resistance management strategies for *Botrytis* include; limiting fungicide use at the multi-seasonal scale, utilising mixtures, alternating MOA and ideally the addition of novel MOAs to spray programs (Fillinger and Walker 2016). For example, for anilinopyrimidines and SDHIs, the Fungicide Resistance Action Committee (FRAC) recommends a maximum of three sprays per season. In Australia, a maximum of two single site fungicide sprays are recommended per season (The Australian Wine Research Institute). In Australia, MRLs (minimum residue levels) restrict the use of fungicides on grapes used for export wine.

The aim of this research was to provide current data on fungicide sensitivity levels in *B. cinerea* Australian populations to a group of seven fungicides classes widely used in wine grape production. A nationwide collection of isolates was screened via a combination of high-through put discriminatory

concentration assays and molecular analyses. In addition, the presence of MDR1 was investigated in selected isolates.

Materials and Methods

Fungal Isolates.

During 2013 - 2016, mono-conidial *B. cinerea* isolates were isolated from 74 commercial wine grape vineyards and 1 commercial table grape vineyard across Australia (Fig. 1, Table S2). These isolates came from across six states: Western Australia (WA), South Australia (SA), Victoria (VIC), Tasmania (TAS), New South Wales (NSW) and Queensland (QLD). Sampling covered a number of wine growing regions; WA – Manjimup, Margaret River, Geographe, Great Southern, Perth Hills, Swan District, Pemberton; SA – Coonawarra, Adelaide Hills, McLaren Vale, Langhorne Creek, Padthaway, Barossa valley, Riverland; VIC – King Valley, Yarra Valley, Beechworth, Rutherglen, Gippsland; NSW – Tumbarumba, Cowra, Southern Tablelands, Hunter Valley, Mudgee, Orange and QLD – Granite Belt (Fig. 1, Table S2). Isolates were maintained as mycelial plugs on yeast soluble starch (YSS) medium, with addition of 1.25% w/v agar (Fillinger et al. 2008), in distilled water at 4 ^{o}C.

Establishing baseline EC_{50} values for azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole using a microtiter assay.

To establish baselines, 53 *B. cinerea* isolates sampled during 2013 – 2015 seasons, were randomly selected for testing against technical grade azoxystrobin (Grace), boscalid (Sigma-Aldrich), fenhexamid (Bayer), iprodione (Sigma-Aldrich), pyrimethanil (Bayer) and tebuconazole (Bayer) within a microtiter assay system (Table S1). Only five isolates (Bc-7, Bc-279, Bc-287, Bc-385 and Bc-410) from the subset of 53 isolates, were selected for microtiter testing against technical grade fludioxonil (Sigma-Aldrich). Induction of sporulation and the harvesting of conidia for all isolates was carried out as previously described by Harper et al. (2019), with two modifications. YSS agar (YSSA) was used for culturing instead of PDA and the conidial suspension was adjusted to 10^5 conidia mL^{-1}, instead of 10^7 conidia mL^{-1}. All types of liquid media mixtures were as described by Mair et al. (2016b) except with the

addition of tween20 at a final concentration of 0.05%. The type of liquid media used in the microtiter assays depended on the fungicide tested. Sensitivity to technical grade azoxystrobin, fenhexamid, fludioxinil, iprodione and tebuconazole was assessed using YSS medium, with the following fungicide concentrations: for azoxystrobin: 0, 0.01, 0.025, 0.05, 0.1, 0.5, 1 and 5µg mL^{-1}, for fenhexamid: 0, 0.025, 0.05, 0.075, 0.1, 0.15, 0.25 and 1 µg mL^{-1}, for iprodione: 0, 0.4, 1, 1.5, 2, 3, 5, and 10 µg mL^{-1}, for fludioxinil: 0, 0.05, 0.1, 0.3, 0.6, 1, 1.5, 2, 2.5, 3, 5 and 10 µg mL^{-1}, and for tebuconazole: 0, 0.2, 0.3, 0.5, 0.75, 1, 2, and 3 µg mL^{-1}. Salicylhydroxamic acid (SHAM) (Sigma-Aldrich) was also included in the media for azoxystrobin testing at a final concentration of 400 µM. Sensitivity to pyrimethanil was assessed using the amended YSS medium minus yeast extract, with the following fungicide concentrations: 0, 0.05, 0.1, 0.2, 0.25, 0.3, 0.4 and 1 µg ml^{-1}. Sensitivity to boscalid was assessed using Yeast Bacto Acetate (YBA) medium (Stammler and Speakman 2006) with the following fungicide concentrations: 0, 0.01, 0.02, 0.03, 0.04, 0.075, and 0.1 µg mL^{-1}. 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; for azoxystrobin: 0, 0.5, 1, 5, 15, 25, 35 and 50 µg mL^{-1} or 0, 1, 5, 25, 50, 75, 100 and 150 µg mL^{-1}, for boscalid: 0, 0.5, 1, 2, 3, 5, 7 and 10 µg mL^{-1}, for fenhexamid: 0, 5, 10, 20, 30, 50, 75 and 100 µg mL^{-1}, for iprodione: 0, 0.5, 1, 2, 3, 5, 7 and 10 µg mL^{-1} or 0, 1, 2, 3, 5, 6, 8 and 10 µg mL^{-1} or 0, 2, 4, 5, 7, 10, 25 and 50 µg mL^{-1}, for pyrimethanil: 0, 0.2, 0.5, 1, 1.5, 3, 5 and 10 µg mL^{-1} or 0, 0.2, 0.5, 1, 3, 5, 10 and 25 µg mL^{-1}, for tebuconazole: 0, 0.25, 0.5, 1, 2, 3, 4 and 5 µg mL^{-1}. The loading of conidia and media, reading of the microtiter plate, and the calculations of EC_{50} values was carried out as previously described by Mair et al. (2016b), except that all plates were incubated for 72 h before reading at 450 nm with the exception of pyrimethanil plates that were read after 96h.

Amplification and sequencing of the target genes; *CytB*, *SdhB*, *Erg27*, *Bos1*, *Mdl1*, *Pos5*, *Cyp51*, and the MDR1-related transcription factor; *Mrr1.*

To investigate mutations involved in resistance identified in the microtiter screen assays, all isolates that exhibited a significant reduction in sensitivity were genotyped for their respective resistance

associated genes. The target genes for azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole were cytochrome B (*CytB*); succinate dehydrogenase subunit B (*SdhB*) and succinate dehydrogenase subunit B (*SdhD)*, 3-keto reductase (*Erg27*); histidine kinase (*Bos1*); mitochondrial ABC transporter (*Mdl1*) and mitochondrial NADH kinase (*Pos5*), and lanosterol 14 alpha-demethylase (*Cyp51*), respectively. To test candidate MDR1 isolates that exhibited constitutive over-expression of the ABC transporter atrB, the *Mrr1* gene was sequenced. The promoter for *Cyp51* was also sequenced for isolates exhibiting a reduction in sensitivity to tebuconazole. Three sensitive strains (Bc-7, Bc-385 and Bc-410; Table S1) were genotyped for all target genes for comparative purposes. Only Bc-385 was genotyped for the MDR1-related transcription factor gene *Mrr1*. MDR1 candidate isolates Bc-128 and Bc-391 were genotyped for *Mrr1* as they grew on the fludioxonil discriminatory concentration (DC) of 0.1 ug/mL and were subsequently designated flu^{MR} (Figure 8, Table S4). DNA was extracted from isolates as previously described by Harper et al. (2019). The primers used to amplify promoter and gene regions, and their respective annealing temperatures and extension times are shown in Table S5. All PCR reactions were carried out in an Eppendorf thermocycler model 5344. The *CytB* gene was amplified in a 50 µl reaction containing 2 µl of DNA, 10 µl of 5x HF reaction buffer, 0.16 mM of each dNTP, 0.5 M of each primer and 1U of Phusion polymerase (New England Biolabs). The subsequent thermal profile was as follows: initial denaturation was at 98 °C for 30 s followed by 35 cycles at 98 °C for 10 s, 59 °C for 30 s, and 72 °C for 3 min, and a final extension at 72 °C for 3 min. *SdhB, Erg27, Mrr1, Bos1, Mdl1, Pos5, Cyp51,* and the *Cyp51* promoter, were amplified using a MyTaq (Bioline) reaction mixture and thermal profile as previously described by Harper et al. (2019), except with the specific annealing temperatures and extension times as described in Table S5. Amplified gene products were confirmed on a 1% agarose gel and then sent to Macrogen Korea for sequencing. Consensus gene sequences were aligned to the following reference sequences: AB262969 (*CytB*), AY726618 (*SdhB*), GQ253439 (*SdhD*), AY220532 (*Erg27*), *B. cinerea* B05.10 chromosome 5; CP009809 (*Mrr1*), AF435964 (*Bos1*), *B. cinerea* B05.10 chromosome 10; CP009814 (*Pos5*), *B. cinerea* B05.10 chromosome 16; CP009820 (*Mdl1*), AF279912 (*Cyp51*), *B. cinerea* T4 contig; FQ790352 (*Cyp51* promoter). Alignments

were carried out as described by Mair et al. (2016b). The nucleotide sequences generated in this study have been deposited in GenBank and are listed in Table S3.

Cleaved amplified polymorphic sequence analysis of *Bos1* gene.

The cleaved amplified polymorphic sequence (CAPS) test utilising the restriction enzyme *Taq* I to identify the I365N/R/S mutant in *Bos1* was carried out as described by Oshima et al. (2006). In this study, the internal sequencing primers os-1 F and os-1 R (Table S5) were used to amplify a 1133 bp fragment encompassing the I365S and Q369P + N373S alleles. The fragment was amplified using the MyTaq protocol described above and with the annealing temperature and extension time described in Table S5. The digestion of the product was carried as per Oshima et al. (2006). The same amplified fragment was also used to test for the presence of the Q369H/P allele, which required digestion with *Sma* I.

Development of a fungicide resistance discriminatory concentration agar assay.

Minimum inhibitory concentration (MIC) values identified in the microtiter assay (Table S1) were used to design a DC agar screen to test the remaining 672 isolates in the collection. The discriminatory doses for the agar assay were as follows: azoxystrobin – 5 µg ml^{-1}, boscalid – 1 µg ml^{-1}, fenhexamid – 1 µg ml^{-1}, fludioxinil – 0.1 µg ml^{-1}, iprodione – 3 µg ml^{-1}, pyrimethanil – 0.4 µg ml^{-1} and tebuconazole – 3 µg ml^{-1}. For all fungicides, the DC screening was carried out using YSS agar, except for boscalid which used YBA agar and pyrimethanil which used YSSA minus the yeast extract. SHAM was added at a concentration of 400 µM for the plates containing azoxystrobin. Mycelial colonies were grown as described by Harper et al. (2019). Two 4 mm plugs per isolate were taken from the edge of actively growing colonies and then placed on plates containing the appropriate media and fungicide combination. Eight isolates were tested per plate, and plates were incubated at room temperature in the dark for three days and scored on their ability to grow on each fungicide.

RT-qPCR analysis of *atrB* and *Cyp51*.

To identify MDR1 phenotypes and further characterise isolates lacking mutations in *Cyp51*, the expression levels of *atrB* and *Cyp51* were assessed, respectively. Isolates Bc-128, Bc-130, Bc-279, Bc-287, Bc-385 and Bc-391 were selected for atrB expression analysis. Bc-130 was selected for atrB analysis to investigate if MDR1 could contribute to a teb^{LR} phenotype. Bc-287 was selected for atrB expression analysis as it exhibited a group S haplotype (Fig. 8) that has previously been associated with MDR1 (Fernández-Ortuño et al. 2014; Leroch et al. 2013; Li et al. 2014b). Isolates were incubated as described by Li et al. (2014b), with some modifications. Culture growth was carried out in 50 ml of PDB in a 250 ml flask. For atrB expression analysis, Bc-279, Bc-287, and Bc-385 were induced with 1 µg ml^{-1} pyrimethanil for 0.5 h. For Cyp51 expression analysis, Bc-130 and Bc-385 were induced with 0.5 µg ml^{-1} tebuconazole for 1 h. Harvesting of biomass, extraction of RNA, production of cDNA, and qPCR was carried out as described by Mair et al. (2016b) with actin used as the endogenous control (BC1G_08198.1).

Statistical analysis.

All statistical analyses were conducted in IBM SPSS software (IBM, New York, U.S.A.). To account for heteroscedasticity, all fungicide sensitivity data were log transformed before analysis. To separate EC_{50} mean values between sensitive and resistant isolates characterised in the 53 isolate microtiter subset, an independent samples t-test (P = 0.05) or Mann-Whitney U-test (P = 0.05) was used depending on whether the data set had a normal or non-normal distribution. Mean values for gene expression analyses were analysed using one-way ANOVA, with means separated by independent samples t-test (P = 0.05)

Results

Resistance identified across seven chemical classes in a subset of Australian *B. cinerea* isolates

Monoconidial *B. cinerea* isolates collected between 2013 and 2016 were tested by microtiter assay to determine EC_{50} values to the fungicides azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole (Table 1 and S1, Fig. 2). Additionally, EC_{50} values were calculated in a subset of five isolates (Bc-7, Bc-279, Bc-287, Bc-385 and Bc-410) to fludioxonil (Tables S1). Except for iprodione and tebuconazole, all isolates with an EC_{50} >0.5 µg mL^{-1} were classified as resistant. For iprodione and tebuconazole, isolates were considered resistant when their EC_{50} were higher than 2.5 µg mL^{-1} and 0.75 µg mL^{-1}, respectively (Tables 1 and S1, Fig. 2). The EC_{50} ranges of the sensitive isolates for azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole, were 0.04 – 0.37, 0.03 – 0.10, 0.05 – 0.22, 0.75 – 2.05, 0.09 – 0.22 and 0.21 – 0.72 µg mL^{-1}, respectively (Tables 1 and S1, Fig. 2). The EC_{50} ranges of the resistant isolates for azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole were >50, 2.74 – 2.90, 25.47 – 27.33, 5.16 - >50, 0.75 – 5.90 and 1.06 – 1.80 µg mL^{-1}, respectively (Tables 1 and S1, Fig. 2). The resistance factor (RF) ranges of the resistant isolates for azoxystrobin, boscalid, fenhexamid, iprodione, pyrimethanil and tebuconazole were >413, 55 – 58, 255 – 273, 4.3 - >42, 5 – 42 and 2.5 – 4.2, respectively (Table 1). For the subset tested against fludioxonil, the sensitive range was 0.09 – 0.17 µg mL-1 (Table S1). The fludioxonil resistant isolate Bc-279 exhibited an EC_{50} of 0.93 µg mL^{-1} (Table S1). EC_{50} values for the sensitive and resistant populations for all fungicides were significantly difference from each other (P = 0.05) (Tables 1 and S1, Fig. 2). Resistant isolates for azoxystrobin (azo), boscalid (bos) and fenhexamid (fen) were all characterised as R isolates (Tables 1 and S1, Fig. 2). Resistant isolates for the fungicides iprodione (ipr), fludioxinil (flu) and pyrimethanil (pyr) were divided into medium resistance (MR) or high resistance (HR) isolates (Tables 1 and S1, Fig. 2). Resistant isolates for the fungicide tebuconazole (teb) were characterised as low resistance (LR) (Tables 1 and S1, Fig. 2).

Resistance frequencies for seven chemical groups in Australian grape growing regions.

A total of 672 isolates collected between 2013 and 2016 from major grape-producing regions of Australia were screened for resistance using a DC agar assay as described above. Combining microtiter and DC agar assay data, the total resistance frequency for azoxystrobin, boscalid, fenhexamid, fludioxonil, iprodione, pyrimethanil and tebuconazole was 5, 2.8, 2.1, 6.2, 11.6, 7.7 and 2.9%. (Fig. 3). Thirty different resistant profiles, with 24 of these showing resistance to at least 2 MOA, were identified (Table 2). The total number of resistant isolates for each fungicide in WA, SA, VIC and NSW, ranged from 0 – 30, 3 – 14, 1 – 26 and 0 – 3, respectively (Fig. 4).The frequency of resistant isolates in each of the states was 15.4%, 25.3%, 61.9%, 36.9%, and 5.8% across WA, SA, TAS, VIC and NSW (Table S2). The frequency of vineyards with resistance to 1 – 5 MOA were 19.5%, 7.8%, 6.5%, 10.4% and 2.6%, respectively (Fig. 5). No resistance was found in QLD.

Fungicide resistance is associated with mutations in the fungicide target genes *CytB*, *SdhB*, *Erg27*, *Bos1*, *Pos5*, *Mdl1* and *Cyp51*.

Relevant target site genes were sequenced in all resistant isolates found in the microtiter assay and compared to target gene sequences from the three sensitive reference isolates (Bc-7, Bc-385, Bc-410) (Table S1, Fig. 6). Mutations in archetype species are stated below if the proposed archetype is not *B. cinerea* or if the mutations are not exclusively characterised in *B. cinerea (Mair et al. 2016a)*. Azo^R isolates all showed the nucleotide change G3628C resulting in the amino acid sequence change G143A (*G143A* in the archetype *Z. tritici*) (Table 3, Fig. 6). Sequencing of *CytB* from the sensitive isolate Bc-385 revealed the presence of an intron at the G143 site (Fig. 6). PCR amplification of *CytB* from Bc-111 revealed the presence of two fragments, indicating the presence of the G143 intron (data not shown). Subsequently, intron specific primers (Table S5) were used to confirm the presence of this intron in Bc-111 (data not shown).

Sequencing of the *SdhB* gene in the two boscalid bos^R isolates identified in the microtiter analysis showed the nucleotide change A871G resulting in the amino acid change H272R (*H277Y* in the

archetype *Pyrenophora teres f. sp. teres*) (Table 3, Fig. 6). Additional *SdhB* genotyping was carried out on nine boscalid resistant isolates identified in the DC assay (Table 3). Eight of these isolates contained the H272R mutation, while the remaining isolate showed the nucleotide change C870T resulting in the amino acid change H272Y (Table 3, Fig. 6). Sequencing of the *SdhD* gene in the two boscalid bos^{R} isolates (Bc-111, Bc-181) and comparative sensitive isolates revealed no non-synonymous changes (data not shown).

All three fen^{R} isolates and two sensitive isolates (Bc-7 and Bc-410) showed a polymorphism at amino acid position 238 (CCT to TCT) that resulted in a change of a proline to a serine (P238S) (Fig. 6). Fen^{R} isolates showed the nucleotide change T1314C resulting in the amino acid change F412S. (Table 3, Fig. 6).

All sensitive and resistant isolates genotyped for *Bos1* had the nucleotide change G4153A resulting in the amino acid change A1259T (Fig. 6). All ipr^{MR} isolates had the nucleotide changes T1214G and G3729A resulting in the amino acid changes I365S (*I365S*) and V1136I (*V1136I*), respectively (Table 3, Fig. 6). All ipr^{HR} isolates either contained the nucleotide changes A1226C + A1238G or T1214G + G2592A resulting in the amino acid changes Q369P + N373S or I365S + D757N, respectively. The I365S + D757N ipr^{HR} haplotype (Bc-396) had a significantly lower iprodione sensitivity (EC_{50} value of 25.10 µg mL^{-1}) when compared to ipr^{MR} isolates which had the I365S + V1136I haplotype (Bc-39, Bc-128, Bc-179, Bc-343; mean EC_{50} value of 8.07 ± 1.58 µg mL^{-1}, (data not shown) (Table 3 and S1, Fig. 6). Twenty-nine iprodione resistant isolates, identified in the DC assay, were tested using the CAPS method described in Oshima et al. 2006 (*Taq* I – I365N/R/S) and in this study (*Sma* I – Q369H/P) (Table 3). From this analysis, eighteen and eleven isolates were characterised as I365N/R/S and Q369H/P, respectively (Table 3).

Two putative target genes were sequenced in the case of pyrimethanil, *Pos5* and *Mdl1*. Four of the pyr^{MR} isolates (Bc-39, Bc-128, Bc-177, Bc-279) collectively showed an EC_{50} range of 0.75 – 2.28 µg mL^{-1} and exhibited no changes in *Pos5* (Table S1, Fig. 6). All pyr^{HR} isolates (EC_{50} range of 4.66 – 5.90 µg mL^{-1})

showed either changes C1066G or G1347T resulting in the changes P319A or L412F respectively (Table 3, Table S1, Fig. 6).

With respect to the *Mdl1* gene, all isolates tested showed the nucleotide changes T85C and A2370G resulting the amino acid changes S29P anf K790R, respecting (Fig. 6).. One sensitive isolate (Bc-385), one pyr^{MR} isolate (Bc-177) and two pyr^{HR} isolates (Bc-297, Bc-289) all showed the nucleotide change G2339A resulting the amino acid change D780N (Fig. 6). One pyr^{MR} isolate (Bc-177) and two pyr^{HR} isolates (Bc-287, Bc-289) showed the nucleotide change G196A resulting in the amino acid change T66A (Fig. 6).

The teb^{LR} isolate Bc-475 showed the nucleotide change C1153T resulting in the amino acid change P347S (*K354* in the archetype *Z. tritici*) (Table 3, Fig. 6). No changes in the *Cyp51* gene were found in the other teb^{LR} isolate Bc-130. Sequencing of a 973 bp region directly upstream of the *Cyp51* start codon in Bc-130, revealed two SNPs (G > A at -365 and A > T at -169) when compared to the sensitive comparative isolate Bc-385 (data not shown). *Cyp51* expression analysis in Bc-130 revealed small constitutive and inducible over expression values (~1.5-fold), compared to the comparative teb^{S} isolate Bc-385 (Fig. S2) ($P < 0.05$). The nucleotide change G70A resulting in the amino acid change V24I was found in the sensitive strain Bc-385 (Fig. 6). The nucleotide change G181A resulting in the amino acid change V61I was found in the sensitive strain Bc-7 (Fig. 6).

D354Y, S611N and D616G mutations in the *Mrr1* transcription factor may contribute to constitutive overexpression of *atrB* in fludioxonil^{LR} isolates

The *atrB* gene was found to be expressed constitutively at a significantly higher level in flu^{MR} isolates Bc-279, Bc-128 and Bc-391 compared to the flu^{S} isolates Bc-385 and Bc-287 ($P < 0.05$) (Fig. 7). The relative increases in expression were 6.3, 7.3 and 9.6-fold for Bc-279, Bc-128 and Bc-391, respectively (Fig. 7). Bc-130 did not exhibit overexpression of atrB compared to Bc-385 (Fig. 7). Under pyrimethanil treatment, Bc-279 but not Bc-287, showed a significantly higher relative expression (5.9-fold) relative to Bc-385 ($P < 0.05$) (Fig. S1). Sequencing of the MDR1-related *Mrr1* gene in the flu^{LR} isolates Bc-279,

Bc-128, Bc-391 and the flu^{S} isolates Bc-385, Bc-130 and Bc-287 revealed the presence of several mutations and indels (Table S4, Fig. 8). Of these mutations, V227I + S611N, D616G and Δ6bp (nt 67 – 72, or 73 – 78, or 79 – 84) + D354Y were only found in Bc-279, Bc-128 and Bc-391, respectively (Table S7, Fig 8).

Discussion

Data on fungicide resistance in Australian grapevine *B. cinerea* populations is very limited, with only a few reports having been published (Nair et al. 1997; Sergeeva et al. 2002b). To produce an extensive and contemporary data set on the fungicide sensitivity status in *B. cinerea* grapevine populations, a nation-wide screening was undertaken.

This study characterised EC_{50} values and RFs that were consistent with previous wine and table grape reports that used a range of different microtiter or agar-based methods (Table 1, Table 4, and Table S1). A similar trend was observed when the data was compared with non-grape *B. cinerea* studies for azoxystrobin (Banno et al. 2009; Weber and Hahn 2011; Yin et al. 2015) and fludioxonil (Fernández-Ortuño et al. 2014; Weber and Hahn 2011) (Table 4).

The resistance frequencies found in this study were generally low (2.1 – 11.6%) but within the values previously reported for *B. cinerea* in wine and table grapes by other authors (Table 4, Fig. 3). Frequency discrepancies between this study and previous reports could be the result of differences in phenotyping methodology. This study used microtiter and YSSA based mycelial growth methods to calculate overall frequencies, while previous reports used either microtiter (Campia et al. 2017; Leroch et al. 2011; Toffolatti et al. 2020) or a non-YSSA based mycelial growth methods (Avenot et al. 2018; Banno et al. 2009; Beresford et al. 2017; Latorre and Torres 2012; Lu et al. 2016; Sergeeva et al. 2002a; Yin et al. 2015) (Table 4).

Besides the methodological approach, the frequencies found may be influenced by a number of contributing factors. Historically varied chemical programs across different wine growing countries could promote differences in frequencies between reports. The current recommended limit of two sprays per season per MOA in Australian wine grape production could play a role in limiting frequency levels across all chemical groups. Leroch et al. (2011) and Walker et al. (2013) characterised low iprodione resistance frequencies in *B. cinerea* and suggested that this was the consequence of limited use of these fungicides in German and French vineyards, respectively. In Australia, the relatively recent

registration of fenhexamid and boscalid, together with the drastic reduction in use of the latter due to maximum residue limit (MRL) restrictions, could also contribute to maintaining low resistance frequency levels for these fungicides. Conversely, the earlier introduction of pyrimethanil and iprodione could be associated with increased exposure to these chemicals and support the relatively higher resistance frequencies found. The lack of independent selection pressure for particular fungicides, e.g., azoxystrobin, tebuconazole and fludioxonil, due to their availability only as mixtures with other MOAs in Australia, could also be associated with low resistance frequency levels to these chemicals. The relatively higher cost of a particular fungicide (e.g. fenhexamid) may also have affected resistance frequencies in Australia, as applications of costly fungicides are often less frequent compared to more cost-effective ones. The presence of fitness costs in fenhexamid and fludioxonil resistant field isolates (Billard et al. 2011; Ren et al. 2016; Saito et al. 2013; Zhao et al. 2010; Ziogas et al. 2005) could also play a significant role in maintaining low resistance frequencies in these fungicides. On the contrary, no significant fitness costs have been reported in iprodione or pyrimethanil resistant isolates, which may also support the relatively higher resistance frequency found for these two chemicals (Bardas et al. 2008; Beever and Brien 1983; Cui et al. 2004; Fernández-Ortuño et al. 2013; Grabke et al. 2014; Oshima et al. 2006).

Resistance to multiple MOA (2 – 5) was recorded in 43.7% of the resistant isolates (Table 2). Multi-resistance has been previously reported in several wine grape *B. cinerea* studies (Campia et al. 2017; Gullino et al. 2000; Latorre and Torres 2012; Leroux et al. 2010; Sergeeva et al. 2002a). This phenomenon could be the result of overexposure of *Botrytis* populations to different MOAs in a sequential manner (Li et al. 2014a). Weekly applications of a single MOA or mixtures of single-site actives appear to have selected for multi-resistant *B. cinerea* strains in blackberry and strawberry (Li et al. 2014a). Resistance to one fungicide in a multi-resistant isolate could be indirectly selected by the application of another fungicide for which resistance already exists, also known as “selection by association” theory (Hu et al. 2016; Weber and Entrop 2011). In this report, the characterisation of multi-resistant isolates resistant to boscalid could be an example of this process as recent selection

pressure from this fungicide is now essentially non-existent in *B. cinerea* populations. The absence of any fitness penalties could also assist this process, as numerous reports have failed to identify any significant fitness penalties in *B. cinerea* (Bardas et al. 2008; Beever and Brien 1983; Cui et al. 2004; Fernández-Ortuño et al. 2013; Grabke et al. 2014; Oshima et al. 2006). The establishment of MRLs may also increase the number of MOA rotated over several seasons as particular fungicides are avoided (e.g. boscalid) or have a more limited use.

Resistance identified in this study was associated in most cases with target-site mutations reported elsewhere (Fig. 6). The *CytB* G143A mutation previously found in QoI highly resistant (RF = 69) *B. cinerea* isolates (Banno et al. 2009) was identified in all azo^{R} isolates (Table 3, Fig. 6). An intron found at position 143 in an azo^{S} and azo^{R} isolates (Fig. 6) had previously been described in sensitive (Banno et al. 2009; Ishii et al. 2009; Jiang et al. 2009; Leroux et al. 2010) and QoI resistant *B. cinerea* isolates (Asadollahi et al. 2013). The presence of this intron prevents the G143A mutation occurring as splicing will be affected, reducing respiratory function due to lower *CytB* levels (Banno et al. 2009; Grasso et al. 2006; Jiang et al. 2009; Vallières et al. 2011). Banno et al. (2009) reported a high number of azoxystrobin sensitive isolates carrying this intron, which suggested a lack of evident fitness penalty associated with the presence of the intron. G143A has been associated with fitness penalties in *B. cinerea* laboratory mutants (Markoglou et al. 2006). In our study, the presence of this intron in an isolate (Bc-111) that also contains mutant (G143A) copies of *CytB*, could suggest the presence of a "fitness balance", whereby intron and G143A *CytB* copies are balanced to maintain the lowest possible fitness cost. Mitochondrial heteroplasmy with respect to the G143A mutation has been confirmed in numerous fungal plant pathogens; *B. cinerea* (Hashimoto et al. 2015; Ishii et al. 2009), *Venturia inaequalis* (Michalecka et al. 2011; Villani and Cox 2014), *Corynespora cassiicola* (Ishii et al. 2007), *Mycovellosiella nattrassii* (Ishii et al. 2007), *Colletotrichum gloeosporioides* (Ishii et al. 2007), and various powdery mildews (Fraaije et al. 2002; Lesemann et al. 2006; Mosquera et al. 2019; Vielba-Fernández et al. 2018). The widespread occurrence of mitochondrial heteroplasmy in fungal plant pathogens could be a mechanism by which some QoI resistant isolates overcome potential fitness

costs associated with G143A. Further studies are required to identify the role of the 143/144 intron in QoI resistant isolates carrying G143A.

Mutations H272R/Y, previously found in the gene encoding for the SdhB subunit of the SDHI target in highly resistant (RF = 40) *B. cinerea* (Leroux et al. 2010; Stammler et al. 2008), were identified in all bos^R isolates genotyped in this study (Table 3, Fig. 6). Sequencing of *Erg27* in fen^{HR} isolates revealed the presence of the F412S mutation (Table 3, Fig. 6), which had been previously associated with a high level of fenhexamid resistance (RF = >30) in *B. cinerea* isolated from grapes (Fillinger et al. 2008).

The P238S mutation identified in fenhexamid sensitive and resistant isolates in this study (Fig. 6), has been found in both sensitive and resistant isolates in a number of grape and non-grape studies, which suggests that P238S may not be associated with resistance (Albertini and Leroux 2004; De Miccolis Angelini et al. 2014; Esterio et al. 2011; Grabke et al. 2012; Toffolatti et al. 2020).

The occurrence of multi-single site resistance phenotypes in a population can disguise the presence of MDR phenotypes. Resistance to fludioxinil, has previously been shown to be associated with overexpression of the ABC transporter atrB (Fernández-Ortuño et al. 2014; Kretschmer et al. 2009; Leroch et al. 2013; Li et al. 2014b). The presence of MDR1 isolates in this *B. cinerea* population was confirmed by measuring the expression of the *atrB* gene in isolates exhibiting different sensitivity levels to fludioxonil (Fig. 7). Three different *Mrr1* haplotypes were found in MDR1 isolates (Bc-128, Bc-177 and Bc-279) overexpressing the ABC transporter AtrB (Fig. 7). The *Mrr1 m*utation D354Y was previously described in MDR1 isolates in two strawberry *B. cinerea* studies (Fernández-Ortuño et al. 2014; Li et al. 2014a). The 6bp deletion in Bc-391 *Mrr1* was previously reported in an MDR1 isolate alongside mutation R632I (Leroch et al. 2013). In addition to D354Y, *Mrr1* mutations S611N and D616G were found in MDR1 isolates. Kretschmer et al. (2009) found the *Mrr1* mutation S611R in MDR1 isolates. It is possible that changes in the vicinity of residue 611 could have a similar effect to that of S611R. Characterisation of additional MDR1 isolates that also exhibit these changes would provide further evidence of the association of these mutations with a MDR1 phenotype. Several group S

isolates, that characteristically exhibit 18 and 21 bp insertions in *Mrr1*, have previously been associated with MDR1 (Fernández-Ortuño et al. 2014; Leroch et al. 2013; Li et al. 2014a). Interestingly, the teb^{LR} isolate Bc-130 exhibited a unique group S-like *Mrr1* haplotype, with the 18bp and 21bp insertions present and absent, respectively. This group S-like haplotype did not produce a MDR1 phenotype in Bc-130.

Mosbach et al. (2017) associated mutations in two genes; *Pos5* and *Mdl1*, with AP resistance in grape and strawberry *B. cinerea* field isolates. All pyr^{HR} isolates showed either mutation P319A or L412F in *Pos5*, which is in agreement with a previous grape and strawberry *B. cinerea* study reporting RF values of >10 in relation to these mutations (Mosbach et al. 2017). All pyr^{MR} isolates (Bc-37, Bc-128, Bc-177, Bc-279) showing no changes in *Pos5* (Table S1, Fig. 6). A T66A change in the *Mdl1* gene was found in the pyr^{MR} in isolate Bc-177. Mosbach et al. (2017) characterised AP-resistant field isolates that carried T66A in combination with V273I, P319A or L412V changes in *Pos5*. The same authors also identified AP-resistant field isolates lacking mutations in *Pos5* or *Mdl1*. The overexpression of the ABC transporter AtrB in MDR1 isolates that lack *pos5* or *Mdl1* mutations (Bc-128, Bc-279) could be contributing to the pyr^{MR} phenotype (Fig. 7). Further research is required to identify molecular markers for pyrimethanil resistance in isolates that lack mutations in *Pos5* and *Mdl1*.

The *Bos1* haplotypes I365S + V1136I (ipr^{MR}), I365S + D757N (ipr^{HR}) and Q369P + N373S (ipr^{HR}) identified in this study have been previously associated with medium to high levels of dicarboximide resistance (RF = >3.4) in other grape and non-grape *B. cinerea* reports (Banno et al. 2009; Grabke et al. 2014; Ma et al. 2007; Oshima et al. 2002; Oshima et al. 2006).

Mutations in *Cyp51A* or *Cyp51B* are commonly associated with DMI resistance in various fungal species, however, none are yet to be identified in *B. cinerea* (Mair et al. 2016a). A new mutation P347S was found in the teb^{LR} isolate Bc-475, further evidence would be needed to associate this change to the teb^{LR} phenotype. The teb^{LR} isolate Bc-130 exhibited no changes in the *Cyp51* promoter or coding region (data not shown, Fig. 6). Nonetheless expression analysis of *Cyp51* showed that Bc-130 had a

marginal but significantly higher constitutive and inducible expression level of *Cyp51* when compared to the sensitive isolate Bc-385 ($P < 0.05$) (Fig. S2). It is not clear whether this small difference in constitutive and inducible expression in *Cyp51* would contribute to the reduction in tebuconazole sensitivity observed in isolate Bc-130.

Several resistance mutations identified in this study have successfully been utilised for the development of robust in-vitro detection methods (Alzohairy et al. 2021; Chatzidimopoulos et al. 2014; Fan et al. 2018; Hu et al. 2017; Liu et al. 2019). However, these *in vitro* detection methods are generally time consuming and require the samples to be sent to a laboratory. Future development of simple, mobile in-field detection methods could provide more timely and cost-effective information that can be used to improve resistance management practices.

This study has shown that the majority of single site MOA botryticides registered in Australia for the control of grey mould in grapes are compromised to some degree. Resistance factors are very high for some compounds and many isolates were resistant to multiple MOA across all Australian wine-growing regions. However the frequency of resistance amongst isolates was low. The current recommended resistance management strategy that limits the use of single site fungicides is probably playing a crucial role in maintaining these low frequencies (Walker et al. 2013).

Acknowledgements

The authors would like to thank Steven Chang for single sporing a small subset of the *B. cinerea* population. The authors would like to thank Wesley Mair for designing a selection of the oligonucleotide primers used in this study. This work was funded by Wine Australia (SAR1204, SAR1602 and SAR1701-1.2) and Curtin University (Perth, Australia).

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Table 1 Results summary of the microtiter phenotyping of 53 *B. cinerea* isolates.

		Azoxystrobin	Boscalid	Fenhexamid	Iprodione	Pyrimethanil	Tebuconazole
EC_{50} (µg/mL-1)	Sensitive range	0.04 - 0.37	0.03 - 0.10	0.05 - 0.22	0.75 - 2.05	0.09 - 0.22	0.21 - 0.72
	Sensitive mean	0.12 ± 0.010	0.05 ± 0.002	0.10 ± 0.006	1.20 ± 0.039	0.14 ± 0.004	0.43 ± 0.015
	Resistant range (LR - R)[a]	>50	2.74 - 2.90	25.47 - 27.33	5.16 – >50	0.75 - 5.90	1.06 – 1.80
	Resistant mean[b]	171.26 ± 16.264	2.82 ± 0.076	26.19± 0.578	21.80 ± 4.901	2.99 ± 0.805	1.43 ± 0.372
	LR range	-	-	-	-	-	1.06 – 1.80
	MR range	-	-	-	5.16 – 11.94	0.75 – 2.27	-
	HR or R range	>50	2.74 – 2.90	25.46 – 27.33	>16.78	4.66 – 5.90	-
no. of isolates	Sensitive	48	51	50	43	46	51
	LR-HR	5	2	3	9	7	2
	RF range	>413	55 – 58	255– 273	4.3 – >42	5 – 42	2.5 – 4.2
	Resistance frequency (%)	9.3	3.7	5.7	17	13.2	3.8

[a]LR = low resistance, MR = medium resistance, HR = high resistance, R = resistance

[b]significant differences were found between the resistant and sensitive populations according to independent samples t-test (P = 0.05) or Mann-Whitney U-test (P = 0.05).

Table 2 Frequency of phenotypes characterised across six Australian states.

	WA	SA	TAS	VIC	NSW	QLD	Total (%)
Number of isolates tested	364	95	21	122	103	20	725
Number of vineyards sampled	38	13	1	11	13	1	77
Sensitive	308	71	8	77	97	20	581 (80.1)
Phenotype[a]							
Azo^R	1	1	0	0	1	0	3 (0.4)
Bos^R	2	0	1	0	0	0	3 (0.4)
Flu^R	11	1	0	10	0	0	22 (3)
Ipr^R	14	2	9	9	1	0	35 (4.8)
Pyr^R	1	0	0	5	1	0	7 (1)
Teb^R	5	4	0	1	1	0	11 (1.5)
Azo^R Bos^R	0	2	0	0	0	0	2 (0.3)
Azo^R Flu^R	0	1	0	0	0	0	1 (0.1)
Azo^R Ipr^R	2	0	0	0	0	0	2 (0.3)
Azo^R Pyr^R	2	0	0	1	0	0	3 (0.4)
Flu^R Ipr^R	1	1	0	1	0	0	3 (0.4)
Flu^R Pyr^R	0	0	0	1	0	0	1 (0.1)
Flu^R Teb^R	1	1	0	0	0	0	2 (0.3)
Ipr^R Pyr^R	2	0	0	1	0	0	3 (0.4)
Ipr^R Teb^R	0	0	3	0	0	0	3 (0.4)
Pyr^R Teb^R	0	0	0	1	0	0	1 (0.1)
Azo^R Ipr^R Pyr^R	1	1	0	0	0	0	2 (0.3)
Azo^R Bos^R Pyr^R	3	0	0	0	0	0	3 (0.4)
Azo^R Fen^R Pyr^R	0	0	0	0	1	0	1 (0.1)
Azo^R Flu^R Ipr^R	0	1	0	0	0	0	1 (0.1)
Fen^R Ipr^R Pyr^R	0	0	0	5	0	0	5 (0.7)
Flu^R Ipr^R Pyr^R	2	0	0	2	0	0	4 (0.6)
Azo^R Bos^R Ipr^R Pyr^R	7	2	0	1	0	0	10 (1.4)
Azo^R Fen^R Ipr^R Pyr^R	0	2	0	1	0	0	3 (0.4)
Azo^R Flu^R Ipr^R Pyr^R	0	3	0	0	0	0	3 (0.4)
Bos^R Flu^R Ipr^R Pyr^R	0	1	0	0	0	0	1 (0.1)
Fen^R Flu^R Ipr^R Pyr^R	0	0	0	4	0	0	4 (0.6)
Flu^R Ipr^R Pyr^R Teb^R	1	0	0	2	0	0	3 (0.4)
Azo^R Bos^R Fen^R Ipr^R Pyr^R	0	1	0	0	0	0	1 (0.1)
Azo^R Fen^R Ipr^R Pyr^R Teb^R	0	0	0	0	1	0	1 (0.1)
Total resistant	56	24	13	45	6	0	144 (19.8)

[a]Azo = azoxystrobin, Bos = boscalid, Fen = fenhexamid, Ipr = iprodione, Pyr = pyrimethanil, Teb = tebuconazole.

Table 3 Frequency of target-site mutations associated with resistance identified in this study.

Target gene	Genotype[a]	Phenotype[b]	No. of isolates	Reference
cytB	G143A	R	5	Banno et al. 2009
sdhB	H272R	R	10	Stammler et al. 2008
	H272Y	ND	1	Stammler et al. 2008
erg27	F412S	R	3	Fillinger et al. 2008
bos1	I365S	MR	5	Oshima et al. 2002
	I365N/R/S	ND	18	Oshima et al. 2002
	I365S + D757N	HR	1	Lu et al. 2016
	Q369P + N373S	HR	4	Cui et al. 2004, Oshima et al. 2006
	Q369H/P	ND	11	Cui et al. 2004
pos5	P319A	HR	2	Mosbach et al. 2017
	L412F	HR	1	Mosbach et al. 2017
mdl1	T66A	MR, HR	3	Mosbach et al. 2017
cyp51	P347S	LR	1	this study

[a]I365N/R/S and Q369P/H indicate CAPS analysis.

[b]LR = low resistance, MR = medium resistance, HR = high resistance, R = resistance. ND = not determined

Table 4 Methodologies and results for EC_{50} and frequency data from wine grape, table grape and non-grape reports for comparative purposes

Fungicide	reference	host	EC_{50} phenotyping method	sensitive EC_{50} range*, mean**, or value***, DC**** (µg mL^{-1})	resistant EC_{50} range*, mean**, value***, RF**** or DC***** (µg mL^{-1})	frequency screening method	total resistance frequency (%)
azoxystrobin	This study	wine grape	microtiter	0.04 - 0.37* (Table 1)	171** (Table 1)	agar - mycelial growth	5 (Fig. 2)
	Banno et al. 2009	non-grape	agar - mycelial growth	0.07 - 0.64**	19.3 – 21*	agar - mycelial growth	3
	Leroux et al. 2010	wine grape	agar - germ tube elongation	0.33**	>75****	agar - germ tube elongation	7
	Weber and Hahn 2011	non-grape	agar - germ tube elongation	0.04 - 0.12**	>100*	ND	ND
	Yin et al. 2015	non-grape	agar - mycelial growth	0.02 - 2.5**	6.1 – 500*	agar - mycelial growth	66
	Lu et al. 2016	non-grape	ND	ND	ND	agar - mycelial growth	31.6
boscalid	This study	wine grape	microtiter	0.03 - 0.1* (Table 1)	2.74 - 2.9** (Table 1)	agar - mycelial growth	2.8 (Fig. 2)
	Stammler and Speakman 2006	wine grape	microtiter	0.01 - 0.21*	ND	ND	ND
	Alberoni et al. 2011	wine grape	microtiter and agar - mycelial growth	0.03 - 0.87*	ND	ND	ND
	Leroch et al. 2011	wine grape	ND	ND	ND	microtiter	2, 8.3, 12.2, 26.7
	Campia et al. 2017	wine grape	microtiter	ND	ND	microtiter	1
	Toffolatti et al. 2020	wine grape	microtiter	<1.2***	>1.2***	microtiter	3
fenhexamid	This study	wine grape	microtiter	0.05 - 0.22* (Table 1)	25.46 - 27.33* (Table 1)	agar - mycelial growth	2.1 (Fig. 2)
	Leroux et al. 1999	wine grape	agar - germination, germ-tube elongation, and mycelial growth	0.008 - 0.06*	0.06 - ≥5*	ND	ND
	Albertini and Leroux 2004	wine grape	agar - mycelial growth	<0.4****	>0.4*****	ND	ND
	Fillinger et al. 2008	wine grape	agar - germination and mycelial growth	<0.1***	>5***	ND	ND
	Latorre and Torres 2012	table grape	agar - mycelial growth	<0.05 - 0.2*	0.2 - >2*	agar - mycelial growth	27.1
	Beresford et al. 2017	wine grape	agar - mycelial growth	0.63**	ND	agar - mycelial growth	0
	Toffolatti et al. 2020	wine grape	microtiter	<0.5***	0.55 -1.15*	microtiter	0.1
fludioxonil	This study	wine grape	microtiter	0.09 - 0.17 (Table S1)	0.93 (Table S1)	agar - mycelial growth	6.2 (Fig. 2)
	Weber and Hahn 2011	non-grape	agar - germ tube elongation	0.01 - 0.178*	0.30 - 0.65*	ND	ND
	Latorre and Torres 2012	table grape	agar - mycelial growth	<0.05 - 0.97*	1 - >5*	agar - mycelial growth	44.8
	Fernández-Ortuño et al. 2014	non-grape	agar - germination	0.02 - 0.05*	0.33 - 3.1*	ND	ND
	Toffolatti et al. 2020	wine grape	microtiter	<0.1***	0.2 – 8*	microtiter	5
iprodione	This study	wine grape	microtiter	0.75 - 2.05* (Table 1)	5.16 - >50* (Table 1)	agar - mycelial growth	11.6 (Fig. 2)
	Leroux et al. 1999	wine grape	agar - germination, germ-tube elongation, and mycelial growth	0.6 - 2.5*	3 - >25*	ND	ND
	Leroch et al. 2011	wine grape	ND	ND	ND	microtiter	1.1 - 6.1
pyrimethanil or cyprodinil	This study	wine grape	microtiter	0.09 - 0.22* (Table 1)	0.75 - 5.90* (Table 1)	agar - mycelial growth	7.7 (Fig. 2)
	Gulliano et al. 2000	wine grape	agar - mycelial growth	<0.1***	0.2 – 20*	ND	ND
	Latorre et al. 2002	table grape	agar - mycelial growth	<0.1 - 0.23*	5.66 - >10*	agar - mycelial growth	38.5
	Sergreeva et al. 2002	wine grape	agar - mycelial growth	ND	1.5 – 2*	agar - mycelial growth	0 - 12
	Latorre and Torres 2012	table grape	agar - mycelial growth	<0.05 - 0.94*	1.1 - >5*	agar - mycelial growth	62.7
	Beresford et al. 2017	wine grape	agar - mycelial growth	<1***	1 – >10*	agar - mycelial growth	28
	Campia et al. 2017	wine grape	microtiter	ND	ND	microtiter	3.4
	Avenot et al. 2018	wine grape	agar - mycelial growth	<1***	>1***	agar - mycelial growth	17
	Toffolatti et al. 2020	wine grape	microtiter	<3.2***	>3.2***	microtiter	4%
tebuconazole	This study	wine grape	microtiter	0.21 - 0.72* (Table 1)	1.06 - 1.8* (Table 1)	agar - mycelial growth	2.9 (Fig. 2)
	Chapeland et al. 1999	wine grape	agar - germ tube elongation	0.2 - 0.4*	1.5 - 2.0*	ND	ND
	Leroux et al. 1999	wine grape	agar - germ tube elongation	0.15 - 0.5*	1.0 - 2.0*	ND	ND

Figure legends

Figure 1 Distribution of the screened population (n = 725) across various wine Australian growing regions.

Figure 2 EC_{50} distribution of the 53 isolates phenotyped in the microtiter assay against azoxystrobin (a), boscalid (b), fenhexamid (c), iprodione (d), pyrimethanil (e), and tebuconazole (f). LR, MR, HR and R indicate isolates designated as exhibiting low resistance, medium resistance, high resistance, or resistance, respectively. *indicates significant difference between the means of the sensitive and resistant populations according to an independent samples t-test (P = 0.05) or Mann-Whitney U-test (P = 0.05).

Figure 3 Frequency (%) of resistant isolates in the population (n=725) on a per fungicide basis. Azo = azoxystrobin resistance, Bos = boscalid resistance, Fen = fenhexamid resistance, Pyr = pyrimethanil resistance, Ipr = iprodione resistance, teb = tebuconazole resistance. Percentage for each fungicide is shown above each column. These results do not consider resistance to more than one MOA.

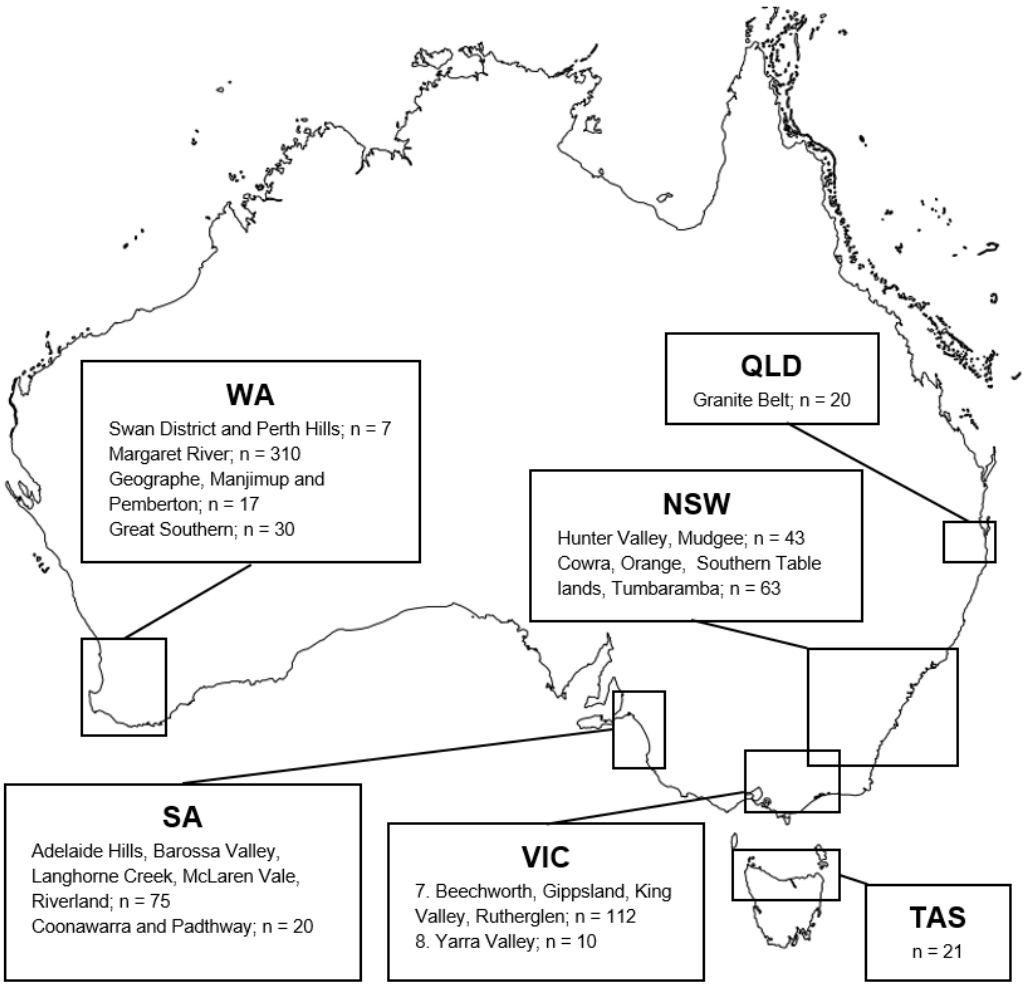
Figure 4 Number of resistant isolates in each state on a per fungicide basis. A = WA, B = SA, C = VIC, D = NSW. TAS and QLD were omitted due to small sample size (≤ 21). Azo = azoxystrobin, Bos = boscalid, Fen = fenhexamid, Ipr = iprodione, Pyr = pyrimethanil, Teb = tebuconazole.

Figure 5 Frequency (%) of vineyards with no resistance and with resistance from one to five modes of action (MOA). Percentage for each grouping is shown above each column.

Figure 6 Amino acid sequence alignment of fungicide target genes; *CytB* (azoxystrobin), *SdhB* (boscalid), *Erg27* (fenhexamid), *Bos1* (iprodione), *Pos5* (pyrimethanil), *Mdl1* (pyrimethanil), *Cyp51* (tebuconazole), for three sensitive comparative isolates and all resistant isolates characterised in the 53 isolate microtiter assay screen. For each target gene, isolates are ordered from most sensitive to the least sensitive in a descending order. LR, MR, HR and R indicate isolates designated as exhibiting low resistance, medium resistance, high resistance, or resistance, respectively. Black boxes represent non-synonymous changes found when compared to the reference sequence. The *CytB* G143 intron is underlined in Bc-385. *K354* indicates the position in *Cyp51B* in the archetype *Zymoseptoria tritici* as proposed by Mair et al. (2016a).

Figure 7 Expression analysis of *atrB* in the sensitive comparative strain Bc-385 and resistant isolates Bc-279 (flu^{MR} pyr^{MR}) and Bc-287 (flu^{S} pyr^{HR}), Bc-279 (flu^{MR} pyr^{MR}), Bc-128 (flu^{MR} pyr^{R}), and Bc-391 (flu^{MR} pyr^{R}). Values indicate expression levels relative to the comparative strain Bc-385 without fungicide treatment (Bc-385 untreated expression was normalised to 1). *indicates significant difference compared to Bc-385 and Bc-287 according to an independent samples t-test (P = 0.05).

Figure 8 Non-synonymous changes (vertical lines) found in *Mrr1* amino acid sequences of the sensitive isolate Bc-385 and MDR1 candidate isolates; Bc-128, Bc-130, Bc-279, Bc-287 and Bc-391. All non-synonomous changes (vertical lines), insertions (+) and deletions (Δ), are indicated as compared to the reference strain B05.10. All changes unique to each isolate compared to other isolates in this study are labelled.



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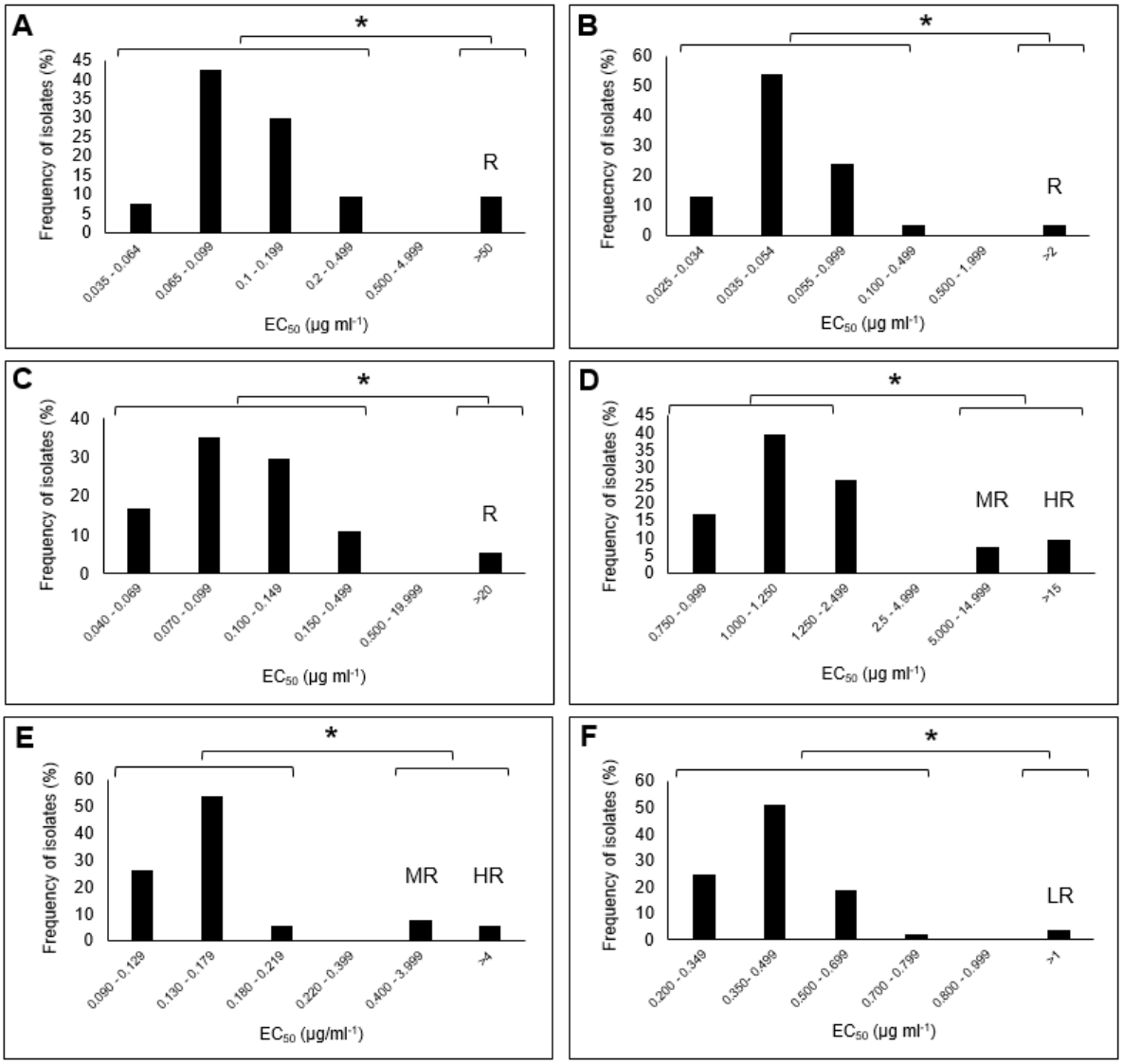

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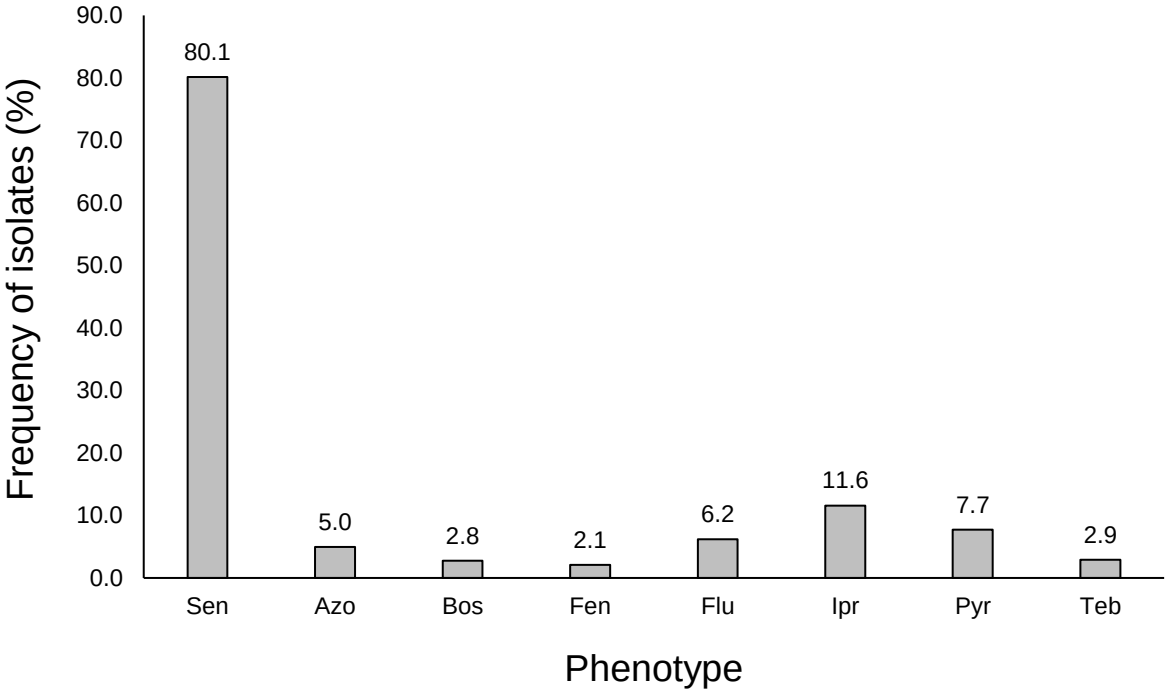

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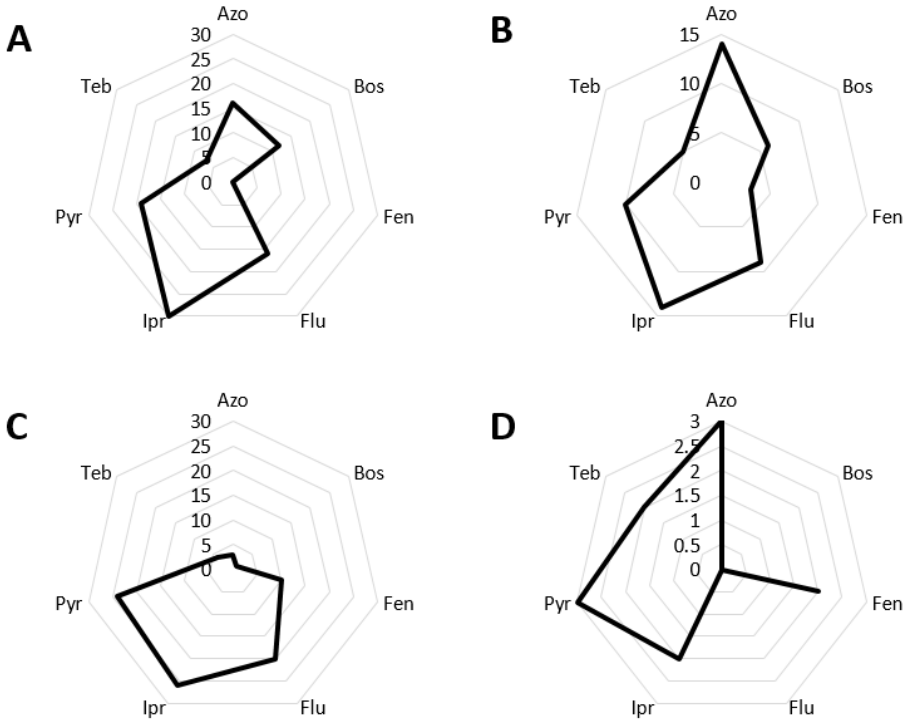

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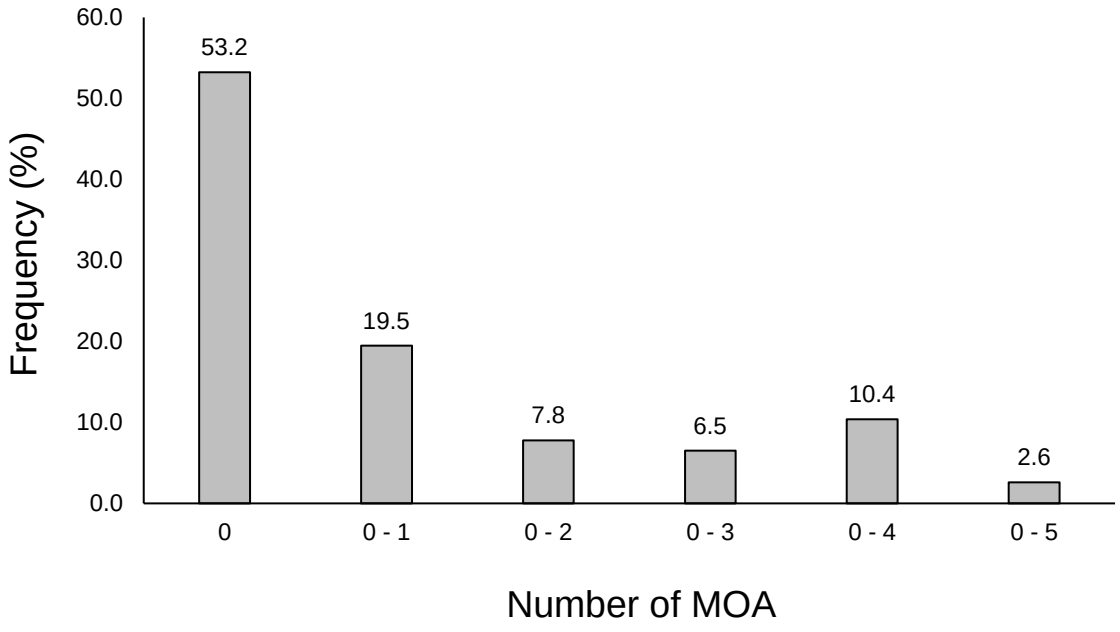

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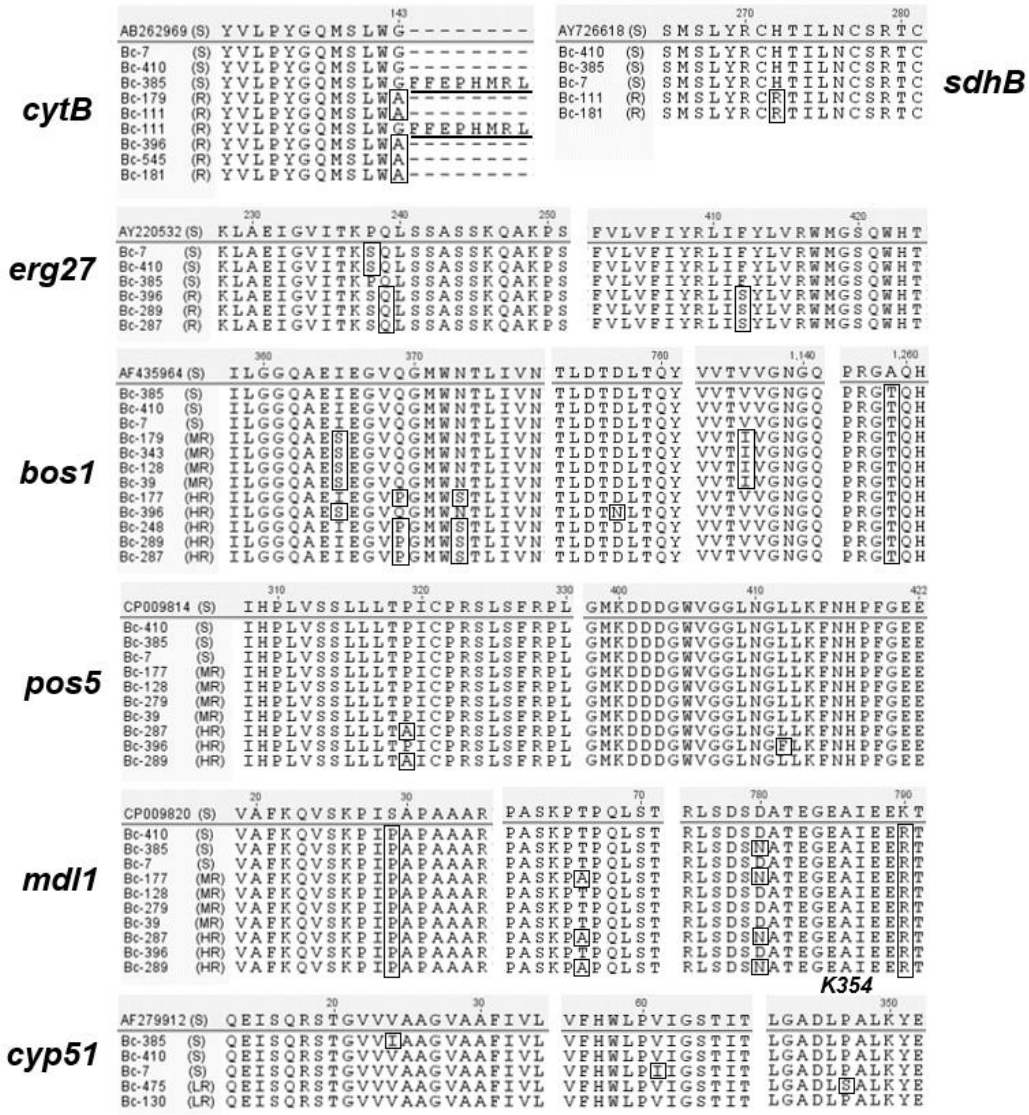

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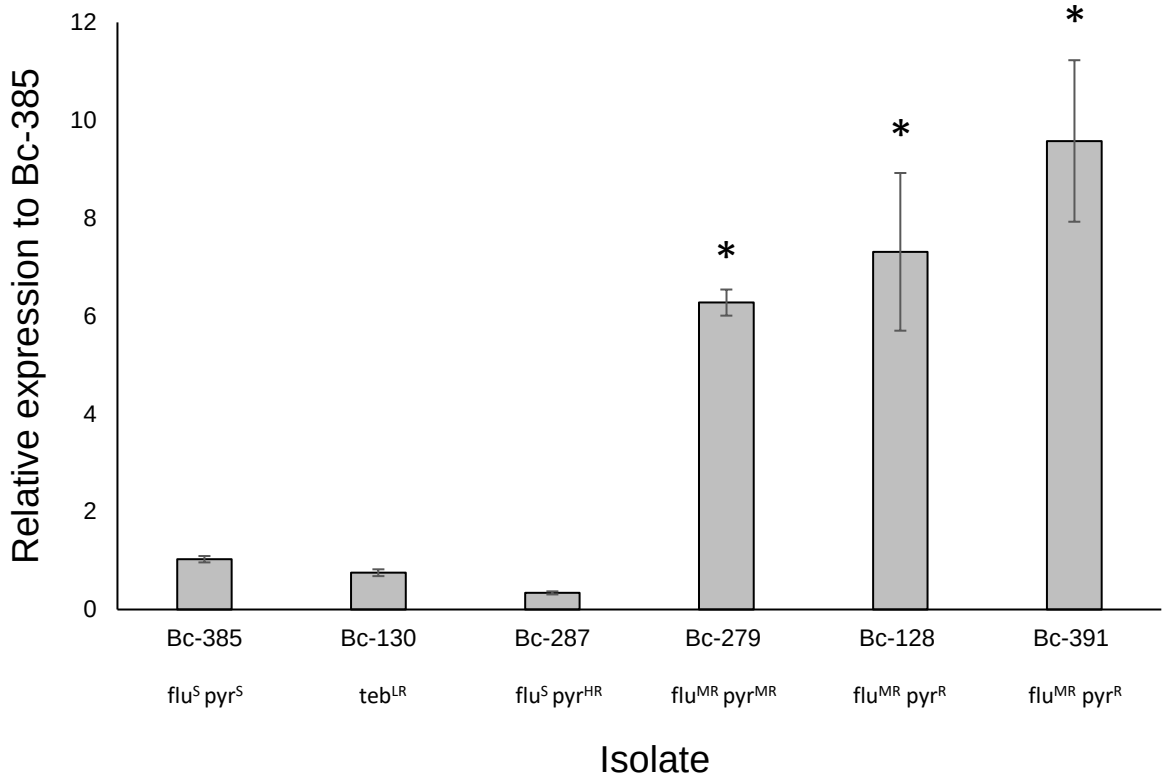

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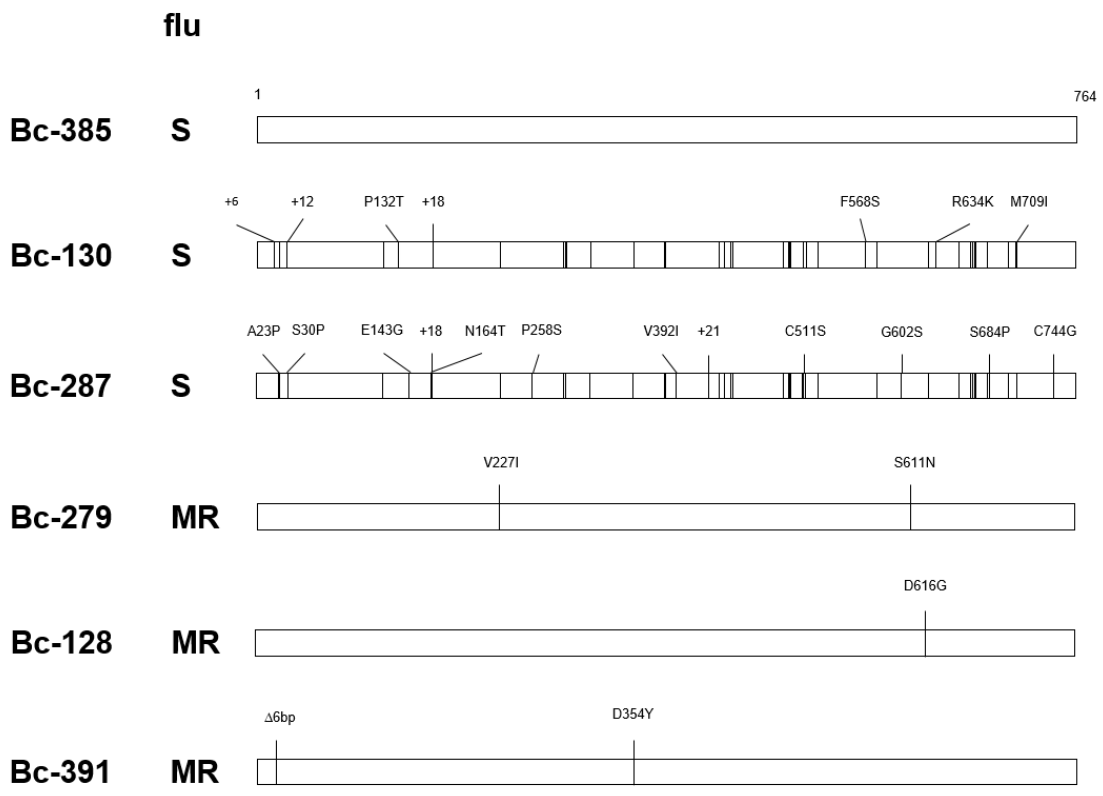

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