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Monitoring grapevine fungicide resistance in Australia

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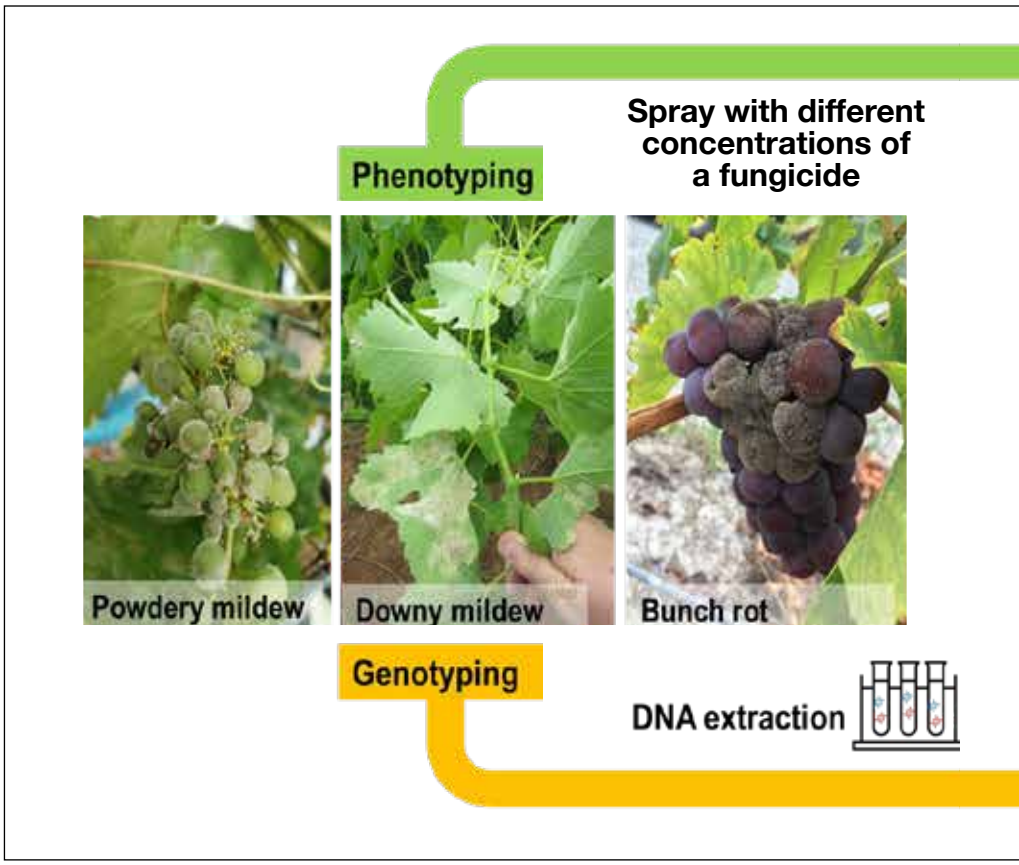
Powdery mildew, downy mildew and Botrytis bunch rot are the most significant diseases in grapevine. Frequent use of fungicides to manage these diseases can lead to the development of resistance, which decreases efficacy and results in field failure causing significant economic impacts. A collaborative project between the Cooperative Research Centre for Solving Antimicrobial Resistance in Agribusiness, Food, and Environments (SAAFE CRC) and Wine Australia is monitoring fungicide resistance. This article provides an update on the status of fungicide resistance in Australia using conventional phenotypic and molecular techniques to support industry to make informed decisions about spray programs and disease management.

INTRODUCTION

Powdery mildew (*Erysiphe necator*), downy mildew (*Plasmopara viticola*) and



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Botrytis bunch rot (*Botrytis cinerea*) are the three most important diseases on grapevines in Australia and worldwide. Growers rely on fungicides for disease management; consequently fungicide resistance is becoming an emerging issue, with reports of fungicide resistance in Australian vineyards (Harper, 2021, McKay *et al.*, 2021). Fungicide resistance adds to the cost of controlling these diseases due to reduced fungicide efficacy which adversely affects the quality and the quantity of the crop and wine production.

Fungicide resistance can be detected by phenotyping (testing sensitivity of isolates to fungicides using a plant bioassay). In addition, when the mechanism of resistance is known, genotyping, which is the molecular detection of mutations associated with fungicide resistance, can be used. There are three mutants associated with fungicide resistance for *E. necator*; G143A - Quinone outside Inhibitors (QoIs, gp 11), Y136F - Demethylation inhibitors (DMIs, gp 3) and H242x - Succinate dehydrogenase inhibitors (SDHIs, gp 7). For *P. viticola*, two mutants are associated with fungicide resistance; G143A - QoIs (gp 11) and G1105S - Carboxylic Acid Amides (CAAs, gp 40). For *B. cinerea*, mutations in *Pos5* (V273I/L, P293S, P319A, L412F/V/S) and *Mdl1* (E407K and G408R) are associated with resistance to Anilinopyrimidines (APs, gp 9) and *Erg27* (F412C/I/S/V) with resistance to Hydroxyanilides (gp 17).

Isolates of *E. necator*, *P. viticola* and *B. cinerea* have been collected from Australian wine regions between 2022 and 2024. The aim of this research was to determine phenotypic sensitivity to a range of fungicides using bioassay, and identify the mutations associated with resistance for several groups of fungicides using molecular detection.

METHODS

Sample collection

Samples were collected during the 2022/23 and 2023/24 seasons by growers and sent by mail to the laboratory. Powdery and downy mildew obligate pathogens were isolated in the laboratory using detached leaves and spores were bulked up for testing (Figure 1). A total of 24 powdery mildew samples were received from regions in NSW and SA and 21 downy mildew samples received from regions in SA, Tas and Vic. The Botrytis bunch rot pathogen was isolated by transferring spores from swabs to media, followed by isolation and bulking. A total of 184 single spore isolates across 23 vineyards were collected from WA, SA, NSW, ACT and Vic.

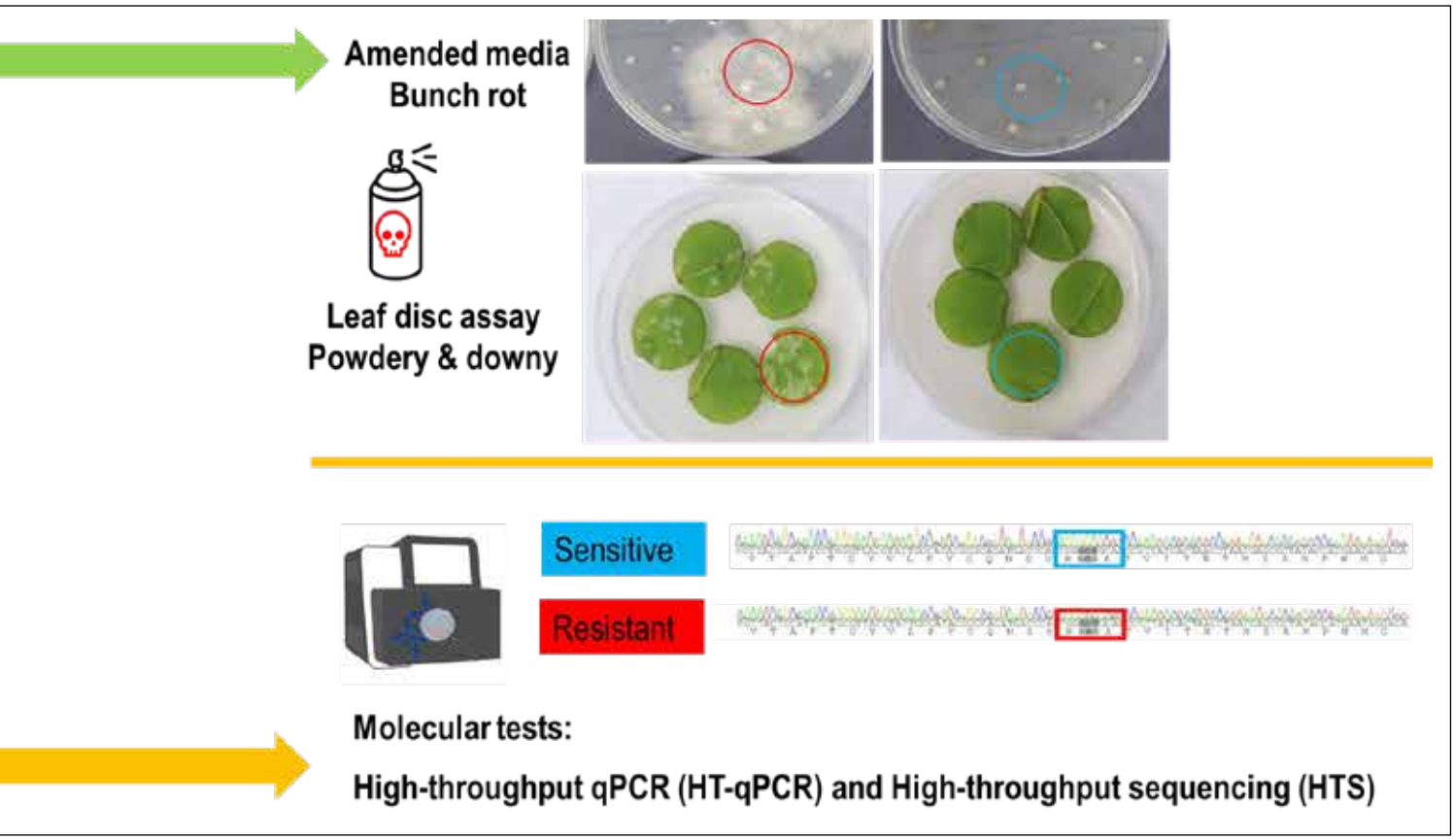

Figure 1. Workflow chart for assessing fungicide resistance: (A) infected plant material collected from the field, (B) phenotyping test for *B. cinerea* using fungicide amended media, (C) phenotyping test for *P. viticola* or *E. necator* using a leaf disc bioassay sprayed with fungicide and (D) genotyping assay using high-throughput qPCR and sequencing.

Table 1. List of fungicide groups and active ingredients used for phenotyping, and genes and mutations used in genotyping to identify resistance.

Disease	Group	Active ingredient (phenotyping)	Gene/mutation (genotyping)
Powdery mildew	Demethylation inhibitor (DMI, group 3)	penconazole difenoconazole	Cyp51/Y136F
	Amine (group 5)	spiroxamine	Unknown
	Succinate dehydrogenase inhibitor (SDHI, group 7)	boscalid pydiflumetofen	SdhB/H242X
	Quinone outside inhibitor (QoI, group 11)	pyraclostrobin azoxystrobin	Cytb/G143A
	Aza-naphthalene (group 13)	proquinazid quinoxyfen	Unknown
Downy mildew	Phenylamide (PA, group 4)	metalaxyl	Unknown
	Quinone outside inhibitor (QoI, group 11)	pyraclostrobin	Cytb/G143A
	Carboxylic acid amide (CCA, group 40)	mandipropamid dimethomorph	cesA3/G1105S
Botrytis bunch rot	Anilinopyrimidines (APs, group 9)	pyrimethanil	Pos5/numerous, Mdl1/E407K/G466R
	Phenylpyrroles (PPs, group 12)	fludioxonil	Not tested
	KetoReductase Inhibitors (KRIs, group 17)	fenhexamid	Erg27/F412C/I/S/V

Phenotyping

Powdery and downy mildew

A leaf-disc bioassay, modified from (Erickson & Wilcox, 1997) was used to test fungicide sensitivity. Young leaves from the 3rd or 4th node were collected from disease free grapevines grown in a controlled environment room and surface disinfested with bleach.

Fungicide suspensions were made using the commercial fungicide products outlined in Table 1, at 0, 1, 10 µg/mL and field rate of active ingredient. Each leaf was sprayed on the adaxial and abaxial surfaces, using a hand spray until run-off. Leaves were dried; 10 mm diameter discs were excised using a cork borer and placed on top of water agar in Petri dishes, with five discs per plate and two or three replicate plates per fungicide concentration/isolate combination.

Inoculation with *E. necator* spores was conducted by tapping an infected leaf over the discs. For *P. viticola* spore suspensions were made in water and sprayed onto leaf discs using an atomiser.

Petri dishes were covered with lids and kept in plastic trays on a laboratory bench for 10-14 days after inoculation. The percentage of each disc covered in sporulating powdery or downy mildew was assessed under a dissecting microscope at x100 magnification. Sensitivity was measured based on growth, an isolate was considered to have reduced sensitivity when it grew more than 5% on 1 or 10 µg/mL of active ingredient, and resistant if it grew more than 5% at field rate.

Botrytis bunch rot

The phenotypic analysis of samples involved placing each isolate on yeast soluble starch agar (YSSA) or YSSA minus yeast extract for testing against groups 12/17 or group 9 fungicides, respectively (Fillinger *et al.*, 2008). Two 4 mm plugs were taken from the edge of actively growing colonies and placed on YSSA plates amended with 0.4 µg mL-1 pyrimethanil (group 9), 0.1 µg mL-1 fludioxonil (group 12) or 1 µg mL-1 fenhexamid (group 17). Plates were incubated for 3 days in the dark, and then scored on their ability to grow on each fungicide concentration.

Genotyping

DNA was extracted from samples of *E. necator*, *P. viticola* and *B. cinerea* using the SARDI proprietary DNA extraction method (Ophel-Keller *et al.*, 2008).

For *E. necator* and *B. cinerea*, assays for the specific detection of QoI (gp 11) and AP (gp 9) respectively, resistance have been developed and implemented to suit a molecular diagnostic high throughput qPCR (HT-qPCR) platform (Sosnowski *et al.* 2023).

DNA from each *E. necator* sample was also subjected to high throughput sequencing (HTS) analysis for resistance allele testing regions of the *Cyp51* (gp 3), *SdhB* (gp 7) and *CytB* (gp 11) genes that potentially contained resistance mutations (Sosnowski *et al.* 2023). For *P. viticola*, DNA was amplified using specific primer pairs surrounding the known resistance mutation hotspots for either *CytB* (gp 11) or *Ces3A* (gp 40).

For *B. cinerea*, DNA was extracted from sporulated biomass using quick DNA extraction method (Dodhia *et al.*, 2021). Genotyping techniques included, qPCR, sanger sequencing and cleaved amplified polymorphic sequence (CAPS) testing (Sosnowski *et al.* 2023).

RESULTS AND DISCUSSION

Powdery mildew

Of the 24 samples collected and phenotyped, resistance was recorded for five of the nine fungicides tested (from 11-67%; Figure 2). Reduced sensitivity was also identified for a further four fungicides (from 16-69%). Multi fungicide resistance was identified in a number of samples, with resistance reported to four fungicides with one isolate (isolate 327, Table 2).

Phenotyping results show that aza-naphthalene fungicides (gp 13) have the highest resistance percentages followed by QoIs (gp 11). It was noted that some of the samples came from vineyards where the same fungicides were sprayed more times than recommended by Croplife

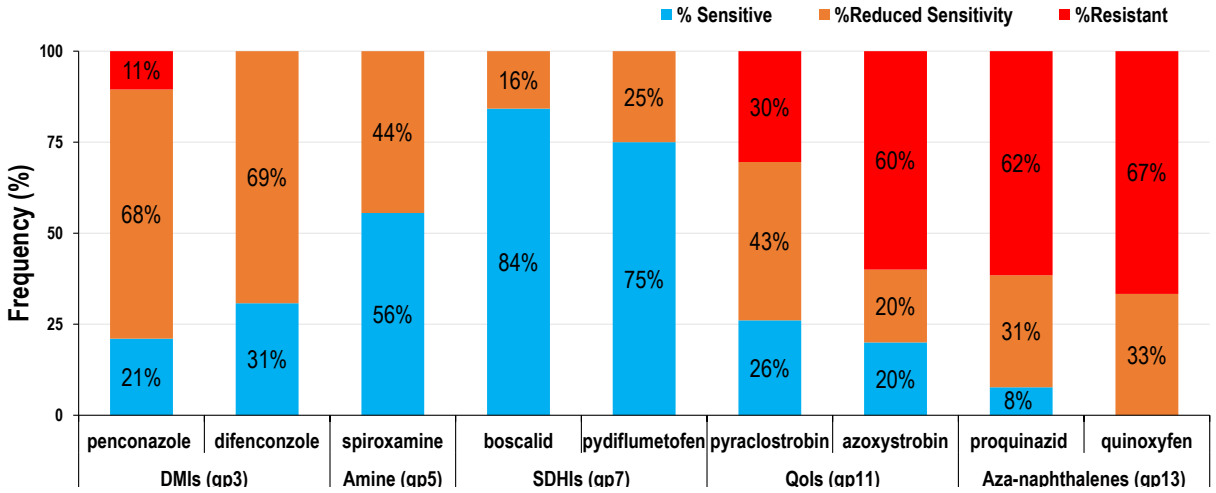

Figure 2. Sensitivity of powdery mildew (*E. necator*) samples to fungicides.

Table 2. Detailed sensitivity of each powdery mildew (*E. necator*) sample received to different fungicide groups and active constituents. Sensitive (S), reduced sensitivity (RS), resistant (R) and not tested (NT)

State	Region	Sample code	Demethylation inhibitors (gp3)		Quinone outside inhibitors (gp11)		Succinate dehydrogenase inhibitors (gp7)		Amine (gp 5)	Aza-naphthalenes (gp13)	
			difenconzole	penconazole	pyraclostrobin	azoxystrobin	boscalid	pydiflumetofen	spiroxamine	proquinazid	quinoxyfen
NSW	Riverina	312	NT	NT	S	NT	NT	NT	NT	NT	NT
		314	S	S	RS	NT	RS	NT	RS	R	NT
		323	RS	RS	RS	R	S	RS	RS	RS	R
SA	Adelaide Hills	318	NT	R	RS	R	S	S	NT	R	NT
		332	NT	RS	RS	R	S	S	S	R	NT
		333	RS	NT	R	NT	NT	NT	NT	NT	R
		334	NT	RS	R	R	S	S	NT	NT	NT
		309	NT	NT	RS	NT	NT	NT	NT	NT	NT
		310	NT	NT	R	NT	NT	NT	NT	NT	NT
	Barossa Valley	301	NT	RS	NT	NT	S	NT	NT	NT	NT
		317	NT	S	RS	NT	S	NT	S	NT	NT
	Limestone Coast	315	S	RS	R	R	S	NT	S	RS	NT
		316	NT	S	S	NT	S	NT	S	NT	NT
		326	RS	NT	NT	RS	S	RS	S	RS	NT
		327	RS	RS	R	R	S	S	S	R	R
	Langhorne Creek	324	RS	RS	R	RS	S	S	RS	R	RS
		325	RS	RS	R	R	NT	S	RS	R	NT
		328	RS	RS	S	S	S	RS	RS	RS	NT
		329	NT	NT	RS	R	S	NT	NT	NT	NT
	McLaren Vale	319	RS	RS	S	S	RS	S	RS	R	NT
		320	S	RS	S	S	RS	S	RS	R	R
		321	RS	RS	RS	RS	NT	S	S	S	RS
	Riverland	303	NT	RS	RS	NT	S	NT	RS	NT	NT
		305	NT	S	S	NT	S	NT	S	NT	NT

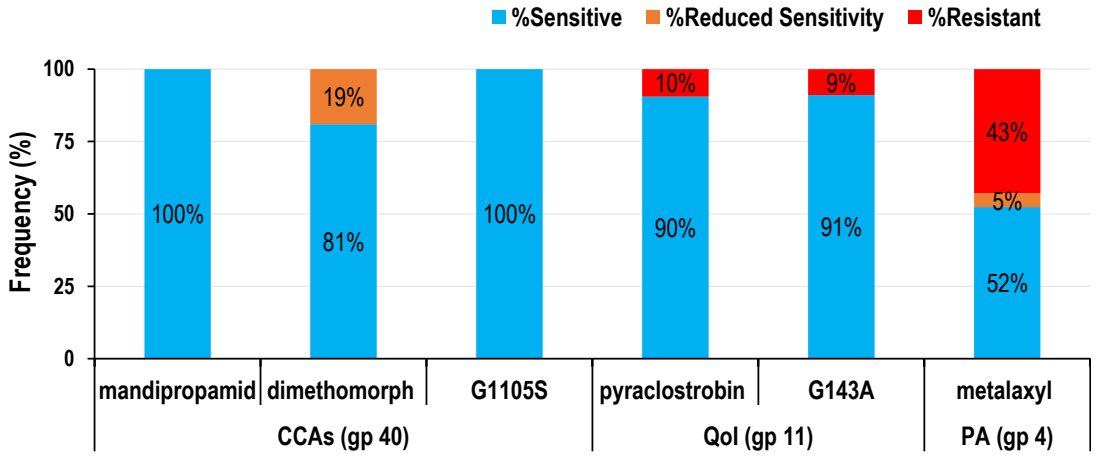

Figure 3. Sensitivity of downy mildew (*P. viticola*) samples to fungicides, and presence of genetic mutations G1105S and G143A associated with CCA and QoI resistance, respectively.

Table 3. Detailed sensitivity of each downy mildew (*P. viticola*) sample received to different fungicide groups and active constituents. Sensitive (S), Reduced sensitivity (RS) and Resistant (R)

State	Region	Sample code	Phenylamide (gp4)	Quinone outside inhibitor (gp11)	Carboxylic acid amides (gp40)	
			metalaxyl	pyraclostrobin	dimethomorph	mandipropamid
SA	Barossa Valley	SA18	S	S	S	S
		SA19	S	R	S	S
		SA21	S	S	S	S
		SA22	S	S	S	S
		SA31	S	S	S	S
		SA32	S	S	S	S
	Langhorne Creek	SA15	S	S	S	S
		SA17	R	S	RS	S
		SA27	R	S	S	S
		SA28	S	S	S	S
		SA33	S	S	S	S
		SA36	R	R	RS	S
		SA40	R	S	S	S
		SA41	R	S	S	S
		SA42	RS	S	S	S
		SA43	R	S	S	S
	McLaren Vale	SA23	S	S	S	S
		SA24	S	S	S	S
	Adelaide Hills	SA49	R	S	S	S
Tas	Pipers River	Tas5	R	S	RS	S
Vic	Yarra Valley	Vic24	R	S	RS	S

and outlined in the AWRI Agrochemical Booklet in the same season. Resistance has been reported for aza-naphthalene (gp 13) by the Fungicide Resistance Action Committee (FRAC) and it is generally understood that cross-resistance exists between the group 13 fungicides (FRAC, 2020, Vojinovic *et al*., 2023). QoI resistance and cross resistance between group members were recorded in several places around the world (Zito *et al*., 2024). Cross resistance has been detected for both aza-napthalenes and QoIs in Australian samples (Table 2). DMI (gp 3) resistance was also recorded in 11% of the samples, however no cross resistance was identified between DMI group members.

Genotyping revealed a high percentage of G143A (59%) was detected and was closely associated with phenotypical resistance to Qols (gp 11). It is well established that the presence of G143A indicates the presence of resistance to QoI fungicides (Ghule *et al*., 2018, Zito *et al*., 2024). High throughput sequencing showed that all powdery mildew samples had the Y136F mutation but only 11% of them showed phenotypical DMIs (gp 3) resistance suggesting that there are other resistance mechanisms involved. Europe wide sensitivity studies indicated that DMIs are only slightly and differently affected by the mutation Y136F, being frequently detected in the cyp51 gene (Kunova *et al*., 2021, Zito *et al*., 2024). None of the samples collected in Australia contained the H242X mutant, which is linked with SDHI fungicide resistance, consistent with the lack of phenotypic resistance detected.

Downy mildew

Resistance to metalaxyl was detected in 43% of the 21 samples received (Figure 3, Table 3), with six of 10 isolates from Langhorne Creek showing resistance. Results showed that 10% of samples were resistant to pyraclostrobin and 19% had reduced sensitivity to dimethomorph. All samples were sensitive to mandipropamid.

The majority of *P. viticola* samples were sensitive to all tested fungicides, however those from high disease pressure regions in SA and Vic, had a high percentage of resistance to metalaxyl. Downy mildew disease was more prevalent than usual in the 2022/23 and 2023/24 seasons due to the above average rainfalls. High humidity conditions are conducive to downy mildew disease development, which leads to increased fungicide applications, and selection of resistant populations. Metalaxyl resistance has been recorded in several regions in Australia (Ismail *et al*., 2024, Wicks *et al*., 2005) and around the world (Fourie, 2004, Sun *et al*., 2010).

The G143A mutant, linked to resistance to QoI (gp 11) fungicides, was detected in most samples that showed phenotypic resistance to pyraclostrobin, and all phenotypically sensitive samples had no mutant (Figure 3). This result concurs with previous reports, and so provides a benchmark for QoI resistance (Salcedo et al., 2021). The G1105S mutant, linked to CAA (gp 40) fungicide resistance was not detected in any samples.

Botrytis bunch rot

Resistance frequencies for isolates sampled across the 2022/23 and 2023/2024 seasons were 21, 13 and 11% for group 9, 12 and 17, respectively. Overall resistance frequencies in ten different regions are shown in Table 4. Mutations in *Pos5* (V273I, G408V and L412F/V) or *Mdl1* (E407K) and *Erg27* (F412C/I/S/V) were associated with resistance to group 9 and 17 fungicides, respectively (Table 5).

Overall resistance frequencies on a per mode of action basis were higher in the combined 2022/23 and 2023/2024 data than previously reported (Sosnowski *et al.*, 2023). This is primarily due to high resistance frequencies found at some of the highly sampled regions e.g. Pemberton and Tumbarumba. As for other wine regions, the resistance frequency ranged from 0 to 86%. However, several regions with high frequencies only had low sample numbers. In general, the overall resistance frequency is still low, but the results illustrate the need for further

Table 4. Resistance frequency for pyrimethanil (gp 9), fludioxinil (gp 12) and fenhexamid (gp 17) for Botrytis bunch rot (*B. cinerea*) isolates sampled from different states.

State	Region	Vineyards sampled	Isolates tested	Sensitive	Resistant								Frequency (%)
					9	12	17	9 + 12	9 + 17	12 + 17	9 + 12 + 17	Total	
WA	Margaret River	1	17	15	-	1	-	-	1	-	-	2	13
	Pemberton	1	11	0	4	2	-	1	1	1	2	11	100
SA	Coonawarra	1	5	5	-	-	-	-	-	-	-	0	0
	Currency Creek	1	22	22	-	-	-	-	-	-	-	0	0
	Langhorne Creek	1	14	14	-	-	-	-	-	-	-	0	0
Vic	Alpine Valleys	1	7	1	3	-	-	-	1	1	1	6	86
	King Valley	6	54	44	2	5	-	-	3	-	-	10	21
	Gippsland	1	3	1	-	-	-	1	-	-	1	2	67
	Rutherglen	2	12	8	-	3	-	-	1	-	-	4	33
NSW	Orange	1	3	1	2	-	-	-	-	-	-	2	67
	Tumbarumba	4	23	12	1	3	-	-	6	-	1	11	48
ACT	Canberra District	3	13	9	4	-	-	-	-	-	-	4	31
	Total (%)	**23**	**184**	**132**	**16**	**14**	**0**	**2**	**13**	**2**	**5**	**52**	

Table 5. Number of resistant samples characterised with a particular Botrytis bunch rot (*B. cinerea*) genotype across wine regions in different states.

State	Region	Group 9						Group 17	
		Isolates tested	Pos5				Mdl1	Isolates tested	Erg27
			V273I	G408V	L412F	L412V	E407K		F412C/I/S/V
WA	Margaret River	1	-	-	1	-	-	1	1
	Pemberton	8	-	-	4	2	2	4	4
Vic	Alpine Valleys	5	2	-	3	-	-	3	3
	King Valley	5	-	-	2	-	3	3	3
	Gippsland	2	2	-	-	-	1	1	
	Rutherglen	1	-	-	1	-	-	1	1
NSW	Orange	2	-	-	1	-	1	-	-
	Tumbarumba	8	1	1	6	-	-	7	7
ACT	Canberra District	5	-	-	5	-	-	-	-
	Total (%)	**35**	**5 (14)**	**1 (3)**	**23 (62)**	**2 (5)**	**6 (16)**	**20**	**20 (100)**

monitoring, especially in wine regions where some vineyards are showing very high frequencies. Genotyping results showed the commonly found mutations associated with group 9 (L412F/E407K) and 17 (F412C/I/S/V) resistances. No new changes were found in the target genes.

Conclusion

Reduced sensitivity and resistance have been detected in powdery mildew, downy mildew and Botrytis bunch rot for some of the major groups of fungicides used to control grapevine diseases. Therefore, resistance management strategies are required to minimise and delay resistance, and protect available chemistry. These strategies include regular monitoring, rotating chemistries, applying fungicides only when necessary, using multi-site fungicides and other alternatives such as inorganic fungicides (sulfur and copper) and biologicals (*Aureobasidium pullulans*). In the case where resistance is detected, withdrawal of the fungicide from the spray program is recommended.

The G143A mutation is an ideal candidate for high throughput and in-field quantitative PCR detection and high throughput sequencing for regular monitoring of both powdery and downy mildew fungicide resistance. It is a reliable, efficient and cost-effective method for regularly monitoring fungicide resistance and has been adapted for use with spore trapping (Ismail et al., 2022). Research is continuing to develop more molecular tests for other fungicide groups.

Current research is striving to increase sample numbers across a wider range of wine regions in order to improve our understanding of fungicide resistance in Australian viticulture. Growers are encouraged to participate by submitting samples to contribute to the national database, and in turn receive results for their own vineyards at no cost. All individual details remain confidential with results reported at regional level. For sampling details please contact: ismail.ismail@sa.gov.au.

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