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Research Article

Received: 8 November 2023 Revised: 4 March 2024 Accepted article published: 18 March 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.8089

Sensitivity of *Plasmopara viticola* to selected fungicide groups and the occurrence of the G143A mutant in Australian grapevine isolates

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Abstract

BACKGROUND: Grapevine downy mildew, caused by *Plasmopara viticola*, is an economically important disease in Australia and worldwide. The application of fungicides is the main tool to control this disease. Frequent fungicide applications can lead to the selection of resistant *P. viticola* populations, which has negative impacts on the management of the disease. Identification of resistance and its prevalence is necessary to inform resistance management strategies.

RESULTS: A total of 86 *P. viticola* isolates were collected between 2017 and 2022 from vineyards in 15 growing regions across Australia for four fungicide groups; phenylamide (PA, group 4), carboxylic acid amide (CAA, group 40), quinone outside inhibitor (QoI, group 11) and quinone outside inhibitor stigmatellin binding type (QoSI, group 45). Decreased phenotypic sensitivity was detected for all four groups, and resistance to metalaxyl-M (PA) and pyraclostrobin (QoI), was detected. Genetic analysis to detect the G143A (QoI) and G1105S (CAA) mutations using amplicon-based sequencing was performed for 239 and 65 isolates collected in 2014–2017 and 2017–2022, respectively. G143A was detected in 8% and 52% of isolates, respectively, with strong association to phenotypic resistance. However, G1105S was not detected in any isolates.

CONCLUSION: *Plasmopara viticola* isolates in Australia with resistance to at least two fungicide groups have been detected, therefore it is necessary to adopt resistance management strategies where resistance has been detected. Vineyards should continue to be monitored to improve management strategies for downy mildew.
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Keywords: downy mildew; metalaxyl; pyraclostrobin; fungicide resistance

1 INTRODUCTION

Plasmopara viticola (Berk. et Curt.) Berl. and De Toni, an obligate biotroph causing grapevine downy mildew disease, originates in North America, but was introduced several times into Europe in the late 1870s. After the pathogen introduction, *P. viticola* spread rapidly through most of continental Europe during the 1900s, becoming one of the most economically important grapevine diseases around the world.[1,2] The first Australian report of *P. viticola* causing downy mildew was in several commercial vineyards in Rutherglen, Victoria (VIC) in 1917.[3] The disease is now widespread across Australian viticultural regions, and is considered economically important in regions that receive frequent summer rain with high humidity and night time temperatures. Epidemics across wider growing areas, occur sporadically when weather conditions are favourable.[4] Genetic analysis shows that Australian and South American populations of *P. viticola* are more closely related to the European population than the North American population,[5] highlighting the link with the movement of grapevine material from Europe.

The most recent comprehensive estimate of the cost of management of, and yield loss to, downy mildew in Australia is A$63 million per year, calculated in 2009.[6] Chemical control is the most effective measure used to protect grapes from downy mildew. However, *P. viticola* is an oomycete pathogen characterized by high mutation rates, sexual recombination and high asexual sporulation efficiency; a set of factors that likely contribute to the propensity of *P. viticola* to rapidly adapt to single-site fungicides.[7] According to the Fungicide Resistance Action Committee (FRAC), *P. viticola* is considered a plant pathogen with a high risk of developing fungicide resistance (frac-pathogen-list-2019, https://www.frac.info). Resistance to single-site fungicides is one of the main issues in downy mildew management and resistance to several fungicide classes has been reported.[8–10]

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Phenylamides (PAs, group 4) are one of the oldest groups of single site chemistries registered to provide specific control of oomycete pathogens, with several compounds being introduced between 1977 and 2007.[11] Having long-lasting preventative activity, systemic mobility, curative potential and excellent crop safety, PAs are considered a valuable tool for disease management across several crops.[11–13] Within this group, metalaxyl and its active isomer metalaxyl-M (mefenoxam) have been available for use in grapevines around the world including in Europe, the United States and Australia since the 1970s.[12,14] Metalaxyl-M inhibits the ribosomal RNA polymerase of the target pathogen, and is categorized by the FRAC Code List 2022 (https://www.frac.info) as being at high risk of developing resistance. In France, metalaxyl-resistant *P. viticola* isolates were detected as early as 1981[15] and PA resistance was observed in 1983,[16,17] according to the FRAC list of first confirmed cases of plant pathogenic organisms resistant to disease control agents (https://www.frac.info) and Colcol and Baudoin.[18] In 2004, 92% of 813 vineyard samples tested in South Africa were reported to be resistant to metalaxyl.[19] The first report of resistance in Australia was from the Hunter Valley, New South Wales (NSW) in 2005 where two of the three vineyard samples tested were resistant to metalaxyl.[14] Resistant isolates of *P. viticola* have since been detected in vineyards across several Australian states between 2014 and 2019.[20] In 2020, 37.5% of 160 isolates collected from vineyards in India were reported to be resistant.[21] Despite multiple investigations into the mechanism of resistance to PAs, the resistance gene and mutation remains unknown.[12]

Carboxylic acid amide (CAA, group 40) fungicides include dimethomorph, iprovalicarb, benthiavalicarb, valifenalate and mandipropamid. These fungicides are widely used to control downy mildew due to their ability to bind to the wax layer of the leaf surface, and their highly effective protective, curative and eradicative properties.[22,23] Dimethomorph was the first CAA fungicide developed to control downy mildew[24] and was first registered for use against grapevine downy mildew in Australia in 1997 (https://apvma.gov.au/sites/default/files/publication/13666-prs-dimethomorph.pdf). Mandipropamid is a mandelic acid amide fungicide, introduced in 2011 (https://apvma.gov.au/node/13846), which displays high activity against foliar-infecting oomycetes, including *P. viticola*. All members of the CAA class are considered cross-resistant and are classified by FRAC (https://www.frac.info) as medium-risk fungicides for the development of resistance.[25] *Plasmopara viticola* strains resistant to CAAs have been detected in Japan, India and mainland Europe, including Italy and France.[25] Single amino acid exchanges in the CesA3 protein conferring mandipropamid resistance in *P. viticola* have been identified. The G1105S mutation,[26] which leads to an amino acid change from glycine to serine at codon 1105, and the G1105V mutation,[27] which leads to an amino acid change from glycine to valine at the same codon, were detected in *P. viticola* in 2010 and 2011, respectively.

Quinone outside inhibitor (QoI, group 11) fungicides are effective against oomycetous pathogens.[28,29] They inhibit mitochondrial respiration and interfere with energy-demanding aspects of the pathogen lifecycle, such as spore germination.[28] Two mutations are known to occur in plant pathogens that develop resistance to this group, which result in either G143A (glycine to alanine) or F129L (phenylalanine to leucine) amino acid substitutions within the mature protein.[11,28] The F129L mutation occurs much less frequently than the G143A mutation.[11] Resistance development to the QoI group can be rapid in *P. viticola* populations. QoI fungicides were introduced into France for pest management in viticulture in 1998, and 2 years later, resistance was confirmed via detection of the G143A mutation.[28,30] In Italy, resistance to QoI was detected in 1999 using molecular and biological assays and a high percentage (90%) of QoI resistance was associated with multiple applications of QoI as a single active ingredient compared with lower percentage (30%) when QoI was mixed with active ingredients belonging to different resistance groups.[30,31] In Japan, resistance was also detected in 2012 in an area where QoI fungicides had been introduced to control downy mildew compared with no resistance detected in another region where QoI not yet been introduced.[32] The QoI fungicides azoxystrobin and pyraclostrobin have been used widely in Australian vineyards since introduced in 2003 (https://apvma.gov.au/node/13951). As such, selection for resistance is likely, particularly in regions where conditions are frequently conducive to downy mildew.

Ametoctradin is the only QoI stigmatellin binding type (QoSI, group 45) fungicide.[33] Resistance risk is assumed to be medium to high according to the FRAC Code List 2022 (https://www.frac.info). Resistance to ametoctradin was first detected in France in 2015 and is likely caused by a single point mutation in the cytochrome b gene, leading to the S34L amino acid substitution.[34]

While sporadic testing has been undertaken in the past, a comprehensive assessment of resistance to fungicides used to manage downy mildew in Australian viticulture is lacking. Given the variability of climates among production areas in Australia, and the associated differences in disease pressure and spray regimes, variability in the *P. viticola* population is expected. The aim of this study was to determine the sensitivity of, and presence of associated genetic mutations in, Australian isolates of *P. viticola* collected across multiple growing seasons, in the major wine grape production areas for the four fungicide groups (PA, CAA, QoI and QoSI) used to control downy mildew.

2 MATERIALS AND METHODS

2.1 Samples

For this multi-year study of fungicide resistance of *P. viticola* in Australia, two periods of sampling were collected from 22 regions in six states: NSW, Queensland (QLD), South Australia (SA), Tasmania (TAS), VIC and Western Australia (WA). From 2017 to 2022, 86 samples of downy mildew-affected grapevine leaves of various *Vitis vinifera* L cultivars were collected at different times of the season from commercial vineyards where fungicides had shown issues in controlling downy mildew (Tables 1 and 2). *Plasmopara viticola* was immediately isolated using the 'bag test' for downy mildew of grapes, described by Western Australia Department of Primary Industries and Regional Development (https://www.agric.wa.gov.au). *Plasmopara viticola* sporangia were bulked up as per Fontaine *et al.*[34] Sporangia were harvested by washing the infected leaves in sterilized reverse osmosis (SRO) water, adjusted to 1×10^6 sporangia mL^{-1} for use as inoculum and small volume was placed in 1.5-mL Eppendorf tubes and stored at −20 °C for genetic analysis.[34–36] DNA was extracted using the Mag-Bind Environmental DNA 96 Kit (Omega Bio-tek, Norcross, GA, USA).

From 2014 to 2017, 271 samples were randomly collected from infected leaves of various *V. vinifera* cultivars regardless of their resistance status. Infected sections of leaves were removed by cutting and storing at −20 °C until required.[5] DNA was extracted using the Powerplant Pro DNA isolation kit (Mo Bio Laboratories,

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Table 1. Summary of *Plasmopara viticola* isolates tested using bioassay (B) and amplicon-based sequencing (ABS) from 2014 to 2022[†‡]

State	Region	Tested (ABS only) 2014–2017	Tested (B/ABS) 2017–2022
New South Wales (NSW)	Barooga	—	4/5
	Griffith	—	6/13
	Hunter Valley	40	15/15
	Sydney	3	—
Queensland (QLD)	Granite Belt	20	—
	Mareeba	5	4/4
	Mutchilba	4	—
South Australia (SA)	Adelaide Hills	15	5/8
	Barossa Valley	4	—
	Clare Valley	—	1/0
	Langhorne Creek	6	—
	McLaren Vale	19	1/0
	Riverland	15	—
	Southern Fleurieu	4	—
Victoria (VIC)	King Valley	1	10/4
	Murray Darling	6	—
	Mornington Peninsula	2	—
	Yarra Valley	21	19/13
Western Australia (WA)	Broome	1	—
	Carnarvon	1	—
	Geographe	5	—
	Great Southern	1	—
	Kalumburu	2	—
	Margaret River	13	—
	Pemberton	1	—
	Swan Valley	73	1/3
	Walkaway	1	—
Tasmania (TAS)	Tamar Valley	8	—
Total		271	66/65[§]

[†] Year of harvest, as grape seasons cross the calendar year in the Southern Hemisphere.
[‡] —, No samples were collected.
[§] Only 65 samples were analysed using ABS.

Carlsbad, CA, USA) according to the manufacturer's instructions and stored at −20 °C until sent for genetic analysis (Table 1). Phenotyping was not conducted for these samples.

2.2 Fungicide sensitivity bioassay

The 86 samples, collected from 2017 to 2022, were phenotyped as arrived for each year for fungicide sensitivity using a bioassay. The following commercial products were tested: Cabrio® (pyraclostrobin 250 g active ingredient (a.i.) L^{-1}, Nufarm Australia, Laverton North, Australia); Ridomil® Gold 450 SL (metalaxyl-M 480 g a.i. L^{-1}, Syngenta Australia, Macquarie Park, Australia); Revus® (mandipropamid 250 g a.i. L^{-1}, Syngenta Australia) and Acrobat® (dimethomorph 500 g L^{-1}, BASF Australia, Southbank, Australia). Technical grade ametoctradin (Sigma-Aldrich, Macquarie Park, Australia) was also tested to represent one active ingredient of Zampro® (300 g a.i. L^{-1} ametoctradin and 225 g a.i. L^{-1} dimethomorph, BASF Australia), as ametoctradin alone is not available as a commercial product. The ametoctradin was dissolved in 1 mL of acetone. The fungicides were diluted in SRO water to concentrations of 0, 1 and 10 µg a.i. mL^{-1},[14,37] for use in bioassays and a field label rate was added to the protocol for isolates collected from 2020 to 2022. The field rates (based on the product labels) were 100, 50, 100, 180, and 240 µg a.i. mL^{-1} of pyraclostrobin, metalaxyl-M, mandipropamid, dimethomorph and ametoctradin, respectively. Different numbers of isolates were tested for each fungicide due the viability of the isolates and availability of fungicides at different times during the study.

Fresh leaves were taken from *V. vinifera* cv. Sultana grown in a controlled environment room, surface-disinfested in 0.5% sodium hypochlorite (White King bleach; Pental, Melbourne, Australia) for 60 s and rinsed three times, for 2 min each time, in SRO water. Leaves were blotted dry on sterile paper towel and 10 mm discs were excised using a sterile cork borer. Isolates collected from 2017 to 2018 were tested by floating[15,28,38,39] or dipping[40] leaves in fungicide suspensions, and for those collected from 2019 to 2022 the protocol was amended to spraying fungicides.[26,41]

For the floating method, ten leaf discs (pseudoreplicates) were placed abaxial side up and floated on the surface of the pre-prepared fungicide suspensions contained in each of two replicate 150 mm glass Petri dishes per treatment. A 10µL droplet of sporangium suspension was applied to each leaf disc and SRO water was used as control, dishes were randomized in plastic trays.

For the dipping method, ten leaf discs were placed into sterile beakers containing fungicide suspensions of each concentration, agitated for 1 h,[29] then dried in a fume-cabinet. Five leaf discs were placed abaxial surface upwards in Petri dishes (60 mm diameter) contained 1.5% water agar amended with 2.5 µL mL^{-1} pimaricin (Sigma-Aldrich) and 50 mg L^{-1} streptomycin (Sigma-Aldrich), two Petri dishes (replicates) were used for each treatment. Sporangium suspension was applied by spray on the discs using an atomizer spray bottle and SRO water sprayed as control. Plates were randomized in plastic trays.

For the spraying method, for convenience, whole leaves were sprayed on both surfaces with fungicide solutions using a hand-held atomizer spray bottle until run-off. When the leaves were dry, 10mm discs were then excised and placed on the water agar. Sporangium suspension was applied by spray using an atomizer spray bottle, and SRO water was applied to the non-inoculated controls.[41] Ten discs were used for each treatment with five discs (pseudoreplicates) in each of two replicate 60mm diameter Petri dishes. Dishes were randomized within plastic trays.

For each method, leaf discs were incubated in the dark for the first 24 h then 12 h of natural light at room temperature 22 °C ± 2 °C, and sporulation was assessed after 7 days. Leaf discs were visually assessed under a stereo microscope (×40 magnification) by estimating the percentage area occupied by sporulation on each disc.[35,36,41] An isolate was considered to have reduced sensitivity if substantial sporulation occurred on leaf discs treated with the minimum inhibitory concentration (MIC) of ≥ 1 µg mL^{-1} of active ingredients.[33,34] Based on several pilot studies, a growth percentage threshold of ≥ 5% was considered substantial growth.[42,43] In addition, when *P. viticola* grew when exposed to the field label rate, the isolate was considered resistant.

2.3 Genotyping of *Plasmopara viticola* isolates

All isolates were genotyped. For amplicon-based sequencing of the *cesA3* G1105s region (CAA target) and the *cytb* G143A region (QoI target), DNA from all samples were amplified using primer pairs specific to each gene locus (Table 3) and the region surrounding the known resistance mutation hotspots. To increase the sensitivity of detection, a nested polymerase chain reaction (PCR) approach was used in which a second amplification step was performed using an additional internal pair of gene-specific

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Table 2. List of *Plasmopara viticola* isolates collected from different regions in Australia at different times during the seasons from 2017 to 2022, with phenotyping results for each isolate against different fungicides and amplicon-based sequencing for G143A and G1105S[†]

State	Region	Isolate	Collection date	Metalaxyl-M (group 4)	Dimethomorph (group 40)	Pyraclostrobin (group 11)	% G143A	Ametoctradin (group 45)	Mandipropamid (group 40)	% G1105S
New South Wales (NSW)	Barooga	NSW29	29 November 2021	NT	NT	NT	0	NT	NT	0
		NSW30	29 November 2021	RS	RS	S	0	NT	S	0
		NSW31	29 November 2021	R	RS	S	28.6	NT	S	0
		NSW32	29 November 2021	R	RS	S	0	NT	S	0
		NSW33	29 November 2021	R	RS	S	0	NT	S	0
	Griffith	NSW27	22 November 2021	S	S	S	0	NT	S	0
		NSW28	22 November 2021	S	RS	S	0	NT	S	0
		NSW34	6 December 2021	NT	NT	NT	11.3	NT	NT	0
		NSW35	6 December 2021	NT	NT	NT	13.5	NT	NT	0
		NSW36	6 December 2021	S	RS	S	19	NT	S	0
		NSW37	6 December 2021	S	RS	RS	91.6	NT	S	0
		NSW39	8 November 2021	NT	NT	NT	0	NT	NT	0
		NSW40	8 November 2021	NT	NT	NT	1.2	NT	NT	0
		NSW41	8 November 2021	NT	NT	NT	0	NT	NT	0
		NSW42	6 February 2022	NT	NT	NT	54.3	NT	NT	0
		NSW45	10 February 2022	NT	NT	NT	6.9	NT	NT	0
		NSW47	15 February 2022	NT	NT	NT	55.6	NT	NT	0
		NSW48	15 February 2022	NT	NT	NT	55.6	NT	NT	0
		NSW49	15 February 2022	S	S	RS	NT	NT	S	NT
		NSW50	15 February 2022	S	S	R	NT	NT	S	NT
	Hunter Valley	NSW10	14 December 2020	R	S	RS	2.2	RS	S	0
		NSW11	14 December 2020	S	RS	RS	4.9	S	S	0
		NSW12	14 December 2020	R	S	RS	12.6	RS	S	0
		NSW14	14 December 2020	RS	S	RS	93.4	RS	S	0
		NSW16	14 December 2020	RS	S	RS	0	RS	S	0
		NSW17	14 December 2020	RS	S	S	3.3	S	S	0
		NSW18	14 December 2020	S	S	S	50.8	S	S	0
		NSW19	14 December 2020	R	S	R	44.8	RS	S	0
		NSW2	6 November 2020	RS	S	S	0	S	NT	0
		NSW20	14 December 2020	S	S	S	1.1	S	S	0
		NSW21	14 December 2020	RS	S	RS	0	RS	S	0
		NSW23	14 December 2020	S	S	S	0	S	S	0
		NSW24	14 December 2020	R	S	RS	32.5	RS	S	0
		NSW3	6 November 2020	S	S	S	0	S	S	0
		NSW5	6 November 2020	S	S	S	2.1	S	S	0
Queensland (QLD)	Mareeba	QLD10	25 February 2021	S	S	S	0	NT	S	0
		QLD6	25 February 2021	RS	S	S	4.4	NT	S	0
		QLD7	25 February 2021	RS	S	S	0	NT	S	0
		QLD8	25 February 2021	S	S	S	0	NT	S	0
South Australia (SA)	Adelaide Hills	SA	6 January 2018	S	NT	NT		NT	NT	NT
		SA4	3 February 2022	S	S	S	0	NT	S	0
		SA5	3 February 2022	S	S	S	0	NT	S	0
		SA6	9 February 2022	NT	NT	NT	0	NT	NT	0
		SA7	9 February 2022	NT	NT	NT	0	NT	NT	0
		SA8	9 February 2022	NT	NT	NT	0	NT	NT	0
		SA9	9 February 2022	S	S	S	0	NT	S	0

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Table 2. Continued

State	Region	Isolate	Collection date	Metalaxyl-M (group 4)	Dimethomorph (group 40)	Pyraclostrobin (group 11)	% G143A	Ametoctradin (group 45)	Mandipropamid (group 40)	% G1105S
		SA10	9 February 2022	NT	NT	NT	0	NT	NT	0
		SA11	17 February 2022	NT	NT	NT	0	NT	NT	0
		SARDI	6 January 2018	S	NT	S	NT	NT	NT	NT
		SARDI1	2018	NT	NT	S	NT	NT	NT	NT
	Clare Valley	CV2	10 February 2017	S	NT	S	NT	NT	NT	NT
	McLaren Vale	CV3	10 February 2017	S	NT	S	NT	NT	NT	NT
Victoria (VIC)	King Valley	KV1	6 January 2018	RS	NT	S	NT	NT	NT	NT
		KV3	6 January 2018	RS	NT	S	NT	NT	NT	NT
		KV4	6 January 2018	RS	NT	S	NT	NT	NT	NT
		KV5	6 January 2018	RS	NT	S	NT	NT	NT	NT
		KV6	6 January 2018	RS	NT	RS	NT	NT	NT	NT
		KV7	6 January 2018	RS	NT	RS	NT	NT	NT	NT
		VIC12	8 February 2021	RS	S	S	1.4	NT	S	0
		VIC14	8 February 2021	RS	S	S	0	NT	S	0
		VIC15	8 February 2021	RS	S	S	0	NT	S	0
		VIC16	8 February 2021	R	S	S	0	NT	S	0
	Yarra Valley	YV1	2018	NT	NT	RS	NT	NT	NT	NT
		YV2	2018	NT	NT	RS	NT	NT	NT	NT
		YV3	2018	NT	NT	RS	NT	NT	NT	NT
		YV4	2018	NT	NT	RS	NT	NT	NT	NT
		YV5	2018	NT	NT	RS	NT	NT	NT	NT
		YV6	2018	NT	NT	RS	NT	NT	NT	NT
		VIC10	28 February 2020	RS	NT	RS	10.1	NT	S	0
		VIC11	8 February 2021	NT	NT	NT	0	NT	NT	0
		VIC18	15 February 2022	NT	NT	NT	48.2	NT	NT	0
		VIC19	15 February 2022	RS	S	R	37.7	NT	S	0
		VIC2	15 January 2020	RS	S	RS	100	S	S	0
		VIC20	15 February 2022	R	RS	R		NT	S	NT
		VIC21	15 February 2022	R	RS	R	13.3	NT	S	0
		VIC22	15 February 2022	R	RS	RS	55.8	NT	S	0
		VIC3	6 February 2020	RS	S	RS	NT	S	RS	NT
		VIC4	6 February 2020	RS	S	RS	5	S	S	0
		VIC5	28 February 2020	RS	S	RS	92.8	RS	NT	0
		VIC6	28 February 2020	RS	RS	RS	62.5	RS	S	0
		VIC7	28 February 2020	S	S	S	31.5	S	NT	0
		VIC8	28 February 2020	RS	S	RS	8	S	S	0
		VIC9	28 February 2020	RS	S	RS	17.3	S	S	0
Western Australia (WA)	Swan Valley	WA1	20 November 2019	NT	NT	NT	0	NT	NT	0
		WA2	20 November 2019	NT	NT	NT	0	NT	NT	0
		WA3	20 November 2019	S	NT	S	0	NT	S	0
Total		86		60	48	66	65	23	47	65

† RS, reduced sensitivity (orange); S, sensitive (blue); R, resistant (purple); NT, not tested.

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Table 3. Primers used for amplicon-based sequencing

Primer	Locus	Sequence
Pv_cytB_G143A_292F	*cytB*	TTTAGGGGTTTGTATTACGG
Pv_cytB_G143A_628R	*cytB*	ATGGATTATTTGAACCTACCTC
Pv_cytB_G143A_331F_N701	*cytB*	CCTACACGACGCTCTTCCGATCTTAAGGCGAGAAGCTTTATGGTGTTCAGG
Pv_cytB_G143A_567R_N701	*cytB*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAGGCGATACAAATGGAAAGGTGAAATG
Pv_ces3A_G1105S_3234F	*ces3A*	AATTGGTGGCATTGATTCG
Pv_ces3A_G1105S_3607R	*ces3A*	CACAATTGCATGTCTTTCTCG
Pv_ces3A_G1105S_3340F_N701	*ces3A*	CCTACACGACGCTCTTCCGATCTTAAGGCGACATGGTCAAGATGAGTATCACG
Pv_ces3A_G1105S_3534R_N701	*ces3A*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAGGCGAGCAGCATCCCCAGTTTCG
Illumina Nextera dual-index F primer	NA	AATGATACGGCGACCACCGAGATCTACACxxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATCT
Illumina Nextera dual-index R primer	NA	CAAGCAGAAGACGGCATACGAGATxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

primers. A final step was performed to anneal sequences to each amplicon that were necessary for the Illumina sequencing process. Individual amplicons were pooled at approximately equimolar ratios and sequenced using Illumina chemistry at 2 × 150 bp read length (Ramaciotti Centre for Genomics, Sydney, Australia). Raw sequencing data was processed using an analytical pipeline consisting of quality trimming,[44] adaptor trimming[45] and paired-end read merging,[46] with both Illumina and in-line barcodes used to uniquely identify reads that originated from each biological sample. To score individual sample genotypes, the merged reads were aligned to a reference DNA sequence for each relevant target locus using Novoalign and the proportion of each specific DNA variant present at each base pair in the amplicon was recorded.[47]

3 RESULTS

3.1 Fungicide sensitivity bioassay

3.1.1 *Metalaxyl-M (PA)*

For the 60 *P. viticola* isolates collected and tested between 2017 and 2022, reduced sensitivity and resistance to metalaxyl-M was detected in 26 (45%) and 11 (19%), respectively. Two of the four isolates collected in QLD had reduced sensitivity (Fig. 1(A) and Table 2). In NSW, five and four of the 15 isolates from Hunter Valley had reduced sensitivity and resistance to metalaxyl-M, respectively. Three of four isolates from Barooga were resistant, and the fourth exhibited reduced sensitivity, while all six isolates from Griffith were sensitive. In VIC, nine of the ten isolates from King Valley had reduced sensitivity and one isolate was resistant, and nine and three of the 13 samples from Yarra Valley had reduced sensitivity and resistance, respectively. On average, 15% of all isolates had MIC at 10 μg mL^{-1}, 27% at field label rate (50 μg mL^{-1}) and 25% at > 50 μg mL^{-1} of active ingredient. All samples from SA and WA were sensitive to the fungicide.

3.1.2 *Mandipropamid (CAA)*

Forty-seven isolates, from five states of Australia, were exposed to mandipropamid between 2020 and 2022, and only one isolate from Yarra Valley, VIC had reduced sensitivity with minimal growth (8%) at 1 μg mL^{-1} on leaf discs. The MIC value was 1 μg mL^{-1} for all isolates except the one with reduced sensitivity, which had MIC of 10 μg mL^{-1}, none of the isolates grew at field label rate (100 μg mL^{-1}) (Table 2).

3.1.3 *Dimethomorph (CAA)*

Between 2020 and 2022, 48 isolates from four states were exposed to dimethomorph. For NSW and VIC, all four isolates from Barooga, three of six from Griffith, one of 15 from Hunter Valley and four of 12 from Yarra Valley had reduced sensitivity (Fig. 1(B) and Table 2). However, no reduced sensitivity was detected in isolates from QLD or SA, and no resistance was detected in any region. The MIC value was 1 μg mL^{-1} for 77% of the isolates, 10 μg mL^{-1} for 20%, 180 μg mL^{-1} for 3% and no isolates grew at field label rate (180 μg mL^{-1}).

3.1.4 *Pyraclostrobin (QoI)*

Between 2017 and 2022, 66 *P. viticola* isolates were exposed to pyraclostrobin, of which 40% had reduced sensitivity and 8% were resistant. The highest incidence of reduced sensitivity and resistance was detected in samples from Yarra Valley, namely 79% and 16%, respectively, followed by isolates from Hunter Valley, 47% and 7%, respectively (Fig. 1(C) and Table 2). Of the six isolates from Griffith, two had reduced sensitivity and one was resistant, however, all isolates from other regions were sensitive to pyraclostrobin. The MIC value was 1 μg mL^{-1} for 56% of the isolates, 10 μg mL^{-1} for 9% and 8% of the isolates grew at field label rate (100 μg mL^{-1}).

3.1.5 *Ametoctradin (QoSI)*

Ametoctradin was only tested for 23 isolates in 2020, of which 39% had reduced sensitivity and none exhibited resistance. Reduced sensitivity was detected in seven of the 15 isolates from Hunter Valley, NSW and two of the eight isolates from Yarra Valley, VIC (Fig. 1(D) and Table 2). The MIC value was 1 μg mL^{-1} for 60% of isolates tested, 10 μg mL^{-1} for 17%, 100 μg mL^{-1} for 21% and no isolates grew at field label rate (240 μg mL^{-1}).

Many isolates exhibited resistance to more than one fungicide, for example four isolates (VIC20, VIC21, VIC22, and VIC6) from Yarra Valley in VIC had reduced sensitivity or resistance to metalaxyl-M, dimethomorph and pyraclostrobin, 21 isolates from NSW and VIC showed reduced sensitivity and/or resistance to metalaxyl-M and pyraclostrobin (Table 2). One isolate (VIC3) had reduced sensitivity to three fungicides (metalaxyl-M, pyraclostrobin and mandipropamid), and another isolate (VIC6) had reduced sensitivity to four fungicides metalaxyl-M, dimethomorph, pyraclostrobin and ametoctradin. From the Yarra Valley in VIC, three isolates (NSW19, VIC20 and VIC21) were resistant to metalaxyl-M and pyraclostrobin and another two isolates (VIC5 and VIC6) had

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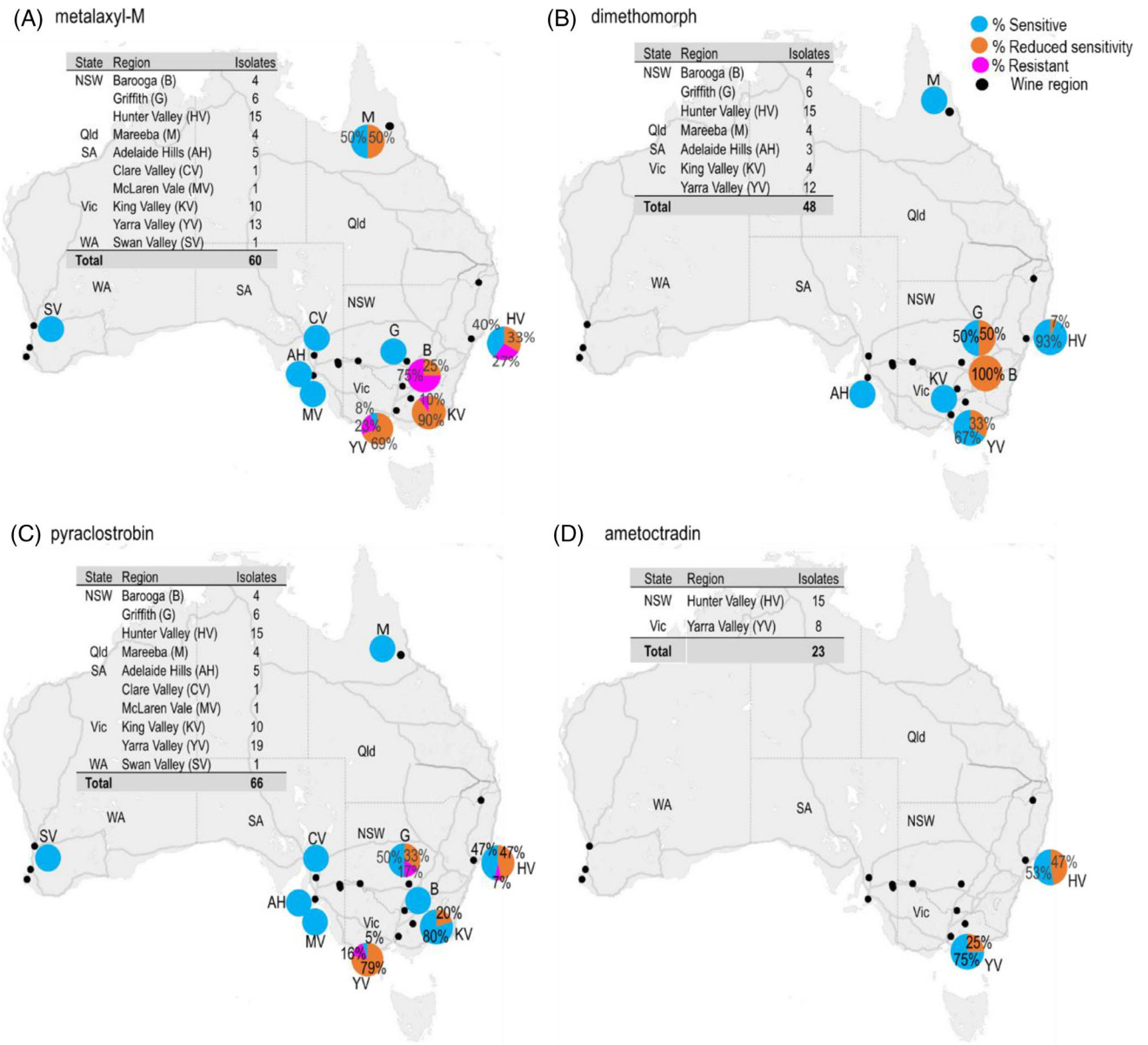

Figure 1. Sensitivity of *Plasmopara viticola* isolates, collected from commercial vineyards in regions of Australia between 2017 and 2022, to (A) metalaxyl-M (group 4), (B) dimethomorph (group 40), (C) pyraclostrobin (group 11) and (D) ametoctradin (group 45) fungicides. Sensitivity was determined based on growth of the pathogen on treated grape leaf discs. Isolates were considered to have reduced sensitivity when they grew at the minimum inhibitory concentration (MIC) of 1 μg mL^{-1} of active ingredients and resistant when they grew at field label rate. Inset table indicates the number of isolates collected from each region.

reduced sensitivity to at least three of the four fungicides (metalaxyl-M, dimethomorph, pyraclostrobin and ametoctradin) (Table 2). From NSW, three isolates (NSW14, NSW16 and NSW21) had reduced sensitivity to metalaxyl-M, pyraclostrobin and ametoctradin, and three isolates (NSW10, NSW12 and NSW24) were resistant to metalaxyl-M, whilst also having reduced sensitivity to pyraclostrobin and ametoctradin (Table 2).

3.2 Genotype testing

Sequences of PCR-generated amplicons of the *P. viticola cytB* and *ces3A* genes were analysed for 239 of the 271 isolates, that is, those deemed reliable reads, collected between 2014 and 2017, and for 65 of the 86 collected between 2017 and 2022. Twenty of the 239 isolates (8%) contained the G143A mutation although this was generally in a mixture with the wild-type allele (Fig. 2(A)). Most isolates carrying the G143A mutation were collected in the Granite Belt, QLD (11 of the 19, 58%) followed by Hunter Valley, NSW (six of the 41, 15%). The G143A mutation was detected in one of the six isolates from the Murray Darling, VIC, and both isolates from Mornington Peninsula, VIC (Fig. 2(A)). The frequency of the G143A mutation was less than 50% in nine isolates and 50–100% in 11 of the 271 isolates tested (Fig. 3(A)).

For isolates collected between 2017 and 2022, 65 of the 86 collected had reliable reads. The highest incidence of isolates with the G143A mutation was from Yarra Valley, VIC (12 of the 13, 92%), then Hunter Valley, NSW (ten of the 15, 67%), Griffith, NSW (nine of the 13, 69%), and mutation was detected in one isolate of the four in Mareeba, QLD and King Valley, VIC and in one

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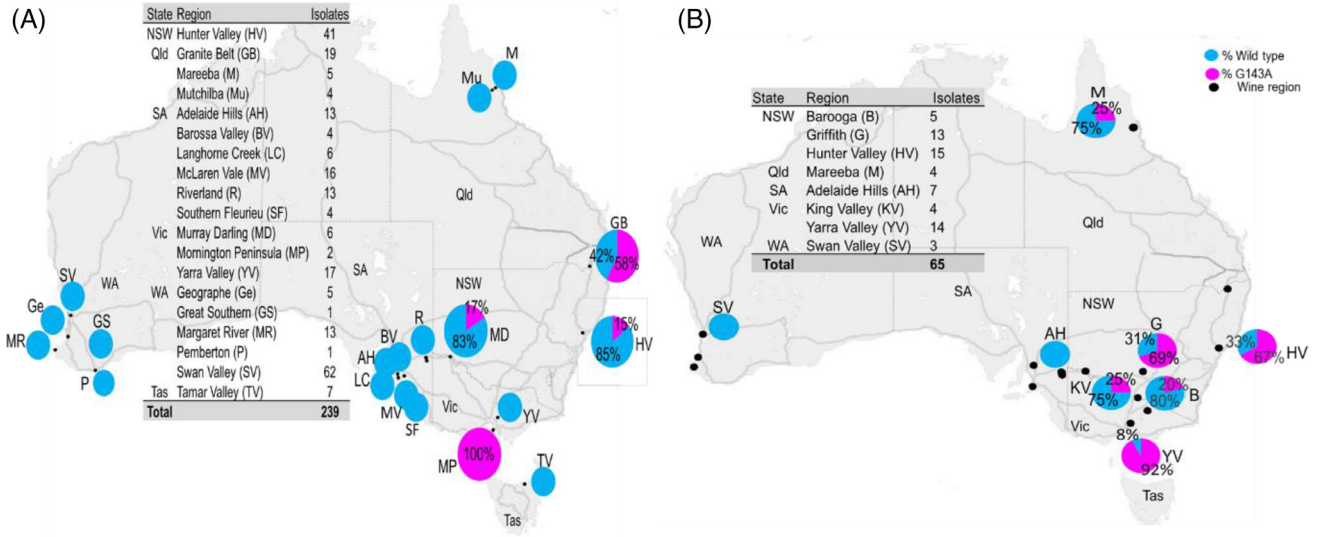

Figure 2. Genetic analysis results for *Plasmopara viticola* samples collected from commercial vineyards in regions of Australia. (A) 2014–2017 and (B) 2017–2022. Amplicon-based sequencing was used to measure the incidence of the G143A mutation and wild type (G143) in each isolate; the percentage of samples with the G143A mutation are displayed. Inset table indicates the number of samples from each region that yielded reliable sequence reads.

isolate of the five in Barooga, QLD (Fig. 2(B)). The G143A mutant was not identified in SA or WA. Overall, 34 isolates (52%) were detected with G143A, with frequency ranging from 1% to 100% (Fig. 3(B)). An association between phenotyping and genotyping was established where 97% of the isolates exhibited reduced sensitivity to pyraclostrobin and carried the G143A mutation (data not shown). The G1105S mutant was not detected in either collection of *P. viticola*.

4 DISCUSSION

Decreased sensitivity to commonly used fungicides was detected among 80 isolates of *P. viticola* collected from commercial vineyards across Australia from 2017 to 2022. Phenotypic sensitivity to metalaxyl-M, dimethomorph, pyraclostrobin and ametoctradin was reduced, and resistance to metalaxyl-M and pyraclostrobin was detected in vineyards where fungicide applications had shown issues in controlling downy mildew. The G143A mutation, which is linked to resistance to the QoI group of fungicides, was detected in 8% of 239 samples collected from 2014 to 2017 and 52% of 65 samples collected from 2017 to 2022. There was an association between phenotypic resistance and genetic detection of G143A in the QoI fungicide group for the latter collection. A six-fold increase in the abundance of the G143A mutation was detected in samples collected from 2017 to 2022 compared to 2014–2017. This increase in detection attributed to the method of sampling, rather than an increase in frequency in the population over time. In 2014–2017, samples were collected randomly regardless of the disease control status, however in 2017–2022, sampling was targeted at vineyards with issues of downy mildew control, and so suspected to have resistance to the fungicides applied.

There appears to have been a shift in the sensitivity of *P. viticola* to metalaxyl since its introduction to viticulture in Australia in the 1970s. When first studied in 1987, growth of 15 isolates collected in SA, NSW, VIC and QLD, was completely inhibited by 1 μg mL^{-1} of metalaxyl.[48] In 2005, resistance to 10 μg mL^{-1} was reported for isolates from two vineyards with poor control in the Hunter Valley, NSW.[14] Hall *et al.*[42] reported reduced sensitivity to metalaxyl-M in 17 of 18 isolates collected in the Hunter and Yarra Valleys between 2013 and 2016. In the current study, 46% of 60 isolates collected from five states in 2017–2022 exhibited reduced sensitivity, and 19% resistance, to metalaxyl-M. Most of the isolates with decreased sensitivity or resistance were obtained from the Hunter Valley in NSW (25%) and the King and Yarra Valleys in VIC (16 and 21%, respectively). All isolates from SA and WA regions were sensitive, most likely reflecting the lower disease pressure due to drier climatic conditions, which are less conducive for infection than the higher rainfall regions of King, Yarra and Hunter Valleys. Although assessment methods have evolved, there has been a decrease in sensitivity of *P. viticola* to metalaxyl-M in Australian vineyards over time, and the current study constitutes the first report of resistance to the field label rate for 11 of the vineyards sampled.

Previous research used concentrations up to 10 μg mL^{-1} as discriminatory doses to classify resistance to metalaxyl-M,[14,19] even though the label rate is equivalent to 48 μg mL^{-1}. Wicks *et al.*[14] and Giraud *et al.*[37] reported resistance to kiralaxyl (also belongs to the PA group and has cross-resistance with metalaxyl) and metalaxyl-M in Australia and Luxembourg, respectively, using only 10 μg mL^{-1}. Whereas, Fourie[19] reported resistance at 100 μg mL^{-1} in South Africa and Ghule *et al.*[21] reported resistance at 200 μg mL^{-1} in India. In the current study, isolates were exposed to 50 μg mL^{-1} (just above the field label rate), to provide a robust test of resistance. It is important that testing conditions are standardized internationally in order to facilitate more meaningful comparisons among countries and over time, and it is proposed that field label rate should be the standard. The mechanism of resistance to metalaxyl is unknown, therefore monitoring for resistance continues to rely on phenotypic analysis.

In the current study, isolates of *P. viticola* were tested for phenotypic sensitivity to two CAA fungicides, mandipropamid and dimethomorph, then for the G1105S mutation which confers resistance.[26,35,36] It was found that 25% of all isolates tested by bioassay had reduced sensitivity to dimethomorph, but none carried the G1105S mutation. Previously in Australia, Hall *et al.*[42]

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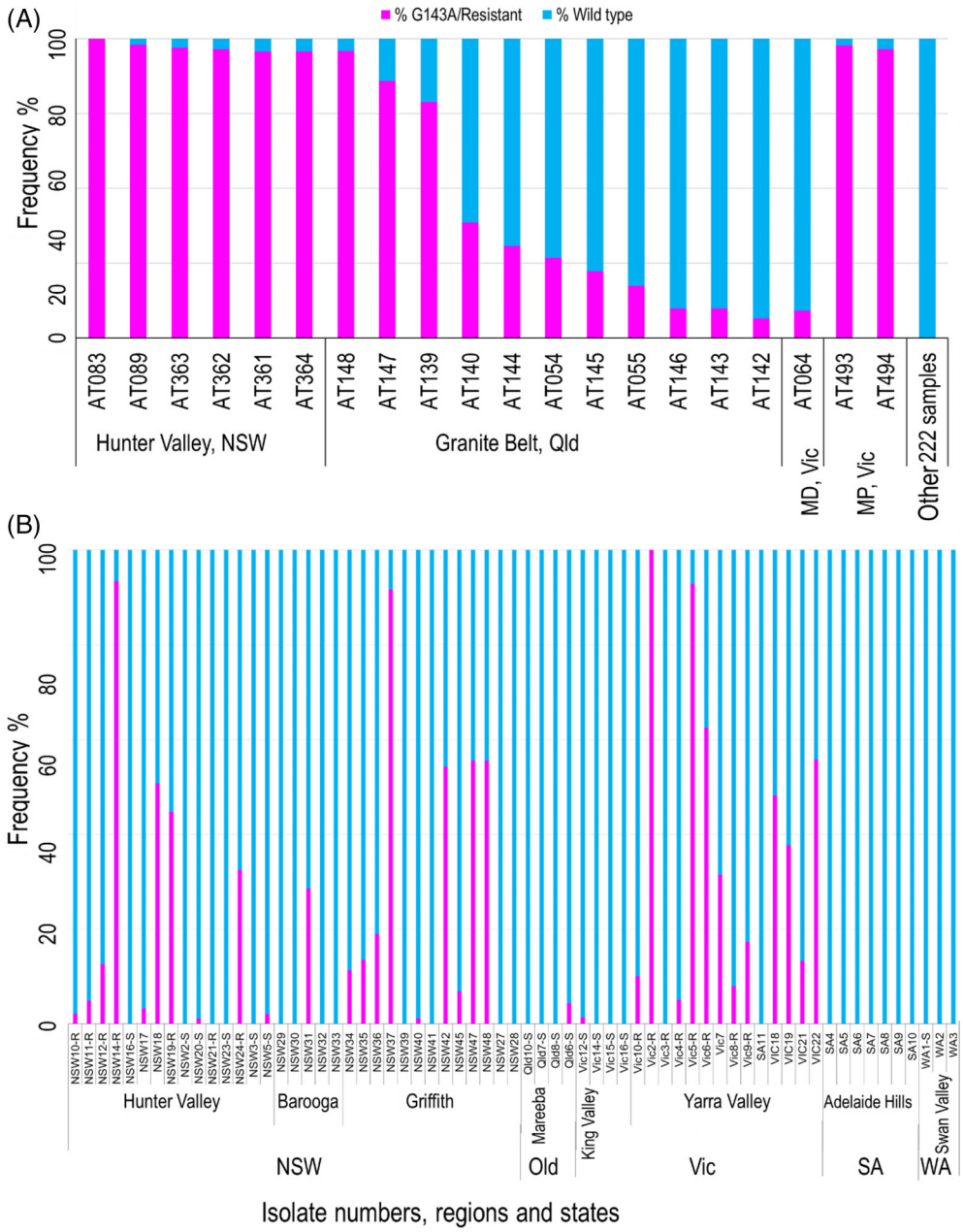

Figure 3. Genetic analysis of the *cytB* (G143A mutant) in *Plasmopara viticola* isolates collected from commercial vineyards in Australia (A) between 2014 and 2017 and (B) between 2017 and 2022. Amplicon-based sequencing was used to measure the frequency ratio of the G143A mutation and wild type (G143) in each isolate. MD, Murray Darling; MP, Mornington Peninsula.

reported reduced sensitivity to mandipropamid when one isolate of the 24 tested exhibited a slightly elevated half maximal effective concentration (EC_{50} 4.95 μg mL^{-1}) compared with sensitive isolates. Gisi *et al.*[24] suggested that isolates with EC_{50} values below 3.6 μg mL^{-1} were considered sensitive to Mandipropamid. Wicks and Hall[22] reported in 1990 that four isolates *of P. viticola* were sensitive to dimethomorph. Genotype analysis was not carried out in any of the previous studies.

Resistance to CAA fungicides has been reported worldwide for *P. viticola*.[24,27] Populations of *P. viticola* resistant to dimethomorph were first reported in France in 1994,[26,49] and since then have been increasing across Europe.[33] In contrast, Sun *et al.*[38] reported in 2010 that all 307 isolates of *P. viticola* tested from China were sensitive to dimethomorph. Gisi *et al.*[24] tested mandipropamid, dimethomorph, iprovalicarb and benthiavalicarb and reported 50% inhibition of growth for 41 European isolates tested at 300 μg mL^{-1}, also reporting cross-resistance between these fungicides. A strong correlation between phenotypic resistance and the presence of the G1105S mutation has been documented[21] and the presence of this mutant allele in Japanese isolates of *P. viticola* has been demonstrated.[35] The current study, which involved both phenotyping and genotyping, showed that 98% of the tested samples were sensitive to mandipropamid, with only one isolate having minimal growth, supporting the genetic analysis. Similar results were found in Georgia in 2020, where phenotypic and genotypic resistance were not detected in any *P.*

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viticola isolates.[40] Mandipropamid is a relatively new fungicide in Australia, and as cross-resistance has been demonstrated between dimethomorph and mandipropamid,[24,25] it is important to continue monitoring for resistance to mandipropamid.

With regard to pyraclostrobin, in the current study, 40% of isolates tested exhibited reduced sensitivity and 8% were resistant; most of these originated in Griffith, NSW and Yarra Valley, VIC. In comparison, none of the isolates tested in the previous study from 2013 to 2016 exhibited reduced sensitivity based on a discriminatory dose of 0.1 μg mL^{-1}.[42] Other authors used different discriminatory doses of pyraclostrobin for *P. viticola* isolates to be resistant, for example Giraud *et al.*[37] reported 3 μg mL^{-1} while Gauthier and Amsden[50] reported 390 μg mL^{-1}. Baseline sensitivities to pyraclostrobin of up to 0.94 μg mL^{-1} were reported by Wong and Wilcox[29] It is likely that the low discriminatory dose used by Hall *et al.*[42] explains the failure to detect reduced sensitivity. Given there is no accepted standard pyraclostrobin concentration for determining reduced sensitivity or resistance, we propose that isolates are considered as having reduced sensitivity when the pathogen grows on at least 1 μg mL^{-1} and being resistant when growth occurs at field label rate. The genetic analysis in this study supported this proposition that there was a strong association between phenotyping and genotyping, with 97% of the isolates that exhibited reduced sensitivity also carried the G143A mutation. Sensitive isolates detected with G143A mutation could be attributed to mixed populations that contained low numbers of individuals with the mutant, but not enough to cause reduced sensitivity or resistance. However, all the wild-type isolates (0% G143A) were sensitive in the phenotyping, except one isolate, suggesting the possibility of another resistance mutation, which needs to be investigated.

Reduced sensitivity to ametoctradin was detected in samples from the Hunter and Yarra Valleys, with 39% of the isolates tested growing on at least 1 μg mL^{-1}, and some of these isolates had reduced sensitivity and resistance to pyraclostrobin and metalaxyl. Resistance to ametoctradin (group 45) has been detected in France, albeit at low frequency, and attributed to a single point mutation in the cytochrome b gene, leading to the S34L substitution.[34] A marker, Pv-Cytb-S34L-F1, has been developed to detect this mutant allele. However, the risk of disease control issues may be reduced in Australian vineyards where the product Zampro is used, which comprises a combination of ametoctradin and dimethomorph, although one isolate in this study had reduced sensitivity to both active ingredients, so continued monitoring will be important.

This study established that many isolates exhibited resistance to more than one fungicide. Due to the nature of phenotype bioassays which are conducted using inoculum generated from mixed populations that may contain resistant and sensitive strains of *P. viticola*, results may have varied for each fungicide test. Therefore, confirmation of multiple resistance will require phenotyping using pathogen inoculum generated from single sporangia. Nevertheless, these results indicate a high risk of resistance developing in Australian *P. viticola* populations.

Genotyping and phenotyping results were closely associated for QoI resistance in this study, which concurs with previous reports that G143A is the most common mutant, and so provides a benchmark for QoI resistance.[28,30,51,52] Therefore, molecular detection can be used alone in future for monitoring QoI resistance in *P. viticola*. The G143A mutation is an ideal candidate for monitoring by amplicon-based sequencing and potentially for high throughput and in-field quantitative PCR detection. Using molecular detection will be more efficient, cost effective, less labour intensive, rapid and can be done in large scale compared with phenotyping. Although the G1105S mutation that is associated with resistance to CAA fungicides has not been detected in Australia, monitoring is still required as resistance may develop in regions where selection pressure is strong. Furthermore, continued monitoring is required for metalaxyl resistance by phenotyping until a resistance mechanism is identified.

Research findings presented here suggest that anti-resistance, integrated pest management strategies should be employed to preserve the effectiveness of all useable fungicides in vineyards or regions where resistance has been detected, and that the frequency of use should be reviewed where reduced sensitivity has been detected. Anti-resistance strategies for viticulture systems[9] include applying fungicides preventatively (e.g., metalaxyl should be applied before the first sign of oilspots), applying fungicide in mixtures, and applying a maximum of two consecutive applications and three sprays per season of any one group.[53] Continued monitoring of vineyards in all regions where downy mildew is managed is required to ensure the continued success and sustainability of the viticulture industry.

ACKNOWLEDGEMENTS

This work was supported by Wine Australia, with levies from Australia's grapegrowers and winemakers and matching funds from the Australian Government. The authors wish to acknowledge Barbara Hall and Suzanne McKay for initiating and managing this research from 2017 until 2019, and Eileen Scott for critically reviewing the manuscript. Thanks to all growers and viticulturists who supplied samples for this study and agrochemical companies that supplied fungicide products.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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