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Resistance of *Plasmopara viticola* to multiple fungicides in vineyards of Maharashtra, India

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Abstract

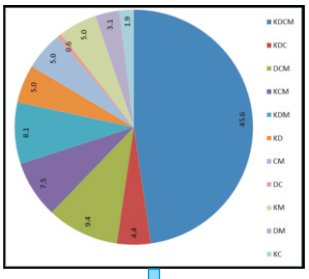
Aim: This study aims to assess the fungicide resistance status of *P. viticola* for developing a future guideline for downy mildew management of grapes in India.

Methodology: One hundred and sixty downy mildew infected grape leaf samples were collected from 16 vineyards located in Maharashtra, India during September 2015 to November 2016. Most of these vineyards had heavy incidence of downy mildew disease, even though several fungicide interventions for its control were made. The fungicide sensitivity was assessed against kresoxim methyl, dimethomorph, Cymoxanil and Metaxyl-M using leaf disc bioassay, and QoI and CAA resistance gene was detected using allele specific multiplex PCR.

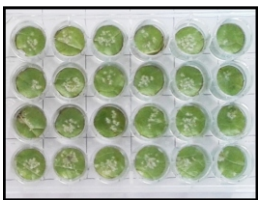
Results: Based on the sensitivity response in leaf disc bio-assay, isolates of *P. viticola* were categorized as, sensitive (S), intermediate (I) or highly resistant (R) to four fungicides. The percentage of sensitive *P. viticola* isolates were 23.75, 19.37, 32.5, 12.5, intermediate were 15.0, 48.12, 48.8, 45.62 and highly resistant were 23.8, 20.6, 29.4, 12.5 to kresoxim methyl, dimethomorph, cymoxanil and metalaxyl-M, respectively. Multiplex allele-specific primer–polymerase chain reaction (ASP-PCR) confirmed G143A mutation in the *cytb* gene in 123 highly and intermediate resistant *P. viticola* isolates to quinone outside inhibitor fungicide and G1105S mutation in the *PvcesA3* gene in 129 highly and intermediate resistant isolates to carboxylic acid amide fungicides. Among these, 101 isolates showed the presence of both G143A and G1005S mutations. Forty-nine isolates showed the presence of either G143A or G1005S mutation, while ten isolates were sensitive to both the fungicides and did not harbor either of the mutations.

P. viticola isolates collected from Indian vineyards

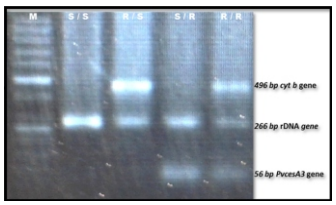
Percentage of *P. viticola* resistant to kresoxim methyl (K), dimethomorph (D), cymoxanil (C) and metalaxyl-M (M)



Determined the fungicide sensitivity against fungicide



ASP-Multiplex PCR used for detection of QoI and CAA resistance gene



Interpretation: Development of multiple fungicide resistant *P. viticola* led to the failure of downy mildew control in vineyards of Maharashtra, India. There is a need for implementing biological control strategies coupled with the use of safer chemicals and reduced application of single target site fungicides.

Keywords: Downy mildew, Fungicide resistance, Fungicides, Grapes, *Plasmopara viticola*

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Introduction

Grape is an important fruit crop grown worldwide, including India. In India, the grape cultivation area had steadily increased from 65 thousand ha in the year 2006-2007 to 136 thousand ha in the year 2016-2017, with almost 80% located in the state of Maharashtra (National Horticultural Board, 2017). The yield and quality of grapes are affected by downy mildew disease caused by oomycete pathogen *Plasmopara viticola* (Berk. and Curt.) Berl and de Toni under moist and warm conditions. Multiple groups of fungicides, classified as high, moderate to low risk fungicides for the development of resistance in *P. viticola,* are the major arsenal to combat this disease. In India, the single target site fungicides of quinine outside inhibitors (QoI); carboxylic acid amides (CAA); phenyl amides (PA) (benalaxyl, metalaxyl-M); cyanoacetamide-oximes (cymoxanil) groups and multi-target site fungicides, chlorothalonil, mancozeb, phosphonates, and copper-based fungicides are registered for downy mildew management in grapes (Anonymous,2018-19; APEDA, 2009).

Quinine outside inhibitor fungicides are high risk fungicides for development of fungicide resistance in pathogens. Point mutations in the cytochrome *b* gene at 2 codons are reported, first mutation at codon 143 (glycine to alanine, G143A) and second mutation F129L (phenylalanine to leucine) at codon 129 (Chen *et al.* 2007). The QoI fungicide resistance in *P. viticola* was detected in the vineyards of Europe, Japan and the United States of America with various levels of sensitivities. The isolates are termed as sensitive if the EC_{50} <1 µgml^{-1} (MIC <10 µg ml^{-1}) and resistant if EC_{50} is >25 µg ml^{-1} (Baudoin *et al*., 2008; Corio-Costet *et al*., 2011).

Carboxylic Acid Amide group is classified as a moderate risk and resistance is due to point mutation in the *PvCesA3* gene at codon 1105 resulting in a change of amino acid from glycine to serine (G1105S) (Blum *et al*., 2010). Sensitivity towards varying levels of CAA fungicides in *P. viticola* populations have been reported in earlier studies from the vineyards of Europe and Asia (Gisi *et al*., 2007; Sawant *et al*., 2017). The EC_{50} of sensitive isolates ranged from 0·03 to 3·6 µg ml^{1} while the resistant isolates showed EC_{50} >300 µg ml^{-1}. In another study, it was reported that the EC_{50} of sensitive isolates was <0.1 µg ml^{1}, while those of resistant isolates was >40.00 µg ml^{-1} (Gisi *et al*., 2007; Sawant *et al*., 2017). Classification of *P. viticola* isolates as sensitive if the MIC was <1.6 µg ml^{-1} and as resistant if the MIC was >100 µg ml^{-1} has been proposed (Zhang *et al*., 2016).

Cymoxanil group is classified as low to moderate risk and its resistance has been reported from European vineyards. The sensitive isolates exhibited MIC value of 3 µg ml^{-1}, while the resistant isolates exhibited MIC values from 10 to >100 µg ml^{-1} (Gullino *et al*.,1997). Phenyl amide group is classified as high risk and *P. viticola* population with MIC values <10, 100, and ≥ 200 µg ml^{-1} were categorized as sensitive, resistant and highly resistant to metalaxyl in French vineyards (Fourie, 2004).

In recent years, the grape farmers from Maharashtra have reported lack of control of downy mildew. Keeping in view the above, the present study was carried out to assess the fungicide sensitivity status of *P. viticola* isolates, especially in those vineyards where downy mildew was not under control even after several applications of different groups of fungicides.

Materials and Methods

Sampling: Downy mildew infected grape leaf samples were collected from 16 vineyards located in Nasik (7), Sangli (6), Solapur (2) and Pune (1) districts of Maharashtra, India. These vineyards were earlier treated with different groups of fungicides, but had reported failure of downy mildew control. Ten leaves with single downy mildew lesion were collected from each vineyard following the procedure of Fourie (2004), with slight modification. Each leaf was kept in a separate plastic zip lock bag and transfered to the laboratory. Sporulation was induced by incubating the leaves overnight in a humid chamber under darkness. The sporangia were used as inoculum for fungicide sensitivity assay.

Leaf disc bio-assay: The sensitivity of *P. viticola* was determined against four groups of fungicides: QoI group: kresoxim-methyl (Tata Rallis, 95.4% Technical grade); CAA group: dimethomorph (BASF, 95.4% technical grade); Cyano-acetoxime group: cymoxanil (Dupont, 95.4% technical grade) and Phenyl Amide group: metalaxyl-M (Syngenta, 95.4% technical grade). To prepare the stock solutions, kresoxim methyl and dimethomorph were dissolved in a small volume of dimethyl sulfoxide (DMSO) while metalaxyl-M and cymoxanil were dissolved in acetone. The working concentrations were prepared using sterile distilled water, and sterile distilled water served as the control. Four replications were maintained for each fungicide concentration. Healthy, fungicide free, fully expanded leaves of susceptible cultivar Thomson Seedless were used for bio-assay. Leaf discs of 15 mm diameter were cut, surface-disinfected and placed upside down in individual wells containing 0.5% sterilized water agar (PCT 0901, Himedia) in 24-well plates. The discs were treated with fungicide solutions and after 24hr they were inoculated by placing 50 µl of *P. viticola* inoculum containing 50,000 sporangia ml^{-1}. The plates were incubated at 22 ºC with alternating periods of 12 hr light and darkness for 7-8 days. Each leaf disc was observed at 20× magnification under a stereomicroscope and scored with a scale, where 0 indicated no visible symptoms; 1 is infection but no sporulation; 2 is little sporulation, lesion size smaller than droplet size; 3 is sporulation, lesion size same as droplet size and 4 is sporulation, lesion size larger than droplet size, respectively. The minimum inhibitory concentration (MIC) was defined as the concentration at which the average rating was < 2 (Fourie, 2004). The isolates were classified as sensitive to fungicides if MIC <10 µg ml^{-1} and resistant if MIC > 500 µg ml^{-1} (kresoxim-methyl), >200 µg ml^{-1} (dimethomorph, cymoxanil and metalaxyl). Isolates with intermediate MIC values were classified as intermediate resistant.

Multiplex ASP-PCR : Allele-specific multiplex PCR was used for simultaneous detection of G143A mutation in *cytb* gene and G1105S mutation in *PvcesA3* gene in conferring resistance to QoI and CAA group of fungicides for each of the 160 *P. viticola* isolates by following the method developed by Aoki *et al*. (2013), with minor modifications. DNA was extracted from 160 isolates using the Extract-N-Amp plant PCR kit (Sigma-Aldrich). For PCR reaction, mixture of 20 µl 50 mM of KCl, 10 mM of Tris-HCl (pH 8.0), 0.2 mM of each dNTP (Sigma Aldrich), 2 µM of each primer, 1 U of Taq DNA polymerase (Sigma Aldrich) and 1 µl of template DNA in the microtube was used. Multiplex PCR was programmed for initial denaturation at 95 ºC for 3 min; PCR amplification with ten cycles of 95 ºC for 30 sec, 65 ºC for 30 sec and 72 ºC for 30 sec and twenty-five cycles at 95 ºC for 30 sec, 60 ºC for 30 sec and 72 ºC for 30 sec and a final extension at 72 ºC for 2 min. PCR products were checked by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining under gel doc instrument.

Results and Discussion

The overall sensitivity of 160 *P. viticola* isolates to four groups of fungicides is shown in Fig. 1, Table S1. Most of the isolates were intermediate resistant or highly resistant to four fungicides. The frequency of highly resistant isolates was 61.25% to kresoxim-methyl, 32.50% to dimethomorph, 21.25% to cymoxanil and 37.50% to metalaxyl-M. Similarly, the frequency of intermediate resistant isolates was 15% to kresoxim methyl, 48.12% to dimethomorph, 46.25% to cymoxanil and 50.00% to metalaxyl-M. Only 23.75, 19.37, 32.50, and 12.50% isolates were sensitive to kresoxim- methyl, dimethomorph, cymoxanil and metalaxyl-M, respectively.

The distribution of highly resistant and intermediate resistant *P. viticola* isolates to all four fungicides varied with vineyards (Fig. 2). High resistance to at least one of the four fungicides was detected in each of the 16 vineyards. Highly resistant kresoxim-methyl isolates were most prevalent and observed in 14 vineyards, while isolates highly resistant to dimethomorph, cymoxanil and metalaxyl-M were detected in 12, 8 and 13 vineyards, respectively. Vineyards Pu-08 and Sa-12 had *P. viticola* isolates highly resistant to only one fungicide, metalaxyl-M and dimethomorph. *Plasmopara viticola* isolates highly resistant to two fungicides were detected in vineyards So-6 and So-7 (kresoxim-methyl and metalaxyl-M) and vineyards Na-10 and Sa-13 (kresoxim-methyl and dimethomorph). *P. viticola* isolates exhibiting high resistance to three fungicides were detected in vineyard Sa-15, Sa-16 (kresoxim-methyl, cymoxanil and metalaxyl-M), vineyard Na-20 and Sa-14 (kresoxim-methyl, dimethomorph and metalaxyl-M). *P. viticola* isolates from other six vineyards, Na-11, Na-17, Na-18, Na-19, Na-23 and Sa-21, showed high resistance to all the four fungicides.

The multiplex allele-specific primer PCR method was successfully performed as seen by 266 bp product of the rRNA gene of *P. viticola* present in PCR reaction of all 160 isolates. The presence of two bands of 496 bp and 56 bp confirmed G143A mutation in *cyt b* gene and G1105S mutation in *PvCesA3* gene, respectively (Fig. 4). Out of 160 *P. viticola* isolates, 123 isolates showed the presence of G143A mutation in *cytb* gene, indicating that 98 highly resistant and 25 intermediate resistant isolates harbored G143A mutation. Similarly, 129 *P. viticola* isolates showed the presence of G1005S mutation in *PvCesA3* gene, revealing that 52 highly resistant and 77 intermediate resistant isolates harbored G1005S mutation. Presence of both G143A and G1005S mutations was detected in 101 isolates. Forty-nine isolates showed the presence of either G143A or G1005S

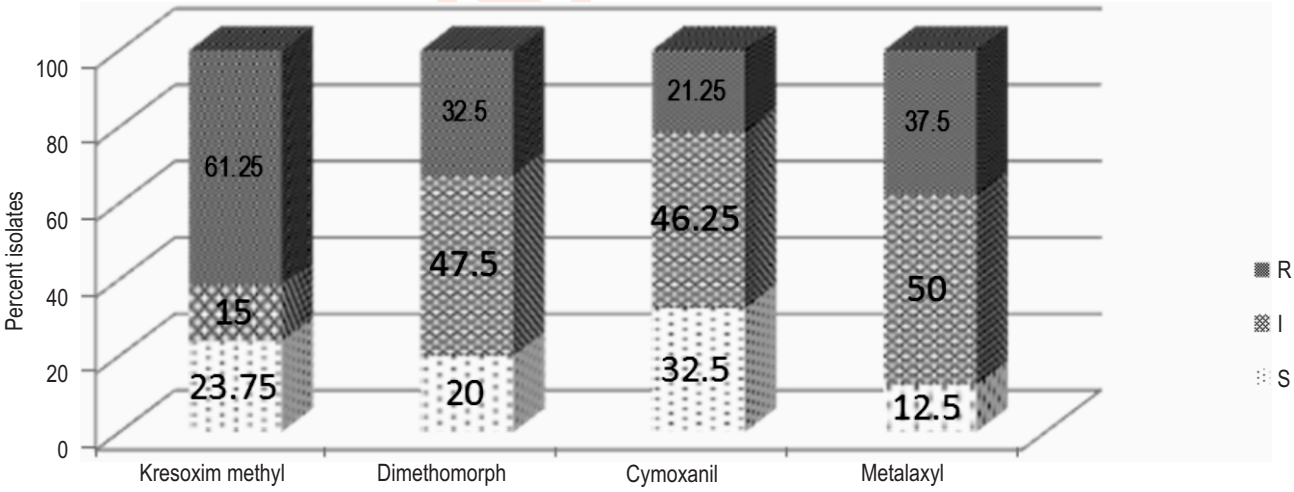

Fig. 1 : Sensitivity of 160 *P. viticola* isolates to kresoxim methyl, dimethomorph, cymoxanil and metalaxyl-M as determined by leaf disc bio-assay. R: highly resistant; I:intermediate resistant and S: sensitive *P. viticola* isolates.

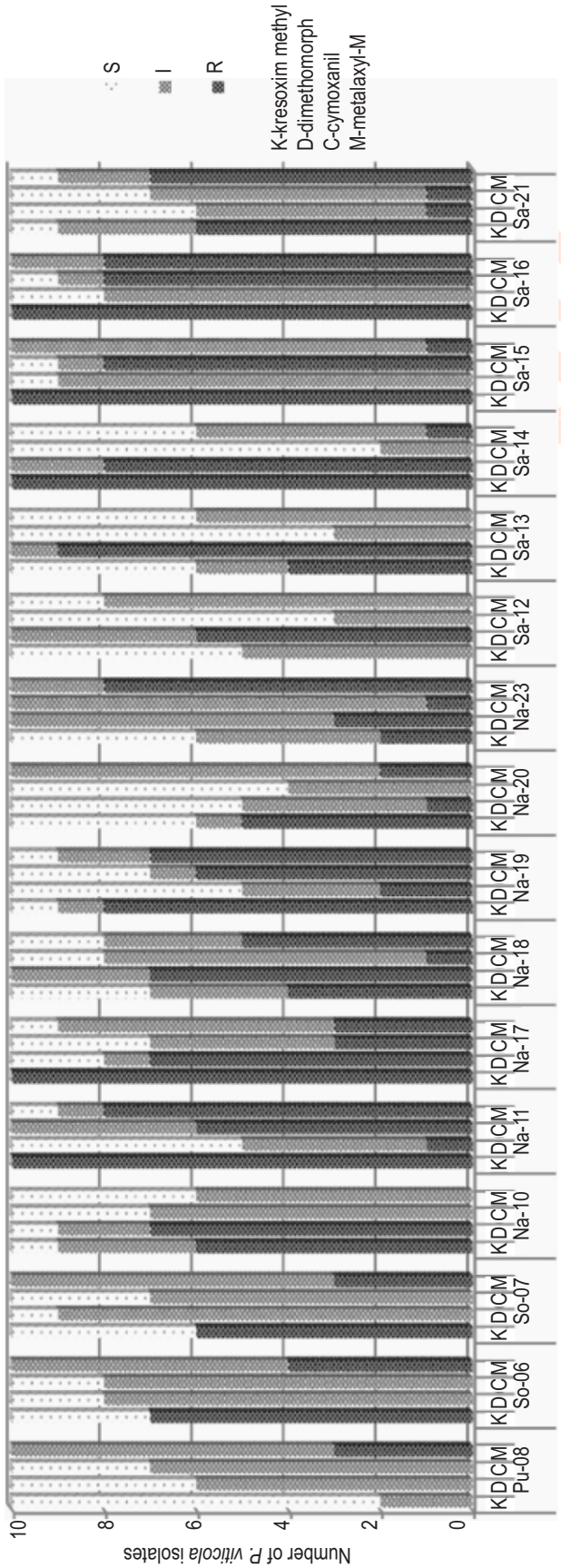

Fig. 2 : Vineyard wise distribution of highly resistant (R), intermediate resistant (I) and sensitive (S) *P. viticola* isolates to kresoxim methyl (K), dimethomorph (D), cymoxanil (C) and metalaxyl-M (M)

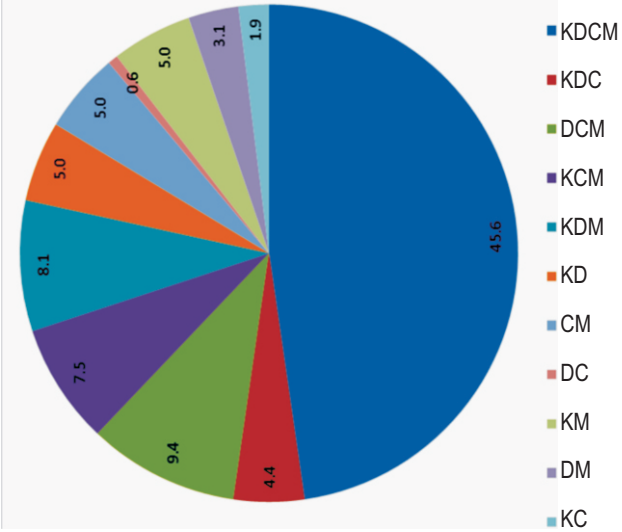

Fig. 3 : Cumulative percentage of highly resistant and intermediate resistant *P. viticola* isolates to kresoxim methyl (K), dimethomorph (D), cymoxanil (C) and metalaxyl-M (M) collected from 16 vineyards of Maharashtra showing multiple fungicide resistance.

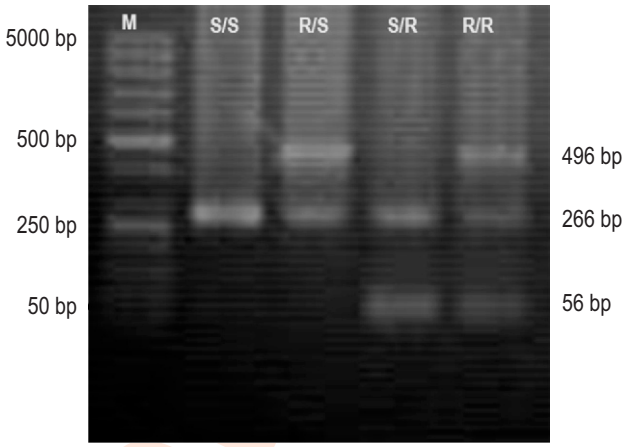

Fig. 4 : Image showing allele specific multiplex PCR product of QoI fungicide resistance gene G143A *cytb* gene 496 bp, CAA fungicide resistant G1105S *PvCes A3* gene 56 bp and internal control 266 bp 160 large rRNA gene of *P. viticola*, M- DNA ladder, S/S- senstive to QoI and CAA fungicide, R/S - QoI resistant /CAA sensitive, S/R - QoI sensitive /CAA resistant, R/R -Resistant to both QoI and CAA fungicide.

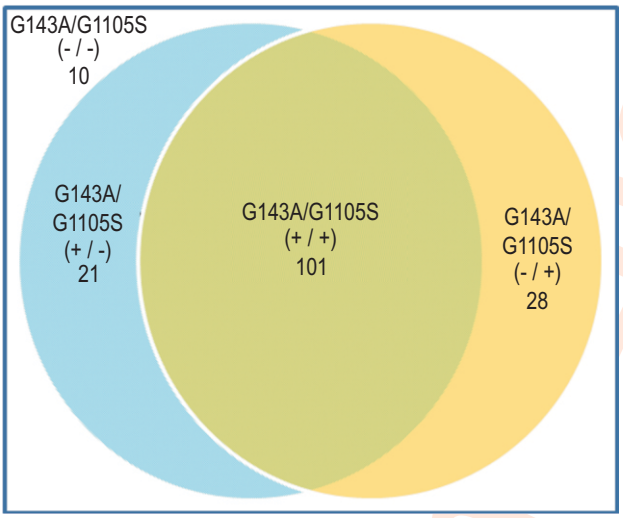

Fig. 5 : Venn diagram: Detection of point mutation *cytb* G143A and *PvCe SA3* gene G1105S in *P. viticola* isolates.

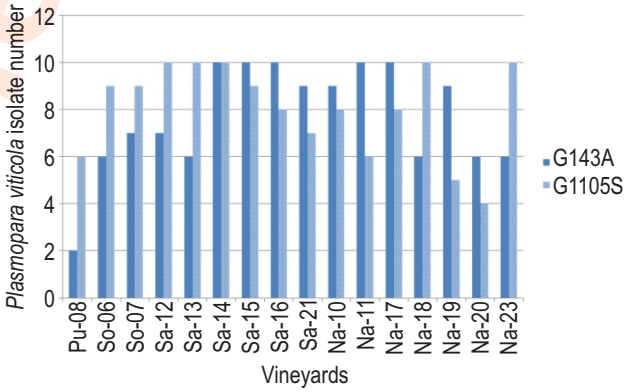

Fig. 6 : Detection of point mutation *cytb* G143A and *PvCeSA3* gene G1105S in *P. viticola* isolates in vineyards.

mutations while ten isolates did not harbor either of the mutations (Table 1, Fig. 5, 6).

This investigation brings forth data on detection of multi-fungicide resistance in *P. viticola* isolates from vineyards in Maharashtra, India. All vineyards showed the presence of intermediate resistant and/or highly resistant isolates to four different fungicide groups. Earlier resistance to at least two fungicides in *P. viticola* was reported (Giraud *et al*., 2013). This result shows similarity with resistance development to two fungicides in *P. viticola* population of Luxembourgian vineyards (Giraud *et al*. 2013).

ASP–PCR method confirmed that QoI and CAA intermediate resistant and highly resistant isolates harbored *cytb* G143A and *PvCesA3* G1105S point mutation whereas, these mutation was not detected in any of the sensitive isolates. In five vineyards cent percent isolates and in another six vineyards more

Table 1 : ASP-PCR detects fungicide resistance gene *cytb* G143A / *PvCesA3* G1105 Smutation in *P.viticola* isolate collected from vineyard of Maharashtra

Vineyard identity	*P. viticola* isolates detected (+) or not detected (-) with G143A / G1105Smutation										Total numbers detected with mutation	
	1	2	3	4	5	6	7	8	9	10	G143A	G1105S
Pu-08	+/-	-/+	-/+	+/+	-/+	-/-	-/+	-/+	-/-	-/-	2	6
So-06	+/+	+/+	-/-	-/+	-/+	-/+	+/+	+/+	+/+	+/+	6	9
So-07	+/+	+/-	-/+	-/+	-/+	+/+	+/+	+/+	+/+	+/+	7	9
Sa-12	-/+	-/+	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	7	10
Sa-13	-/+	-/+	+/+	+/+	-/+	+/+	+/+	+/+	+/+	-/+	6	10
Sa-14	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	10	10
Sa-15	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	10	9
Sa-16	+/+	+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/-	+/+	10	8
Sa-21	+/+	+/+	+/-	+/+	+/+	-/-	+/-	+/+	+/+	+/+	9	7
Na-10	+/+	+/+	+/+	+/+	+/-	+/+	+/+	+/+	-/+	+/-	9	8
Na-11	+/+	+/+	+/-	+/+	+/-	+/-	+/+	+/+	+/-	+/+	10	6
Na-17	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	10	8
Na-18	+/+	+/+	+/+	+/+	-/+	-/+	-/+	+/+	-/+	+/+	6	10
Na-19	+/+	+/+	-/-	+/+	+/+	+/-	+/-	+/-	+/-	+/+	9	5
Na-20	+/+	+/-	-/-	-/-	+/+	-/-	-/-	+/-	+/+	+/+	6	4
Na-23	-/+	+/+	+/+	+/+	-/+	+/+	+/+	-/+	-/+	+/+	6	10
Total	123	129										

Table S1: Number of sensitive, intermediate resistant and highly resistant isolates of *P. viticola* collected from sixteen vineyards against four fungicides

Region	Vineyard identity	Number of isolates of *P.viticola*[a]											
		Kresoxim methyl			Dimethomorph			Cymoxanil			Metalaxyl-M		
		S[b]	I	R	S	I	R	S	I	R	S	I	R
Pune	Pu-08	8	2	0	4	6	0	3	7	0	0	7	3
Solapur	So-06	3	0	7	2	8	0	2	8	0	0	6	4
	So-07	4	0	6	1	9	0	3	7	0	0	7	3
Nashik	Na-10	1	3	6	1	2	7	3	7	0	4	6	0
	Na-11	0	0	10	5	4	1	0	4	6	1	1	8
	Na-17	0	0	10	2	1	7	3	4	3	1	6	3
	Na-18	3	3	4	0	3	7	2	7	1	2	3	5
	Na-19	1	1	8	5	3	2	3	1	6	1	2	7
	Na-20	4	1	5	5	4	1	6	4	0	0	8	2
	Na-23	4	4	2	0	7	3	0	9	1	0	2	8
Sangli	Sa-12	5	5	0	0	4	6	7	3	0	2	8	0
	Sa-13	4	2	4	0	1	9	7	3	0	4	6	0
	Sa-14	0	0	10	0	2	8	8	2	0	4	5	1
	Sa-15	0	0	10	1	9	0	1	1	8	0	9	1
	Sa-16	0	0	10	2	8	0	1	1	8	0	2	8
	Sa-21	1	3	6	4	5	1	3	6	1	1	2	7
Total No.	160	38	24	98	31	77	52	52	74	34	20	80	60
Percentage	23.75	15.0	61.25	19.37	48.12	32.5	32.5	46.25	21.25	12.5	50.0	37.5	

[a]Out of total 10 number of *P.viticola* isolates sampled from each vineyard; [b]Sensitivity response of *P.viticola* isolates; S: sensitive; I: intermediate resistant; R:highly resistant to the fungicide

than 50% isolates were resistant to kresoxim-methyl. Application of five to six QoI fungicides during single-season caused QoI resistance in 90% population as compared to only 10% resistant population in untreated vineyards (Chapman *et al*., 2011*)*.

Long-term, extensive fungicide use also leads to the development of resistance to multiple fungicides as observed in *Venturia inaequalis* causing apple scab disease in orchards of Indiana and Michigan *(*Chapman *et al*., 2011*)*. Stepwise

accumulation of resistance in fungal pathogens and development of multiple fungicide resistance was reported from numerous countries in grapevines and from other crops (Koller and Wilcox, 2001; Rupp *et al.,* 2017; Panebianco *et al*., 2015). Similarly, single fungicide resistant *P. viticola* population present in vineyards can develop multiple fungicide resistance (Sawant *et al*., 2016a; Sawant *et al*., 2017; Rao and Reddy, 1988). Development of resistance to fungicides with different modes of action in *P. viticola* in the vineyards of Maharashtra is a matter of grave concern and should not to be neglected, as it may drastically reduce the effectiveness of these fungicides in disease control (Rupp *et al.,* 2017). *Plasmopara viticola* has the ability for fast adaptation to single-site fungicides and continuous application of such fungicide results in soft selective sweeps towards resistant isolates, leading to the development of several resistant haplotypes (Delmas *et al*., 2017). Resistance mechanism in *P. viticola* against single target site fungicide is due to single point polymorphism in the target gene, however, different levels of sensitivity in intermediate resistant and highly resistant *P. viticola* isolates is still unclear. This may be due to number of point mutations at different codon in the target gene, e.g. *cytb* and PvCeSA3 (Chapman *et al*., 2011).

Continuous use of single target site fungicides causes changes in *P. viticola* population frequency from sensitive to intermediate resistant and finally to highly resistant population, leading to failure of control of downy mildew disease. The development of such resistance against multiple groups of fungicides will pose a serious threat for downy mildew management in Maharashtra, India. A number of options like inducing resistance against downy mildew in grapevines, use of low risk chemical control options and effective biological control agents (Falk *et al*., 1996; Genet *et al*., 2006; Ghule *et al.,* 2018a; Ghule *et al*., 2018b; Sawant *et al.,* 2016b; Manjunath *et al*., 2017) and need based application of single-site fungicides will need to be used judiciously for successful management of downy mildew disease in vineyards. This Centre has prepared a bio-intensive strategy for disease management in grapes and successfully demonstrated it in farmer's fields at different locations in Maharashtra for two consecutive years.

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