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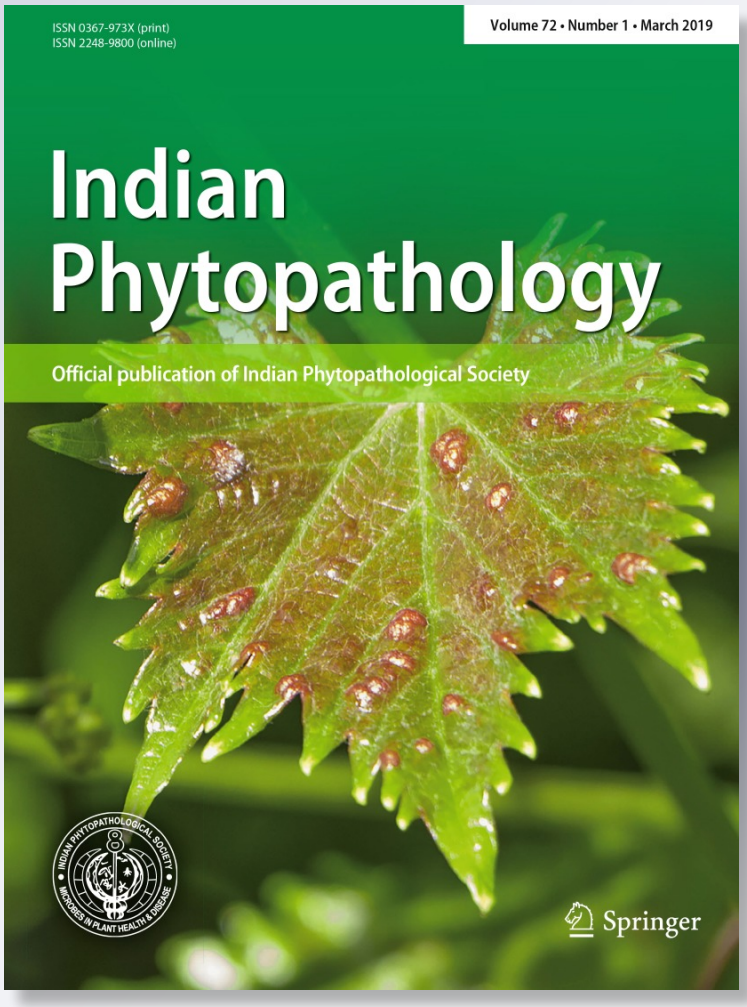

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RESEARCH ARTICLE

Detection of resistance to demethylation inhibitor fungicides in *Erysiphe necator* from tropical India by biological and molecular assays

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Abstract
Fungicides of demethylation inhibitor (DMI) group are used worldwide for the management of *Erysiphe necator* but are associated with medium to high risk of development of resistance in the pathogen. Till date there was no report on the presence of DMI resistance in *E. necator* isolates from the major grape growing regions in tropical India, though there were instances of DMI fungicides providing less than accepted levels of powdery mildew control. In this study, 54 *E. necator* isolates were collected during 2015–2017 from vineyards located in different geographical regions of India. The isolates were tested for their sensitivity to the commonly used DMI fungicide, myclobutanil, using leaf disc bioassay. Four isolates were sensitive (MIC < 1.0 µg/ml), nine were moderately resistant (MIC 1.0 to < 10 µg/ml) and 41 were resistant (MIC > 10 µg/ml) to the fungicide myclobutanil. The resistance factor (RF) ranged from 1.5 to 295. In PCR amplification of a specific allele, the product specific for A495T mutation was produced only in the 43 isolates with RF > 4. The *CYP51* gene sequence analysis confirmed A495T mutation leading to Y136F change associated with high levels of resistance to DMI fungicides. Cross resistance studies among the DMI fungicides showed that 11 out of 13 myclobutanil resistant isolates were also resistant to difenoconazole and tetraconazole. Three myclobutanil sensitive isolates were also sensitive to difenoconazole and tetraconazole. Detection of resistance in *E. necator* isolates from the major grape growing region of tropical India stresses on the need for developing resistance management strategies.

Keywords *CYP51* gene · Fungicide resistance · Maharashtra · Powdery mildew

Introduction

Grapevines are mainly grown in the tropical regions of India, especially in Maharashtra and adjoining regions of Karnataka. In these regions, powdery mildew is a serious disease as it occurs throughout the year, except during the hot summer months (Sawant et al. 2015). In these regions the grapevines do not undergo dormancy and are pruned twice in a year, although the crop is harvested only during October to March which is designated as the fruiting season. Powdery mildew decreases the vine productivity and also affects the fruit quality resulting in lower marketable yield and poor shelf-life of table grapes (Ashtekar et al. 2017).

Various fungicides, and few low risk chemicals and biocontrol agents are available for disease management. However, fungicides are preferred during high risk periods. Until 2016, only four different FRAC groups were available for management of powdery mildew in India. These were the demethylation inhibitors (DMIs) and quinone outside inhibitors (QoIs), dinitrophenylcrotonates and inorganic.

The Fungicide Resistance Action Committee (FRAC) categorizes the fungicides into different groups based on their mode of action. The DMI and the QoI fungicides have a single-site mode of action which is associated with medium to high risk of development of resistance in pathogen populations. The other two fungicides, sulfur and dinocap, are non-systemic fungicides and are classified as low risk fungicides.

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Powdery mildew disease is caused by the biotrophic fungus *Erysiphe necator* (Schwein.) Burrill (earlier *Uncinula necator*). *Erysiphe necator* is also classified as a pathogen with medium or high risk of development of resistance to fungicides by FRAC and the European and Mediterranean Plant Protection Organization (EPPO) respectively (FRAC 2013). The fungus has developed resistance to different fungicide groups, such as methyl benzimidazole carbamates (MBCs, FRAC group 1), demethylation inhibitors (DMIs, FRAC group 3), azanaphthalenes (FRAC group 13) and quinone outside inhibitors (QoIs, or strobilurins, FRAC group 11).

Generally 8–12 fungicide applications are required in a year, depending on the weather conditions (Sawant et al. 2011). The general recommendation is that use of these fungicides should be limited to maximum 2–3 applications per season. The DMIs have been used for management of powdery mildew in Indian vineyards for more than 30 years and in the initial years they have provided effective control of the disease at remarkably low doses (Bhujbal et al. 1982). DMIs such as triademefon, myclobutanil, fenarimol, difenoconazole, tetraconazole, hexaconazole, penconazole and flusilazole were used for powdery mildew management. Except for fenarimol which is a pyrimidine group fungicide, all other fungicides belong to the triazole group. Among these, myclobutanil, difenoconazole and tetraconazole fungicides are commonly used for powdery mildew management because of their low pre-harvest interval (PHI). Hexaconazole and flusilazole have high pre-harvest interval of 60 days and there are residue issues at harvest hence their use is very limited while triademefon and penconazole are no longer in use in India. Thus the triazole group is a major group of fungicides for powdery mildew control in India. They are usually applied in preventive programmes starting as early as the bloom stage (25–30 days after fruit pruning) and are continued until veraison. The other major fungicide group registered for management of powdery mildew is the group QoI which are also registered for downy mildew management. The systemic fungicides are mostly used by growers in early growth stages when risk of potential loss in yield due to downy mildew infection on clusters is high. Resistance in *E. necator* to QoI fungicides in Maharashtra, India has been reported earlier (Ghule et al. 2018).

Due to the frequent use of DMIs, resistance against these fungicides has been reported in several countries, including Australia (Scott 2001), Austria (Steinkellner and Redl 2001), Canada (Northover and Homeyer 2001), Chile (Frenkel et al. 2015), France (Delye et al. 1997; Dufour et al. 2011), Italy (Miazzi and Hajjeh 2011), New Zealand (Beresford et al. 2016), South Africa (Halleen et al. 2000) and the USA (Gubler et al. 1996; Rallos and Baudoin 2016; Frenkel et al. 2015; Colcol et al. 2012). In India reduced sensitivity to triademofon fungicide was reported by Thind et al. (1998). DMI involves the polygenic and quantitative nature of resistance and complete loss of efficacy generally does not occur (Brent and Hollomon 2007).

Quantitative variation in triazole sensitivity is thought to result from multiple mechanisms and multiple genes contributing to resistance. DMIs act by inhibiting the cytochrome P-450 sterol 14α-demethylase (P-45014DM) which is encoded by *CYP51,* a key enzyme in biosynthesis pathway of ergosterol, a precursor of a cell membrane component in fungi (Ma and Michailides 2005). Three different mechanisms of resistance were reported for DMIs resistance in fungi (Ma and Michailides 2005). The first reported mechanism in *E. necator* and other fungal pathogens is the single-point mutation at nucleotide position A495T in the 14 α -demethylase (*CYP51*) gene leading to the amino acid substitution of phenylalanine for tyrosine at codon position 136 (Y136F) as reported by Ma and Michailides (2005), Delye et al. (1997), and Frenkel et al. (2015). The Y136F mutation was detected in triadimenol-resistant isolates with resistance factors higher than five, but was absent in the isolates with resistance factors less than five (Miazzi and Hajjeh 2011; Delye et al. 1997). The second mechanism involves the constitutive overexpression of *CYP51* resulting from mutations in the promoter region upstream of *CYP51 (*Ma and Michailides 2005) or a synonymous mutation in *CYP51* gene, A1119C (Rallos and Baudoin 2016; Frenkel et al. 2015). Third mechanism is overexpression of ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporters genes which are involved in the efflux of these fungicides out of fungal cells (Ma and Michailides 2005).

Recently, grape growers from Maharashtra, reported low disease control in vineyards where DMI fungicides were used for powdery mildew management, indicating the possibility of reduced sensitivity against DMI fungicides in *E. necator*. The present study was undertaken to detect DMI fungicide resistance in *E. necator* isolates from India using biological and molecular methods.

Materials and methods

Sample collection

From July 2015 to December 2017, 54 powdery mildew infected leaf and bunch samples were collected from vineyards in Maharashtra, (9, Solapur; 10, Sangli; 17, Pune; 10, Nasik); Tamil Nadu (6, Theni) and Himachal Pradesh (2, Kinnaur). All vineyards were exposed to DMI fungicides, except the vineyard of Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Kinnaur, HP. Infected samples were collected in individual polypropylene bag and carried to the laboratory. The samples were incubated for 48 h

at 23 °C and 60% RH in a plant growth chamber (Binder, KBW 720, Germany) to favour fresh sporulation.

Culturing and multiplication of *E. necator*

Erysiphe necator is an obligate pathogen hence leaves from the susceptible *Vitis vinifera* cultivar, Thompson Seedless, were used for culturing the pathogen and also for the leaf disc assay. Plants were maintained in a Fibre-reinforced polymer (FRP) house under optimum growth and disease free condition. Fully expanded leaves from the 5th and 6th node from the apex of a growing shoot were harvested and surface sterilized using sodium hypochlorite (4% available chlorine) for 60 s followed by triple rinsing with sterile distilled water. They were placed in plant culture dishes containing approximately 20 ml of solidified 1.5% agar medium with their petioles immersed in agar. The leaves were inoculated by transferring the conidia using a sterile camel hair brush. Plates were incubated in the growth chamber with a 12 h photoperiod at 23 °C for 14 days for mass production of *E. necator* conidia. This sporulating culture was used as inoculum for bio-assay and also for DNA extraction.

Fungicide sensitivity bioassay

The sensitivity of the 54 isolates to a DMI fungicide, myclobutanil, was determined by leaf disc assay using its commercial formulations (Systhane 10% WP, Dow Agro) at 0, 0.1, 1, 4, 10, 40 µg/ml of active ingredient. The fungicide was dissolved in sterile distilled water to prepare 500 µg/ml stock solutions and serially diluted to get the appropriate concentration. Leaf discs of 15 mm diameter were cut from healthy, freshly detached Thompson Seedless leaves and surface sterilized as described above. Four discs were dipped in each fungicide concentration for 1 h, air dried and then placed adaxial side up on 2% water agar plates (50 mm diameter). The discs were left overnight in closed Petri dishes at 25 ± 2 °C and were then inoculated by dusting about 150 *E. necator* conidia from the mass multiplied culture with the help of a camel hair brush. Dishes were incubated as described above for 10 days to induce infection and sporulation.

After incubation, the leaf discs were observed under stereomicroscope for powdery mildew development and each disc was assessed using a rating scale of 0–5; where, 0, no visible mildew development; 1, up to 5%; 2, 6–25%; 3, 26–50%; 4, 51–75%; and 5, > 76% of disc surface covered with powdery mildew (Ishii et al. 2001). Then, disease severity was estimated by converting the rating to the percentage range mid-point for that particular rating [e.g., rating 2, 15.5 (6 + 25)/2] (Bock et al. 2009) and then the relative severity was calculated by dividing the average severity over four replications of fungicide treated discs by average severity on control discs. The EC_{50} was calculated by regressing percent inhibition on the log-transformed fungicide concentration using Microsoft Excel 2007 (Colcol et al. 2012). The minimum inhibitory concentration (MIC), i.e. the fungicide concentration at which powdery mildew growth was completely inhibited, was also determined. The resistance factor (RF) was calculated as EC_{50} of resistant strain/mean EC_{50} of sensitive strains (Delye et al. 1997).

Earlier studies had shown that while sensitive isolates were inhibited at < 1 µg/ml, resistant isolates could grow up to > 10 µg/ml (Beresford et al. 2016; Steinkellner and Redl 2001). Using this analogy, the fifty-four *E. necator* isolates were classified into three classes: sensitive (S), MIC < 1 µg/ml, moderately resistant (MR), MIC 1 to < 10 µg/ml, resistant (R) MIC > 10 µg/ml. Nineteen of these isolates were also tested for their sensitivity to two other DMI fungicides, difenoconazole (Score 25% SC, Syngenta) and tetraconazole (Domark 3.8% EW, Isagro) at 0 µg/ml, 1 µg/ml and 10 µg/ml for detection of sensitive, moderately resistant, and resistant isolates. Cross resistance among the DMI fungicides was determined by correlation analysis of the EC_{50} values performed using Spearman's rank correlation test in SPSS (Ypema et al. 1997) and by construction of a Venn diagram using Microsoft Excel.

Molecular characterization of the *CYP51* gene from *E. necator* isolates

DNA extraction

DNA was extracted from the 15 day old *E. necator* culture grown on leaves as described above. The fungal biomass was collected on a 1 cm^2 office cello tape strip and the strip was transferred to 1.5 ml sterile micro centrifuge tube and was kept at − 20 °C overnight. Then 100 µl of 5% chelex 100 resins (Bio-Rad, USA) and 2–3 sterile glass beads of 2 mm size were added to the sample. It was vortexed for 30 s and incubated at 95 °C for 10 min, vortexed again for 20 s and further incubated at 95 °C for 10 min. The solution was allowed to stand for 20 min and the supernatant was transferred to a sterile micro-centrifuge tube (Brewer and Milgroom 2010). DNA solutions were kept at − 20 °C until further usage.

PCR for amplification of a specific allele (PASA)

All 54 *E. necator* isolates were subjected to nested PCR for amplification of a specific allele (PASA) for detection of A495T mutation in the *CYP51* gene of the *E. necator* (Scott 2001). The first round of PCR was performed using primers U14DM (5′ATGTACATTGCTGACATTTTGTCGG3′) and C14R2 (5′CATCAACCGCATCATTTCCTA3′). This primer pair specifically amplifies a 1432 bp fragment of the *CYP51*

gene (nucleotides 1–1432). In the second round, the PCR product from the first round was added to a reaction mixture containing three primers MU4 (5′TCACAAGTATCG CATTTT3′), MU3R (5′TGGAATTTGGACAATCAA3′) and C14R-2. Primer pair MU4 and C14R-2 were used as an internal control for amplification of a *CYP51* DNA fragment from all isolates, while the primer pair MU4 and MU3R were used for amplification of a DNA fragment encompassing the A495T mutation.

The first round of PCR reaction was performed in 25 µl reaction volume containing 2.5 µl of 10 × *Taq* buffer, 2 µl dNTPs (2.5 mM each), 1 µl each of U14DM and C14R2 primers (10 µM), 1 µl of 1U *Taq* polymerase enzyme (Bangalore Genei, India), 1 µl DNA template and 16.5 µl of sterile nano pure water. The thermal cycling was performed with an initial denaturation of 4 min at 94 °C; 35 cycles with a denaturation step at 94 °C for 1 min, annealing at 65 °C for 2 min, extension at 72 °C for 2 min; and final extension of 7 min at 72 °C. The PCRs were performed on a ABI Gold GeneAmp PCR System 9700 (Applied Biosyatems, USA). The PCR product was resolved in 1.4% TAE agarose electrophoresis gel containing ethidium bromide and visualized using a gel-documentation system (Alpha Ease FC™ version 4.0.1, Alpha Innotech Corporation, USA).

The nested PCR was performed in 25 µl reaction volume containing 2.5 µl of 10 × *Taq* buffer, 2 µl dNTPs (2.5 mM each), 1 µl each of primers MU4, MU3R, C14R-2 (10 µM), 1 µl of 1U *Taq* polymerase enzyme (Bangalore Genei, India), 1 µl DNA template from the first round of PCR and 16.5 µl of sterile nano pure water. The thermal cycling was performed as described above except that the annealing temperature was kept at 51 °C.

Sequencing and analysis of *CYP51* gene

The sequencing of *CYP51* gene was performed for confirmation of A495T mutation. Five resistant, two moderately resistant and three sensitive isolates were selected for *CYP51* gene sequencing. The amplification of partial sequence of *CYP51* was performed using primer pair MU3 and C14R-2. PCRs were performed in 50 µl reaction volume containing 5 µl of 10 × *Taq* buffer, 4 µl dNTPs (2.5 mM each), 2 µl each of MU3 and C14R-2 (10 µM) primers, 2 µl of 1U *Taq* polymerase enzyme, 2 µl DNA template and 33 µl of sterile nano pure water. The amplification was performed as described for nested PCR. The PCR product was resolved in 1.4% TAE agarose electrophoresis gel containing ethidium bromide and visualized using a gel-documentation system. The PCR products were sequenced directly in both senses using primer pair MU3 and C14R-2.

The forward and reverse directional sequences of each isolate were aligned in MEGA 6 and manually edited to generate consensus sequences. Assembled sequences were used for similarity search using BLASTN against NCBI database. Earlier reported *CYP51* gene sequences of *E. necator* were retrieved from NCBI database and were aligned with the sequences of resistant and sensitive isolates of *E. necator* generated in this study. Multiple sequence alignment (MSA) was performed using ClustalW for determination of nucleotide variation(s). The open reading frame (ORF) was determined for partial *CYP51* gene from *E. necator* using NCBI ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder) and yeast mitochondrial genetic code. The putative *CYP51* protein was determined using Smart BLAST available at ORF finder by comparing with GenBank database. The multiple sequence alignment of amino acid sequences was also performed as described for nucleotide sequences.

Results and discussion

Fungicide sensitivity bioassay

The determination of sensitivity of fifty-four *E. necator* isolates, sampled from different geographical regions of India to myclobutanil showed varying levels of sensitivity (Table 1). Based on the MIC values, only four isolates were inhibited at 1 µg/ml myclobutanil showing their sensitivity. Two of these isolates were from the DMI unexposed vineyard at Kinnaur, H.P and the other two sensitive isolates were from a commercial vineyard at Nasik and at Theni. Nine isolates were inhibited at 10 µg/ml myclobutanil showing that they were moderately resistant, while 41 isolates could grow at 10 µg/ml myclobutanil showing that they were resistant to myclobutanil. Thus, more than 96% of the isolates from DMI exposed vineyards had developed resistance to myclobutanil.

The two sensitive isolates from the vineyard at Kinnaur, H.P., were inhibited at 1 µg/ml hence the EC_{50} value could not be determined. This high sensitivity was expected as there is no history of DMI fungicide use in these vineyards. However, the EC_{50} value of the two sensitive isolates from tropical India was 0.131 µg/ml and this value was used to calculate the resistant factor. The EC_{50} of moderately resistant isolates ranged from 0.15 to 1.43 µg/ml, while that of the resistant isolates ranged from 0.53 to 38.64 µg/ml.

In earlier studies from different countries, EC_{50} for myclobutanil of the sensitive population of *E. necator* (wild type population) was described in the range of less than 0.06 or about 0.8 µg/ml (Colcol et al. 2012; Gubler et al. 1996; Northover and Homeyer 2001; Steva and Clerjeau 1990; Wong and Wilcox 2002). While the EC_{50} values for the myclobutanil resistant *E. necator* isolates were in the range of 30–40 µg/ml (Gubler et al. 1996; Colcol 2008).

The continuous distribution EC_{50} values of *E. necator* isolates from this study supports the multigenic, quantitative

Table 1 Origin of *E. necator* isolates and their sensitivity to DMI fungicides myclobutanil, difenoconazole and tetraconazole

Sr.No	Isolate	Location	Myclobutanil			Difenoconazole			Tetraconazole			A495T mutation	RF
			EC_{50}	MIC	Class	EC_{50}	MIC	Class	EC_{50}	MIC	Class		
1.	Hp1	Himachal Pradesh	< 0.01	< 1	S	< 0.01	< 1	S	< 0.01	< 1	S	–	–
2.	Hp2	Himachal Pradesh	< 0.01	< 1	S	0.017	< 1	S	0.011	< 1	S	–	–
3.	NRCG D5-2	Manjari, Pune	12.709	> 10	R	0.695	< 10	MR	0.900	< 10	MR	+	97.02
4.	NRCG D5-3	Manjari, Pune	9.759	> 10	R	0.695	< 10	MR	1.176	< 10	MR	+	74.50
5.	NRCG D5-4	Manjari, Pune	7.698	> 10	R	1.191	> 10	R	0.979	< 10	MR	+	58.78
6.	NRCG D5-5	Manjari, Pune	5.521	> 10	R	1.260	> 10	R	0.518	< 10	MR	+	42.14
7.	NRCG FRP-C	Manjari, Pune	4.721	> 10	R	0.723	< 10	MR	0.151	< 1	S	+	36.03
8.	NRCG Bower-1	Manjari, Pune	3.199	> 10	R							+	24.43
9.	NRCG Bower-2	Manjari, Pune	2.873	> 10	R							+	21.91
10.	NRCG Bower-3	Manjari, Pune	7.655	> 10	R							+	58.47
11.	NRCG Bower-4	Manjari, Pune	0.197	< 10	MR							–	1.53
12.	NRCG Bower-5	Manjari, Pune	0.987	> 10	R							+	7.56
13.	NRCG Bower-6	Manjari, Pune	0.180	< 10	MR							–	1.37
14.	NRCG A1-1	Manjari, Pune	1.242	> 10	R							+	9.47
15.	NRCG A1-2	Manjari, Pune	5.010	> 10	R							+	38.24
16.	NRCG A1-3	Manjari, Pune	2.851	> 10	R							+	21.76
17.	Yavat 1	Yavat, Pune	13.462	> 10	R							+	102.75
18.	Yavat 2	Yavat, Pune	20.267	> 40	R							+	154.73
19.	Baramati-1	Baramati, Pune	21.314	> 40	R							+	162.67
20.	Nasik A1	Pimpalgaon, Nasik	0.422	< 10	MR							–	3.21
21.	Nasik A2	Pimpalgaon, Nasik	5.293	> 10	R							+	40.38
22.	Nasik A3	Pimpalgaon, Nasik	0.533	> 10	R							+	4.05
23.	Nasik A4	Pimpalgaon, Nasik	15.039	> 40	R							+	114.81
24.	Nasik K1	Kothure, Nasik	0.166	< 10	MR	0.048	< 1	S	0.255	< 10	MR	–	1.30
25.	Nasik K2	Kothure, Nasik	0.137	< 1	S	0.038	< 1	S	0.151	< 1	S	–	–
26.	Nasik K3	Kothure, Nasik	0.832	> 10	R	0.407	< 10	MR	0.868	< 10	MR	+	6.34
27.	Nasik K4	Kothure, Nasik	1.029	> 10	R	1.290	> 10	R	0.489	< 10	MR	+	7.86
28.	Sula 1	Sula vineyards, Nasik	0.864	< 10	MR							+	6.56
29.	Sula 2	Sula vineyards, Nasik	1.431	< 10	MR							+	10.92
30.	Karkambh C1	Pandharpur, Solapur	5.414	> 10	R	1.131	> 10	R				+	41.30
31.	Karkambh C2	Pandharpur, Solapur	0.259	< 10	MR	0.015	< 1	S				–	1.98
32.	Karkambh C4	Pandharpur, Solapur	5.991	> 10	R							+	45.73
33.	Karkambh C5	Pandharpur, Solapur	9.759	> 10	R				5.602	> 10	R	+	74.50
34.	Karkambh F2	Pandharpur, Solapur	6.770	> 10	R	2.041	> 10	R				+	51.68
35.	Karkambh H5	Pandharpur, Solapur	0.559	> 10	R				3.280	> 10	R	+	4.27
36.	SOA1	Nannaj, Solapur	10.206	> 10	R							+	77.94
37.	SOA2	Nannaj, Solapur	12.182	> 40	R							+	92.98
38.	SOA5	Nannaj, Solapur	20.781	> 40	R							+	158.63
39.	Borgaon A1	Borgaon, Sangli	2.712	> 10	R	0.695	< 10	MR	2.302	> 10	R	+	20.69
40.	Borgaon A2	Borgaon, Sangli	5.703	> 10	R	1.437	> 10	R	4.020	> 10	R	+	43.51
41.	Borgaon A3	Borgaon, Sangli	5.524	> 10	R	4.367	> 10	R	2.968	> 10	R	+	42.14
42.	Borgaon A5	Borgaon, Sangli	5.509	> 10	R	1.066	< 10	MR	0.926	< 10	MR	+	42.06
43.	Walva 1	Walva, Sangli	9.142	> 10	R							+	69.77
44.	Walva 2	Walva, Sangli	38.643	> 40	R							+	294.96
45.	Nimani A1	Nimani, Sangli	10.810	> 10	R	1.296	< 10	MR	1.048	> 10	R	+	82.52
46.	Nimani A2	Nimani, Sangli	3.931	> 10	R							+	30.00
47.	Nimani A4	Nimani, Sangli	0.436	< 10	MR							–	3.36
48.	Nimani A5	Nimani, Sangli	0.864	> 10	R				1.424	< 10	MR	+	6.56

Table 1 (continued)

Sr.No	Isolate	Location	Myclobutanil			Difenoconazole			Tetraconazole			A495T mutation	RF
			EC_{50}	MIC	Class	EC_{50}	MIC	Class	EC_{50}	MIC	Class		
49.	TNB 2	Theni, Tamil Nadu	5.963	> 10	R							+	45.50
50.	TNB 4	Theni, Tamil Nadu	0.146	< 10	MR							–	1.15
51.	TNB 6	Theni, Tamil Nadu	3.730	> 10	R							+	28.47
52.	TNB 9	Theni, Tamil Nadu	2.586	> 10	R							+	19.77
53.	TNB 10	Theni, Tamil Nadu	9.306	> 40	R							+	71.07
54.	TNB 11	Theni, Tamil Nadu	0.125	< 1	S							–	–

S Sensitive, *MR* moderately resistant, *R* resistant, *RF* Resistance factor for myclobutanil

pattern of resistance against DMI fungicides (Delye et al. 1997; Erickson and Wilcox 1997; Gubler et al. 1996). The resistance factor (RF) for the 50 fifty myclobutanil resistant isolates ranged from 1.15 to 295. High resistance factor of 378 and 104 in myclobutanil resistant isolates was reported by Colcol et al. (2012) and Rallos and Baudoin (2016) respectively.

The sensitivity assay for difenoconazole and tetraconazole showed that the MIC of isolates from Kinnaur was < 1 µg/ml indicating their sensitivity to these two fungicides, too. As this was a DMI unexposed region, development of resistance to DMI fungicides was not expected. Of the 17 isolates from tropical India evaluated against difenoconazole and tetraconazole, three isolates were sensitive while seven each were moderately resistant and resistant to difenoconazole; while two isolates were sensitive, nine were moderately resistant and six were resistant to tetraconazole (Table 1). The EC_{50} values of these isolates ranged from 0.015 to 4.367 µg/ml for difenoconazole and from 0.151 to 5.602 µg/ml for tetraconazole.

Cross resistance

Correlation analysis using EC_{50} values showed that all the three correlation coefficients were positive, and were statistically significant at $P < 0.05$ (Fig. 1a–c) indicating cross resistance between the three DMI fungicides. The correlation observed in this study was only partial as some of the isolates had a high EC_{50} to one fungicide and much lower values for others. Of the 12 myclobutanil resistant isolates, five and four isolates were resistant to difenoconazole and tetraconazole respectively while the remaining isolates exhibited moderate resistance to these two fungicides (Table 1). One myclobutanil resistant isolate showed moderate resistance to difenoconazole but was sensitive to tetraconazole, while another isolate was moderately resistant to myclobutanil and tetraconazole but was sensitive to difenoconazole. The partial nature of positive correlations was also reported in earlier studies (Erickson and Wilcox 1997; Steva and Clerjeau 1990). This indicates an isolate resistant to one DMI does not always have increased resistance to another. This difference in cross resistance pattern between different DMI fungicides is due to the intrinsic activity of individual DMIs (Gubler et al. 1996).

The Venn diagram constructed based on the MIC values, showed that 11 out of 13 myclobutanil resistant isolates were also resistant to difenoconazole and tetraconazole exhibiting cross resistance (Fig. 1d). The remaining two isolates were resistant to either difenoconazole or tetraconazole. The three myclobutanil sensitive isolates were also sensitive to difenoconazole and tetraconazole. Two different types of mechanisms for DMI resistance were speculated, fungicide-specific mechanism which confers resistance to one or more but not all DMI fungicides, and group-specific mechanism confer resistance to all DMI fungicides (Colcol et al. 2012). The results from this study suggest that both types of mechanisms are present in *E. necator* population from tropical India.

Due to the incomplete nature of spray history information obtained and few numbers of isolates from many of the individual vineyards, it was difficult to correlate relationships between EC_{50} and spray history. However, the six isolates from the research vineyards of this Center where myclobutanil and difenoconazole were used more frequently as compared to tetraconazole, the EC_{50} values were higher for myclobutanil and difenoconazole compared to tetraconazole.

PCR amplification of a specific allele (PASA) in the *CYP51* gene of *E. necator*

In the first round of PCR, all the 54 *E. necator* isolates produced the amplification product of 1432 bp size. In the second round of PCR, all the myclobutanil resistant and two moderately resistant isolates produced two bands of size 1362 and 409 bp. The detection of 409 bp product indicates an A495T mutation in *CYP51* gene associated with reduced sensitivity to myclobutanil (Colcol et al. 2012; Rallos and Baudoin 2016). The EC_{50} value of these isolates was > 0.8 µg/ml. Seven moderately resistant and all the four sensitive isolates produced only one product of 1362 bp

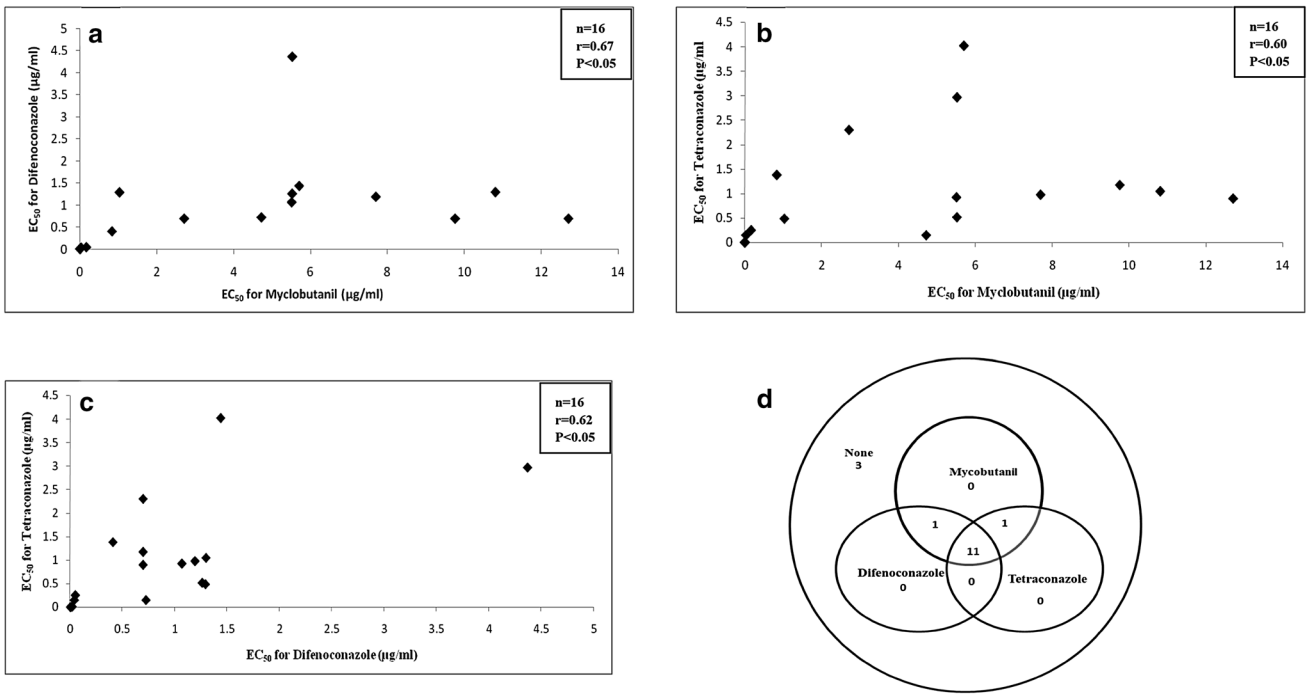

Fig. 1 Cross resistance pattern of sixteen *E. necator* isolates for three DMI fungicides, myclobutanil, difenoconazole and tetraconazole. Scatter plot illustrating the correlation among the EC_{50} values relative to **a** myclobutanil and difenoconazole, **b** myclobutanil and tetraconazole, **c** difenoconazole and tetraconazole and **d** Venn diagram showing three-way comparison of resistance to myclobutanil, difenoconazole and tetraconazole. The large circle represents all the sixteen isolates; each smaller circle represents the isolates resistant to the fungicide(s). Numbers within the circles indicate the number of individual isolates within each indicated subset

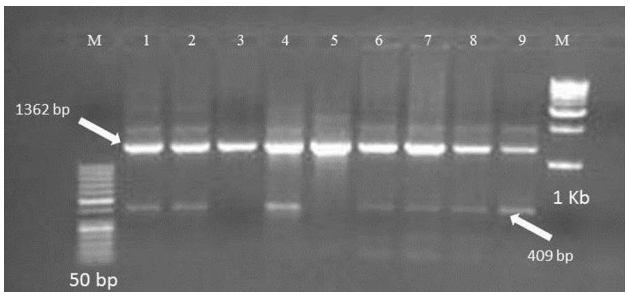

Fig. 2 PCR amplification of a specific allele (PASA) from *CYP51* gene from seven myclobutanil resistant *E. necator* isolates; Lanes 1, 2, 4, 6–9 denote NRCG A1-1, NRCG Bower-6, TNB 10, Nasik A3, Sula 1, Borgaon A1 and SOA5 isolates respectively. Lanes 3 and 5 denote two sensitive isolates Hp1 and TNB 11 respectively. M denotes DNA markers of 50 bp (L) and 1 kb (R). Absence of 409 bp product indicates the absence of A495T mutation

indicating the absence of an A495T mutation (Fig. 2). The EC_{50} value of these isolates was much lower and ranged from 0.15 to 0.44 µg/ml.

The 409 bp product was detected in *E. necator* isolates with resistant factors higher than four and was absent in isolates with resistance factors less than four. Earlier, this mutation was reported in *E. necator* isolates with resistant factors higher than five and was absent in isolates with resistance factor less than five against triademofon (Miazzi and Hajjeh 2011; Delye et al. 1997). This shows that the lower level of resistance is governed by some other mechanisms which need to be elucidated. DMI resistance is not mono-allelic and there may be other mechanism operating in addition to the existing A495T mutation. Further study needs to be done to confirm the polygenic trait using large number of population for other mechanisms such as *CYP51* expression and expression of ABC transporter and MFS homologs.

Sequencing and analysis of *CYP51* gene for detecting the A495T mutation

The *CYP51* gene sequences of *E. necator* from this study were subjected to MSA alignment with the *CYP51* sequences of resistant and sensitive isolates of *E. necator* retrieved from GenBank. The A495T mutation was observed only in the resistant isolates but not in the sensitive isolates and the moderately resistant isolates with resistance factor less than four (Table 2). In MSA alignment of amino acid sequence the A495T mutation resulted in Y136F change. Our isolates did not exhibit the A1119C mutation which is correlated with high expression of *CYP51* gene conferring DMI resistance as described in other studies (Frenkel et al. 2015; Rallos and Baudoin 2016). The two haplotypes of *CYP51* gene

Table 2 Nucleotide alignment of sequences of *CYP51* gene in Indian *E. necator* isolates with varying levels of sensitivity to myclobutanil with reference sequences of resistant and sensitive isolates retrieved from GenBank

Haplotype	Isolates/accession number	Nucleotide sites						References
		323	324	492	495	1119	1170	
CYP51+	EF649776	A	C	T	A	A	G	Scott (2001)
CYP51+	KM077178	A	C	T	A	A	A	Frenkel et al. (2015)
CYP51^{A1170G}	KM077180	A	C	T	A	A	G	
CYP51$^{A495T/A1170G}$	KM077179	A	C	T	**T**	A	G	
CYP51$^{A1119C/A1170G}$	KM077181	A	C	T	A	C	G	
CYP51^{A1119C}	KM077182	A	C	T	A	C	A	
CYP51+	KR106192	T	A	T	A	A	G	Rallos and Baudoin (2016)
CYP51^{A495T}	KR106193	T	A	T	**T**	A	A	
CYP51^{A495T}	Yavat 2 (R)	T	A	C	**T**	A	G	This study
CYP51^{A495T}	Walva 2 (R)	T	A	C	**T**	A	G	
CYP51^{A495T}	SOA5 (R)	T	A	C	**T**	A	G	
CYP51^{A495T}	Nimani A1 (R)	T	A	C	**T**	A	G	
CYP51^{A495T}	TNB 2 (R)	T	A	C	**T**	A	G	
CYP51+	Karkambh C2 (MR)	T	A	C	A	A	G	
CYP51+	Nasik A1 (MR)	T	A	C	A	A	G	
CYP51+	Hp1 (S)	T	A	C	A	A	G	
CYP51+	Nasik K2 (S)	T	A	C	A	A	G	
CYP51+	TNB 11 (S)	T	A	C	A	A	G	

Note the nucleotide substitution from A to T at position 495 shown in bold fonts. Mutation causes an amino acid substitution from tyrosine (Y) to phenylalanine (F) at codon 136. Mutation Y136F is present in resistant isolates. Nucleotide sites are relative to the reference *CYP51* sequence in GenBank accession EF649776. Additional nucleotide substitutions found at positions 1119 and 1170 in the United States are absent in Indian isolates. *CYP51+* indicates wild type mutant while numbers in superscripts represent mutation at that particular site. The sensitive, moderately resistant and resistant isolates from this study are indicated by (S), (MR) and (R) respectively

sequences, from a resistant and a sensitive isolate, have been deposited in GenBank under accession numbers MH352351 and MH352350 respectively.

Additionally, all our isolates harbored a T492C mutation in the *CYP51* gene which was absent in the sequences retrieved from NCBI database. This mutation resulted in a V135A change in amino acid sequence. Presence of this mutation in both sensitive and resistant isolates indicates that this mutation is not involved in DMI resistance. Earlier, too, a A1170C mutation in *CYP51* gene was reported but was not found to be involved in DMI resistance (Frenkel et al. 2015; Rallos and Baudoin 2016).

The study points the urgent need for monitoring for DMI fungicide resistance in *E. necator* populations in India as is being initiated in many other countries (Beresford et al. 2015) and development of mitigating strategies. It will be important to determine if cross resistance to other chemical groups, especially to QoI fungicides (Dufour et al. 2011) is widespread in Indian vineyards as some of our isolates were resistant to both DMI and QoI fungicides. Thus, a good strategy will depend on not more than two consecutive DMI applications as preventive and not more than three–four application per season in mixture or alternation with non-cross resistant fungicides (FRAC 2017). Interspersing of fungicide applications with bio-control agents and low risk chemicals is reported to enhance powdery mildew control and shelf-life of grapes (Ashtekar et al. 2017; Sawant et al. 2017) and may help in resistance management. A decrease in myclobutanil resistance and better powdery mildew control was observed in vineyards when it was used in rotation with the non-systemic fungicide, sulfur, compared to its use as solo or as tank mix with sulfur (Gubler et al. 1996; Ypema et al. 1997).

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