P9: Mutation in the 14α-Demethylase Gene of *Erysiphe necator*

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Introduction

Powdery mildew, caused by the obligate fungus *Erysiphe necator* (Schw.) Burr., is one of the most common and severe diseases of grapevine wherever the crop is intensively grown. It costs millions of dollars annually to vine growers, due to crop losses and intensive usage of fungicides for its control.

Sterol demethylation inhibitors (DMIs) fungicides became very important in grapevine protection since their discovery in the late 1960's, due to their broad spectrum of activity, systemic movement in the plant, potential curative activity, and high effectiveness.

Acquired resistance to DMIs in *E. necator* has been reported after 10 years of the DMIs usage in Portugal (Steva *et al*., 1988) in France (Steva *et al*., 1989), in Italy (Garibaldi *et al*., 1990), and in California (Miller and Gubler , 2003).

One single-point mutation in the *CYP51* gene coding for a cytochrome P450, causing an amino acid change from tyrosine to phenylalanine at position 136 of the native protein, was found responsible of low resistance to triadimenol in *E. necator* (Délye *et al*., 1997a) and in *Erisiphe graminis* (Délye and Corio-Costet, 1998). Several single-point mutations in the *CYP51* gene at amino acid positions 129, 132, 405, 464 and 467 confer low resistance to DMIs in *Candida albicans* (Sanglard *et al*., 1998).

The present work is aiming at assessing the base line sensitivity of *E. necator* to DMIs fungicides and to study the molecular bases of DMIs resistance in *E. necator*.

Material and methods

Fungicide testing: Leaves from *in vitro*-grown grapevine plantlets were used to assess the sensitivity of *E. necator* isolates to fungicides. Leaves production and maintenance of used isolates were as described by Miazzi *et al*.(1997).

Commercial formulations of the following fungicides were used: tebuconazole (Folicur®, Bayer CropScience), and triadimenol (Bayfidan®, Bayer CropScience). Fungicides were suspended in sterile water at the final concentrations of 0.1, 0.3, 1, 3, and 6 μg ml^{-1} of active ingredient.

Leaves of cv. Baresana were used, taking in consideration that they were uniform and of a similar sizes as possible to minimize any possible influence on the growth of *E. necator* colonies. Fungicides were applied just before usage by immersion of leaves into glass beakers, containing the fungicide suspension at appropriate concentration for 1 min under gentle shaking; leaves immersed in water were the untreated check.

Single leaves were then placed in 55-mm-diam Petri dishes containing 10 ml of the substrate B0/2 as described by Miazzi *et al*.,.(1997). Petri dishes were left closed in a laminar flow cabinet for 24 hr before inoculation. Each leaf was inoculated on three points with 15-30 conidia. Leaves were then kept in a growth chamber at 21±1°C and exposed 16 hours per day to the light produced by a combination of 3 Osram L36W Cool White lamps and 3 Silvanya Grolux F36W lamps. At 1-week intervals, diameters of colony were measured with the aid of a stereomicroscope.

When needed, resistance factor (RF) was calculated by using the formula RF = EC_{50} for the resistant isolate / EC_{50} of sensitive isolates; where EC_{50} is the Effective Concentration at 50%.

Molecular assay: Allele-specific PCR amplification of *E. necator* DNA was performed using the primers MUT1 and U14DM, designed on the region of the *CYP51* gene, where the point mutation were responsible for the high resistance to DMIs occurs (Délye *et al.,* 1997b). PCR were performed as described by Délye *et al.* (1997b). Amplification products were separated

by gel electrophoresis as described by Miazzi *et al*.(2008). The primer pairs C14 and C14R that amplify the whole *CYP51* gene (Délye *et al*., 1997b) were used in PCR with resistance and sensitive *E. necator* strains. The PCR conditions were as described for the primers MUT1 and U14DM, with the only exception that the annealing temperature was 65°C. Amplified DNA were cloned and sequenced as described in by Hajjeh *et al*., (2005).

Results

Twenty *E. necator* isolates sampled from 10 different Italian vineyards, with DMIs treatment histories, were used to establish baseline sensitivity to tebuconazole and triadimenol.

The MUT1 and U14DM primers were used to screen 50 isolates sampled in 7 different Italian vineyards. Only 4 isolates (X109, X112, X113 and X115), carrying the point mutation in the *CYP51* gene, hence yielding a DNA band of expected size, were detected (Fig. 1).

The normal response to tebuconazole, established for 20 fungal isolates through an *in vitro* bioassay, was EC_{50} = 0,1-3 μg ml^{-1} and MIC = 1-6 μg ml^{-1}. The isolates X112 and X115 and the isolate X104, not carrying the mutation, were less sensitive than normal (EC_{50} = 6-10 μg ml^{-1} and MIC = 10 μg ml^{-1}), while the isolate X109, although carrying the point mutation (Table 1), was normally sensitive (EC_{50} = 0.3 μg ml^{-1} and MIC = 3 μg ml^{-1}).

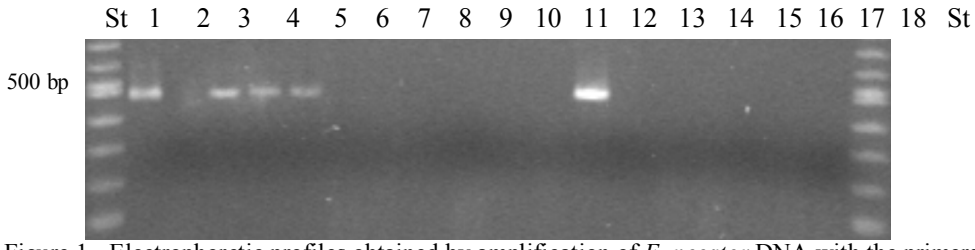

Figure 1 - Electrophoretic profiles obtained by amplification of *E. necator* DNA with the primers MUT1 and U14DM. Notice the presence of the point mutation in the *CYP51* gene in the isolates at lanes 1, 3, 4, 5, 12.

Table 1 – *In vitro* response of 4 putative field mutants of *E. necator* resistant to tebuconazole.

Fungicide concentration		Diameter (mm) of 20-days-old colonies			
(μg ml^{-1})		X104	X109	X112	X115
0		6	7	7	7
0.1		6	5	6	6
0.3		5	3.5	4	6
1		5	2	6	5
3		5	0	4	5
6		4	0	4	4.5
10		0	0	0	0
20		0	0	0	0
100		0	0	0	0
	EC_{50}	6-10	0.3	6-10	6-10
	MIC	10	3	10	10

The primer pairs C14 and C14R that amplify the whole *CYP51* gene were used in PCR with the four strains. Amplified DNA was cloned, sequenced and aligned with the nucleotide sequence of the whole *E. necator CYP51* gene (1,755 nt) available in GenBank (accession number U83840) (Déley *et al*., 1997a). The alignment confirmed that the obtained sequences were actually of the *CYP51* gene and that the strains X109, X112 and X115, but not X104, showed the point mutation A495T. Additional point mutations at different positions on the

CYP51 sequence were detected in X112 (G136T, A344G and G1753T), in X109 (A733G), in X115 (C1304), and in X104 (A732C).

Our results suggest that the point mutation A495T in the *CYP51* gene is not constantly associated with resistance to DMIs in *E. necator*. Further studies are in progress to explore the molecular basis of resistance to DMIs of the grape powdery mildew fungus *E necator*.

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