

## CROSS RESISTANCE AND FITNESS OF ISOLATES OF *VENTURIA INAEQUALIS* TO DMI FUNGICIDES

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### ABSTRACT

Cross resistance was assessed using a colony growth test with nine isolates of *V. inaequalis* and seven demethylation inhibitor (DMI) fungicides. All isolates were cross resistant to eight triazole and one non-triazole DMI fungicides. Resistance to triforine was the most correlated to other products. A leaf disc test in controlled environments was used to estimate the duration of the incubation period, the percentage of diseased area and conidial production of selected sensitive and resistant isolates. More host tissue was colonised, more conidia were produced and the incubation period was reduced at the highest spore concentration applied, but there were no significant differences between any isolates tested. Resistance to DMI products in these isolates was not associated with any reductions in pathogenicity or fitness on host tissues under the test conditions.

**Keywords :** demethylation inhibitors (DMIs), cross resistance, *Venturia inaequalis*, black spot, fitness

### INTRODUCTION

The development of resistance in *V. inaequalis* to DMI products has been reported (Thind *et al.* 1986; Fiaccadori *et al.* 1987) but practical resistance in the field has not yet been shown to be a serious problem except in a few specific locations. Reduced sensitivity of *V. inaequalis* strains to DMIs has been reported in the USA and Germany (Stanis and Jones 1985) and in New Zealand (Whelan *et al.* 1992).

Resistant strains are frequently cross resistant to structurally related chemicals or to chemicals with similar modes of action (Delp 1980). However, some strains resistant to a DMI compound are not cross-resistant to all other DMI compounds (Köller 1988). Mutants which are resistant to a particular fungicide may vary in pathogenicity and fitness. However, they may not differ from the wildtype fungus if mutation to resistance does not show pleiotropic effects with respect to these characters (Köller 1991). Evidence has been presented that some benomyl-resistant isolates of *V. inaequalis* from one apple orchard in Pennsylvania were less fit than the sensitive isolates (Lalancette *et al.* 1987). The objective of this study was to investigate the degree of cross resistance to DMI fungicides in selected isolates and to evaluate some fitness characteristics.

### METHODS

Nine isolates of *V. inaequalis* (Plant Protection Research Unit, Lincoln University) were tested for resistance to penconazole, myclobutanil, fenarimol, triforine, flusilazole, pyrifenoxy and bitertanol in a randomised complete block design with three replications. Colony growth was measured as an indication of relative resistance, expressed as EC<sub>50</sub> values and as a resistance factor (RF) in relation to the most sensitive isolate.

Five different concentrations of each product were used, based on the EC<sub>50</sub> values from previous studies (Whelan *et al.* 1992; Smith *et al.* 1991) and a preliminary test, as follows:

penconazole (Topas, 100 g/litre): 0, 0.04, 0.08, 0.2 and 0.6 mg/litre  
myclobutanil (Systhane, 400 g/litre): 0, 0.03, 0.1, 1, and 4 mg/litre

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fenarimol (Rubigan 12EC, 120 g/litre): 0, 0.03, 0.1, 1 and 3 mg/litre  
 triforine (Saprol, 190 g/litre): 0, 0.5, 5, 10 and 30 mg/litre  
 flusilazole (Nustar, 200 g/litre): 0, 0.05, 0.1, 0.2 and 0.4 mg/litre  
 pyrifenoxy (Dorado, 250 g/litre): 0, 0.05, 0.1, 0.2 and 0.4 mg/litre  
 bitertanol (Baycor, 500 g/litre): 0, 0.05, 0.1, 0.2 and 0.4 mg/litre.

Concentrations of penconazole, myclobutanil, fenarimol and triforine were prepared by dissolving in acetone and diluting to volume with sterile water. Flusilazole, pyrifenoxy and bitertanol were dissolved directly in sterile water. Fungicide suspension, benzylpenicillin (100 mg/litre) and streptomycin sulphate (100 mg/litre) were added to molten PDA at 50°C, mixed well and poured immediately into 90 mm diameter Petri dishes. A 4 mm mycelial plug from the margin of a 21 day-old culture of *V. inaequalis* was placed on amended PDA and incubated at 20°C in the dark. The mean colony diameter of each treatment was measured manually with a calliper micrometer, with two measurements taken at 90° to each other, after 20 days of incubation for all products except triforine. For triforine the measurements were recorded after 11 days of incubation because this chemical was unstable when plates were incubated for long periods (Whelan *et al.* 1992). EC<sub>50</sub> and EC<sub>95</sub> values were calculated from linear regression equations of the colony diameter and log<sub>10</sub> concentration of each fungicide. EC<sub>50</sub> and EC<sub>95</sub> values for bitertanol were excluded from analysis by ANOVA because of high variations of values derived from linear regression equations.

Six isolates of *V. inaequalis* were selected, based on their EC<sub>50</sub> values to myclobutanil, for an investigation of their pathogenicity and fitness. These were classed as medium-resistant types (MR), low-resistant (LR) and sensitive (S) types. Conidia for inoculation were produced by growth on PDA at 20°C for 15 days. Leaf discs were prepared by washing young, fully expanded "Red Delicious" apple leaves with tap water, dipping in a solution of sodium hypochlorite (1% active hypochlorite) for 1 minute and then rinsing three times with sterile distilled water. Leaf discs were cut with a sterile cork borer (20 mm diameter) and then placed in sterile Petri dishes (14 cm diameter) containing sterile wet filter paper. Droplets (40 µl) of conidial suspensions (5x10<sup>3</sup> and 5x10<sup>4</sup> spores/ml) were placed on the adaxial surface of leaf discs. The surface area covered by the inoculum droplets was termed the inoculation site. A randomised complete block design with three replications (four leaf discs/replication) was used. Leaf discs inoculated with droplets of sterile water served as controls. The Petri dishes were sealed with cling wraps to maintain high relative humidity for 72 h. Then the seal was removed, the inoculated leaf discs were incubated at 20 ± 2°C with a 12 h photoperiod until black spot lesions appeared.

The length of the incubation period was measured as the time from inoculation to the first appearance of lesions. Assessment was made every day after 3 days post-inoculation both visually and by binocular microscope. Further disease development was assessed as the percentage area of each disc infected with black spot. Diseased area was estimated visually and by binocular microscope at 12, 18 and 23 days post-inoculation. The density of colonisation was also estimated and, combined with diseased area, was described as disease severity. Conidial production defined as the number of conidia produced per leaf disc, was assessed 24 days post-inoculation. Conidia were removed from lesions by scraping the surface with a hairbrush, and then placed into sterile water, mixed and counted with a haemocytometer.

The data for each variable were analysed by ANOVA. For the incubation period, the effects of isolates and inoculum densities on incubation period were analysed using a factorial ANOVA. Data were log transformed prior to analysis to stabilise variances.

## RESULTS AND DISCUSSION

Different degrees of sensitivity were observed in isolates of *V. inaequalis* to all the DMI fungicides tested, with the lowest sensitivity being to triforine and the greatest sensitivity to flusilazole (Table 1). Cross resistance varied considerably. Resistance factors varied from 2.8 (flusilazole) to 10.7 (triforine) for isolate 1 and from 9.4 (flusilazole) to 163.6 (triforine) for isolate 5 (Table 1). Resistance to the DMI compounds is commonly governed by multiple-gene mutations although a UV-

induced major gene of *Nectria haematococca* var. *cucurbitae* that confers a high degree of resistance to some of the DMIs was reported (Kalamarakis *et al.* 1989). Multiple gene mutations may account for the range in cross resistance observed.

**TABLE 1: Sensitivity and resistance factor of nine isolates of *V. inaequalis* to DMI fungicides (detected by colony growth test).**

Isolate	myclobutanil	EC <sub>50</sub> (mg/litre) and resistance factor					Isolate mean (EC <sub>50</sub> )
		pyrifenoxy	flusilazole	fenarimol	penconazole	triforine	
1	0.157 <sup>1</sup> (9.8) <sup>2</sup>	0.062 (4.4)	0.028 (2.8)	0.10 (6.2)	0.044 (4.4)	2.175 (10.7)	0.428
2	0.016	0.014	0.01	0.016	0.01	0.203	0.045
3	0.022 (1.4)	0.013 (0.93)	0.011 (1.1)	0.023 (1.4)	0.011 (1.1)	0.568 (2.8)	0.108
4	0.073 (4.6)	0.04 (2.86)	0.026 (2.6)	0.127 (7.9)	0.025 (2.5)	4.512 (22.2)	0.8
5	2.209 (138.1)	0.372 (26.6)	0.094 (9.4)	0.435 (27.2)	0.383 (38.3)	33.214 (163.6)	6.118
6	0.177 (11.1)	0.048 (3.4)	0.055 (5.5)	0.329 (20.6)	0.046 (4.6)	9.142 (45.0)	1.633
7	0.019 (1.2)	0.011 (0.8)	0.009 (0.9)	0.025 (1.6)	0.01 (1)	0.486 (2.4)	0.093
8	0.03 (1.9)	0.019 (1.4)	0.013 (1.3)	0.03 (1.9)	0.014 (1.4)	0.523 (2.6)	0.105
10	0.022 (1.4)	0.014 (1)	0.009 (0.9)	0.018 (1.1)	0.009 (0.9)	0.281 (1.4)	0.059
Product mean (EC <sub>50</sub> )	0.303	0.066	0.028	0.123	0.061	5.678	

<sup>1</sup>EC<sub>50</sub>

<sup>2</sup>Resistance factor (in parentheses) = isolate EC<sub>50</sub>/lowest EC<sub>50</sub> (isolate 2)

**TABLE 2: ANOVA of the incubation period duration<sup>1</sup> and conidial production (number of conidia/leaf disc) of six isolates of *V. inaequalis* on apple leaf discs.**

	Assessment	Source	d.f.	Mean square	F-value	P-value
Incubation period	Visually	isolate	5	0.024	0.933	0.477
		inoc. dens. <sup>2</sup>	1	0.837	31.888	<0.001
		iso x inoc. dens.	5	0.065	2.469	0.061
		Error	24	0.026		
Incubation period	Stereo microscope	isolate	5	0.013	0.485	0.784
		inoc. dens.	1	1.033	38.391	<0.001
		iso x inoc. dens.	5	0.041	1.515	0.223
		Error	24	0.027		
Conidial production <sup>3</sup>	Compound microscope	Isolate	5	15918094	1.05	0.412
		inoc. dens.	1	103934624	6.88	0.016
		iso. x inoc. dens.	5	14105860	0.93	0.478
		Rep.	2	13015519	0.86	0.436
		Error	22	15099310		

<sup>1</sup> data were log transformed prior to analysis

<sup>2</sup> inoc. dens. = inoculum density

<sup>3</sup> assessed at 24 days post-inoculation

There was a significant effect of inoculum density on the incubation period and conidial production (Table 2). Increasing the inoculum density from  $5 \times 10^3$  to  $5 \times 10^4$  conidia/ml significantly stimulated faster and more abundant growth and also reduced the incubation period (Table 3). The higher the inoculum density provided, the higher the number of conidia produced (Table 3). The percentage diseased area, disease severity and conidial production were not statistically different between isolates.

The relative fitness of resistant strains is not easily evaluated. Firstly, fitness is a summation of various factors; secondly, the overall fitness could be determined by the particular mechanism of resistance (resistance penalty), or it could be a completely separate trait. In the latter case, the fitness evaluation would have to be based on a large number of phenotypes (Köller 1991). The fitness characteristics of the resistant isolates could be used to predict the direction of the entire population in the future. If the resistant isolates are considerably less fit than sensitive ones, then reversion to sensitivity in a population becomes a realistic consideration. On the other hand, if resistant strains emerge with a higher pathogenicity and fitness than the wildtype fungus, the whole population of a pathogen may become resistant even without selection pressure of the fungicide (Dekker 1977).

**TABLE 3: Incubation period duration<sup>1</sup> (microscope assessment) and conidial production (number of conidia/leaf disc) of six isolates of *V. inaequalis* on apple leaf discs.**

Isolate	Incubation period (days)		Conidial production <sup>2</sup>	
	$5 \times 10^3$ conidia/ml	$5 \times 10^4$ conidia/ml	$5 \times 10^3$ conidia/ml	$5 \times 10^4$ conidia/ml
1	6.1(0.18) <sup>3</sup>	5.2(0.02)	$9.44 \times 10^2$	$6.06 \times 10^3$
2	6.5(0.14)	5.9(0.09)	$7.22 \times 10^2$	$3.39 \times 10^3$
4	7.2(0.19)	8.5(0.23)	$13.33 \times 10^2$	$1.78 \times 10^3$
5	5.3(0.09)	5.7(0.11)	$11.66 \times 10^2$	$9.94 \times 10^3$
6	7.4(0.45)	4.9(0.07)	$13.89 \times 10^2$	$3.28 \times 10^3$
10	6.2(0.05)	5.1(0.26)	$3.33 \times 10^2$	$1.83 \times 10^3$

<sup>1</sup> incubation period of those that emerged

<sup>2</sup> assessed at 24 days post-inoculation

<sup>3</sup> figures in parentheses are standard deviations

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