RESISTANCE OF GRAPE POWDERY MILDEW (*UNCINULA NECATOR*) TO TRIADIMENOL, A STEROL BIOSYNTHESIS INHIBITOR : BIOCHEMICAL CHARACTERISATION OF SENSITIVE AND RESISTANT STRAINS.

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ABSTRACT

Similar sterol compositions were found when mycelium and conidia from two sensitive and three naturally triadimenol-resistant strains of *Uncinula necator* were analyzed. Ergosta-5, 24 (24^1)-dien-3β-ol was the major sterol. Mycelium contained a higher quantity of this compound than conidia. Triadimenol treatment led to eburicol(4, 4, 14-trimethyl-ergosta-8, 24 (24^1)-dien-3β-ol) accumulation in the mycelium only, indicating that C14-demethylase would be the target of the fungicide. Moreover, triadimenol had no significant action on conidia of resistant strains, whereas those of sensitive strains were strongly affected (c. 50% quantitative reduction of all sterols).

INTRODUCTION

Grape powdery mildew, caused by *Uncinula necator* (Schw., Burr.), is a disease of major importance on grapes (*Vitis vinifera*, L.), causing loss of production and reduction of wine quality. A significant step towards better vineyard protection was made in the late 70s, when systemic compounds inhibiting sterol biosynthesis, such as the triazole triadimenol, began to replace sulphur. However, all the sterol biosynthesis inhibitors (SBIs) used against *Uncinula necator* inhibit a single target enzyme, the C14-demethylase (Buchenauer, 1977, Vanden Bossche, 1988). Development of field resistance to SBIs was reported in Portugal in 1988, and in France in 1989 (Steva & Clerjeau, 1990). The aim of this study was therefore to obtain data concerning the sterol biochemistry of *Uncinula necator* and to achieve a better understanding of its resistance to SBIs.

METHODS

The five monoconidial isolates studied were isolated from vineyards in 1992. Inhibition curves for triadimenol were established (Steva & Clerjeau, 1990), and fungal material was obtained from inoculated young vines. Plants were either untreated or sprayed at a concentration of triadimenol inhibiting 30% of growth (IC30) 12 h before inoculation, so that sufficient material could be obtained for sterol extraction. Conidia and mycelium were harvested separately from plants incubated for 14 days, by blowing and scraping, respectively. Freeze-dried fungal material was heated under reflux with methanolic KOH (6%) for 1 h. Neutral lipids were extracted with hexane, separated by tlc, acetylated prior to glc analysis and identified by gc-ms.

RESULTS

Sterol composition of conidia and mycelium of grape powdery mildew

It appeared that although the five strains investigated differed greatly in their sensitivity to triadimenol (table 1) their sterol composition was very similar, being dominated by methylene-24 cholesterol (ergosta-5, 24 (24^1)-dien-3β-ol, E) which comprises over 60% of the total sterols. The remainder was mainly made up of cholesterol (D), sitosterol (F), eburicol (B) and cycloartenol (C) (Table 2). On the basis of their molecular ion and fragmentation pattern, sterols were identified according

to Loeffler *et al.* (1992), Debieu *et al.* (1992) and Rahier & Benveniste (1989).

TABLE 1. Sensitivity of *U. necator* strains to triadimenol.

Strain	Origin	IC30 (mg/l)	IC50 (mg/l)	IC100 (mg/l)
P18 (S*)	Perpignan	0.2	0.3	0.8
P17 (R)	Perpignan	5.0	9.0	14.0
M27 (S)	Madiran	0.3	0.4	0.8
M42 (R)	Madiran	1.6	2.0	4.7
M43 (R)	Madiran	10.0	14.0	>20

*: S: sensitive (IC100 < 1mg/l), R: resistant (IC100 > 1 mg/l)

In order to discriminate plant sterols from fungal sterols, healthy leaves were scraped and the material obtained was extracted in the same manner as the fungus (data not shown).

The sterol profiles of *Uncinula necator* conidia and mycelium did not vary greatly. Nevertheless, the level of methylene-24 cholesterol in the mycelium was found to be slightly higher in resistant strains. Considering the total sterol concentration, it appeared that mycelium contained more sterols than conidia (from 30 to 60% more). This was especially true for methylene-24 cholesterol and plant sterols (A, C, D, F and minor sterols) in all strains. However, the relative amount of eburicol was generally lower in mycelium.

Effect of triadimenol on sterol content of conidia and mycelium

In the conidia produced by the sensitive strains after triadimenol treatment, the amounts of total sterols and methylene-24-cholesterol were drastically reduced (c. 60% of control conidia, Table 2). Conversely, no effect was noted on conidia issued from resistant strains in which the quantities of total sterols and methylene-24 cholesterol remained unchanged. In both cases, although there was a slight increase of plant sterol levels (D, F and other sterols), no accumulation of 14α-methyl sterols such as eburicol was detected.

Considering the mycelium, triadimenol treatment led to an increase from 20 to 75% in total sterol content in all strains. Likewise, amounts of eburicol increased significantly in all strains except M27. A careful examination of the relative proportions of the different sterol categories showed a marked decrease (10 to 30%) in methylene-24 cholesterol. Concurrently, the level of 4,4-dimethyl sterols increased, especially in resistant strains. This increase was due to eburicol and cycloartenol in mycelium. As in the conidia, the plant 4-desmethyl sterol fraction was greater after treatment.

Moreover, in all 4-desmethyl sterol fractions of treated samples, the presence of 14α-methyl-ergosta-8-en-3β-ol was detected by gc-ms.

DISCUSSION

For the first time, we have compared sterol profiles of conidia to those of mycelium. This study provided further data concerning sterol biosynthesis and resistance to SBI fungicides in grape powdery mildew.

The major sterol of *Uncinula necator* was methylene-24 cholesterol, as found in other *Erysiphaceae* by Loeffler *et al.* (1992) and Debieu *et al.* (1994). We detected 4,4-dimethyl sterols such as lanosterol, 4,4-dimethyl fecosterol, episterol (< 0.5% total sterols) and eburicol: this indicates that *Uncinula necator* is able to synthesize its own sterols. However, as shown in Table 2, significant amounts of plant sterols such as 4,4-dimethyl-cyclopropyl sterols (A, C and most of minor sterols) and some 4-

TABLE 2: Effect of triadimenol (IC30) on sterol composition of conidia and mycelium of *U. necator* sensitive and resistant strains.

STEROLS	Percentage of total sterols (untreated(treated)) SENSITIVE STRAINS P18 con.	P18 myc.	M27 con.	M27 myc.	RESISTANT STRAINS P17 con.	P17 myc.	M42 con.	M42 myc.	M43 con.	M43 myc.
4,4 dimethyl sterols										
A	- * (2)	2(3)	-(-)	2(3)	-(1)	2(4)	-(1)	1(3)	-(-)	2(3)
B	16(21)	12(17)	28(28)	10(9)	23(22)	20(20)	23(22)	7(17)	31(22)	8(19)
C	22(8)	18(18)	4(12)	12(20)	5(8)	7(26)	1(3)	6(9)	3(7)	8(22)
other sterols (1)	-(-)	5(6)	-(-)	5(5)	-(-)	7(6)	-(-)	8(5)	-(-)	3(5)
4-desmethyl sterols										
D	1(7)	1(2)	-(1)	1(2)	-(3)	1(3)	-(2)	1(1)	-(1)	2(3)
E	58(56)	56(41)	67(55)	58(48)	70(61)	61(31)	72(63)	70(59)	63(69)	69(43)
F	1(4)	3(11)	-(-)	6(7)	-(2)	2(8)	-(2)	4(4)	1(1)	4(3)
other sterols (2)	1(1)	1(2)	-(4)	4(3)	2(2)	-(2)	2(6)	2(-)	1(-)	2(-)
4,4-dimethyl-	38(31)	37(44)	32(40)	29(39)	28(31)	36(56)	24(26)	22(34)	34(29)	21(49)
4α methyl-	1(1)	2(-)	1(-)	2(1)	-(1)	-(-)	2(1)	1(2)	1(-)	2(2)
4-desmethyl-sterols	61(68)	61(56)	67(60)	69(60)	72(68)	64(44)	74(73)	77(64)	65(71)	77(49)
Total sterol amount (µg/mg dry weight) untreated(treated)										
Total sterols	3.8(2.3)	6.7(11.4	6.4(3.1)	5.3(6.4)	3.8(3.7)	6.3(7.9)	3.1(3.3)	8.4(11.4	3.7(3.1)	5.2(9.2)
methylene-24 cholesterol	2.2(1.3)	3.8(4.6)	4.3(1.7)	3.0(2.6)	2.6(2.3)	3.8(2.5)	2.2(2.1)	5.9(6.7)	2.3(2.2)	3.6(4.0)
eburicol	0.6(0.5)	0.8(1.9)	1.8(0.9)	0.5(0.5)	0.9(0.8)	1.3(1.6)	0.7(0.7)	0.6(1.9)	1.1(0.7)	0.4(1.8)

con.: conidia, myc.: mycelium. *: < 0.5% total sterols.
A: unidentified sterol (RRt=1.275, $[M]^+$= 480), **B**: eburicol (4,4,14α-trimethyl-ergosta-8,24(24^1)-dien-3β-ol; RRt=1.230), **C**: cyloartenol (4,4,14α-trimethyl-9β,19-cyclo-cholest-24-en-3β-ol; RRt=1.312), **D**: cholesterol (cholest-5-en-3β-ol; RRt=1.098), **E**: methylene-24 cholesterol (ergosta-5, 24 (24^1)-dien-3β-ol; RRt=1.185), **F**: sitosterol (stigmast-5-en-3β-ol; RRt=1.273).(**1**): Lanosterol (RRt=1.21), Unidentified sterol (RRt= 1.28, $[M]^+$ = 468); isomer of methylene-24-cycloartenol (RRt= 1.345, $[M]^+$= 482); methylene-24-cycloartenol (RRt= 1.359).(**2**): cholestan-3β-ol (RRt: 1.111); cholest-7-en-3β-ol (RRt= 1.141); 14α-methyl-ergost-8-en-3β-ol (RRt= 1.023), campesterol (RRt: 1.221), stigmasterol (RRt= 1.221), episterol (RRt= 1.241), stigmastan-3β-ol (RRt= 1.283)

desmethyl sterols (D, F and minor sterols) appeared in sterol profiles of both mycelium and conidia. Their presence in mycelium may in part be due to scraping it from plants, as demonstrated by controls. Nevertheless, for conidia which are gently blown from leaves, a selective accumulation of plant sterols by the biotrophic fungus *Uncinula necator* might also be considered.

Indeed, by comparing only the vegetal 4-desmethyl sterol fraction between fungus (cholesterol, stigmasterol and sitosterol) and plant scrapings, we found a difference between their relative amounts, particularly for cholesterol (10 to 15% in plant scraping, 25 to 53% in conidia). This argues in favour of selective cholesterol uptake from the plant, or of potential cholesterol biosynthesis by *Uncinula necator*.

In the presence of triadimenol, the conidia from sensitive strains displayed a drastic decrease in final sterol without precursor accumulation despite the low inhibition level (IC30): this may be due to a breakdown of sterol biosynthesis. However, the accumulation of eburicol in the mycelium of all strains and the presence of 14α-methyl-ergosta-8-en-3β-ol confirms that the C14-demethylase is the target of triadimenol in *Uncinula necator* as shown in other fungi (Hollomon *et al.*, 1990; Pontzen *et al.*, 1990). Furthermore, mycelium of resistant as well as sensitive strains showed an increase in total sterol amounts partly due to the presence of plant sterols. In the conidia, plant sterol quantities were also increased. These facts favour the idea of a greater selective accumulation of plant sterols in response to the treatment.

Our data indicate that unusual sterol biosynthesis (due to C14-demethylase deficiency) does not account for resistance to triadimenol. However, other resistance mechanisms (Hollomon *et al.*, 1990) such as reduced fungicide uptake, detoxification or mutation of the target are still to be considered.

From our results, the only specific feature of resistance seems to be the stability of the amount of methylene-24 cholesterol present in the conidia. As triadimenol has a similar effect on the sterol content of mycelium of all strains, and as conidia are only an extension of mycelium, an hypothesis explaining this fact could be that in resistant strains the mycelium would "attenuate" inhibition by neutralizing triadimenol. Another possibility may be that, in sensitive strains, C14-demethylase inhibition would generate a compound repressing the sterol biosynthesis upstream from the C14-demethylation step. This may be assumed from data in the literature (Kerkenaar et *al.*, 1984; Favata *et al.*, 1987).

In order to complete this work, experiments with stronger inhibition (IC50, IC75) will be done, including additional sensitive and resistant strains from different origins. Furthermore, it would be instructive to analyze carefully the 4α-methyl sterol fraction and the squalene content of treated and untreated samples, and to look for potential 14α-methyl sterol derivatives of *Uncinula necator* after treatment.

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REDUCED SENSITIVITY TO DMI FUNGICIDES IN POPULATIONS OF *MONILINIA FRUCTICOLA* IN NEW ZEALAND STONE-FRUIT ORCHARDS.

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ABSTRACT

Significant reductions in sensitivity to triforine (a demethylation inhibitor, DMI) were detected in *Monilinia fructicola* populations from seven of nine Hawkes Bay orchards in 1992. The mean LC50 of a wild population from unsprayed home garden sites was 3.3 mg/litre triforine (range for individual isolates, 0.5-10.8 mg/litre). In contrast, the mean LC50 for the orchard populations ranged from 4.5 to 7.9 mg/litre triforine (range for individual isolates, 0.4-28.1 mg/litre). Isolates with reduced triforine sensitivity were also significantly less sensitive to three other DMI fungicides. We discuss these findings in relation to the need to reduce DMI selection pressure on *M. fructicola* populations.

INTRODUCTION

Repeated applications of fungicide are required in New Zealand orchards to control brown rot of stone-fruit caused by *Monilinia fructicola* (Wint.) Honey. The occurrence of DMI resistance in several host/pathogen systems (Koller and Scheinpflug, 1987) and the relative ease of selection of DMI resistant strains of *M. fructicola* in laboratory studies (Nuninger-Ney *et al.*, 1989) indicated that the DMI's may be at risk of resistance development.

Current recommendations in New Zealand reflect the need to restrict DMI selection pressure on *M. fructicola* populations and specify no more than three DMI's in any one season with non-DMI's at all other times (Prince *et al*, 1989). In 1990, three orchards in the Hawkes Bay region reported inadequate disease control following DMI applications. In a preliminary investigation significant shifts in triforine sensitivity were detected at two of the three orchards sampled (Elmer *et al,* 1992). In 1992, a greater number of orchards were surveyed to determine the extent of changes in triforine sensitivity in *M. fructicola* populations and to determine the sensitivity of selected isolates to three other DMI fungicides registered for use in New Zealand.

METHODS

Up to 50 fruit infected with *M. fructicola* were sampled randomly from each of nine orchards using a stratified sampling pattern (Delp *et al.*, 1986). A wild population of *M. fructicola* was obtained by sampling unsprayed home garden sites throughout New Zealand.

Triforine sensitivity tests

The sensitivity of each isolate was expressed as an LC50 value calculated from a dose response curve of growth rate (mm/day) to $\log_{10}$ triforine concentration (mg/litre). The mean LC50 of each orchard population was compared with the mean LC50 of a home garden population using analysis of variance (ANOVA).

Other DMI sensitivity tests

Three isolates with reduced sensitivity to triforine (mean LC50 =9.3mg/litre) and five isolates classed as triforine sensitive (mean LC50 = 4.4mg/litre) were used to determine cross-resistance to bitertanol, cyproconazole and flusilazol. For each DMI, the mean LC50 of the sensitive isolates was compared to the mean LC50 of isolates with reduced triforine sensitivity using ANOVA.

RESULTS AND DISCUSSION

The triforine sensitivity of *M. fructicola* populations from seven of nine Hawkes Bay orchards was significantly ($P<0.01$) less than that of the home garden population (Table 1).

TABLE 1. The triforine sensitivity of *M. fructicola* populations from three representative Hawkes Bay orchards in 1992.

Source	Number of isolates	Mean log LC50	Range
Home gardens	27	0.519 (3.3)[a]	(0.5 - 10.8)[a]
Orchard 2	26	0.658 (4.5)	(0.6 - 9.8)
Orchard 8	22	0.860 (7.2)**	(3.1 - 25.3)
Orchard 9	31	0.900 (7.9)**	(2.4 - 15.6)
LSD (5%)		0.143	
LSD (1%)		0.188	

[a] Backtransformed values are presented in brackets.
**, significantly different from the home garden population at $P<0.01$.

The magnitude of the shift in sensitivity was small (resistance factors based on population mean values ranged from 2.2 - 2.4) and it may be debatable whether they should be called resistant sub-populations. Therefore, we have used the term "significantly reduced sensitivity".

Isolates with reduced sensitivity to triforine were also significantly ($P<0.01$) less sensitive to bitertanol, cyproconazole and flusilazol, compared to the standard home garden isolates.

Significant shifts in sensitivity to triforine and other DMI's indicates that there is a need to reduce DMI selection pressure. At one orchard, 11 DMI fungicides were applied in one season, which exceeds current recommendations.

The emergence of populations of *M. fructicola* with reduced sensitivity to DMI's does not necessarily mean that disease control failures will occur. Research to investigate the biological characteristics of isolates with reduced sensitivity to DMI's and the relationship between reduced sensitivity and disease control on host tissues *in vivo* is in progress.

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SENSITIVITY OF *UNCINULA NECATOR* TO PENCONAZOLE IN EUROPEAN COUNTRIES

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ABSTRACT

Demethylation inhibiting fungicides (DMIs) play an important role in the control of *Uncinula necator*, the causal fungus of grape powdery mildew.

A survey was initiated in 1992 to investigate the sensitivity of *U. necator* to penconazole in different European countries. In one part of the study, samples were collected from ornamental vines, which had never been treated with DMIs, in order to establish a "baseline"-sensitivity and to check the prevelance of possible shifts. In the other the sensitivity of samples collected in vineyards showing both good and weak performance of the product was tested, to examine the contribution of changes in sensitivity to disease control problems.

The "baseline"-sensitivity of *U. necator* to penconazole showed only a small variation within and between different European countries. Sensitivity data for samples from treated vineyards support the assumption that many factors are responsible for the variability in the disease control level and that a decrease in sensitivity could be one of them. It was shown that aspects of the safety margin for activity become more important compared to previous years.

INTRODUCTION

Grape powdery mildew, caused by the fungus *Uncinula necator,* is one of the most serious diseases in viticulture. Traditional control measures consisted of treatments with sulfur, which was applied either as dust or as a wettable powder. Since the introduction of the DMI-fungicides (demethylation inhibitors) at the beginning of the 1980's, these compounds have been widely and intensively used in all vine-growing areas for the effective control of this disease.

DMIs are site-specific fungicides and as such changes in sensitivity of a pathogen cannot be excluded. After many years of successful use of DMIs, several authors observed a decrease in sensitivity of grape powdery mildew (Aloi *et al.*, 1991, anonymous, 1987, Steva *et al.*, 1988). All reports mention several factors which were closely related to the reduced efficacy against the pathogen. Performance problems occurred under high disease pressure, on susceptible cultivars and with the exclusive and season-long use of a DMI over a number of years. Other important aspects are related to the application: incorrect doses of the fungicide, inadequate timing and poor spray quality.

The two main objectives of the study started in 1992 were to attempt to establish a "baseline"-sensitivity for *U. necator* to penconazole, in order to detect possible shifts in areas where the compound has been used for many years, and to obtain sensitivity data from vineyards where both good and reduced efficacy of the product had occurred, so that the role of sensitivity shifts in grape powdery mildew control could be investigated (active monitoring).

MATERIALS AND METHODS

Sampling

Samples of mildew infected leaves and bunches were collected during the 1993 season in the following European countries: France, Germany, Italy, Portugal and Switzerland. Each sample consisted of 20 - 30 leaves and bunches. All samples for the active monitoring originated from trial plots or commercially treated vineyards. In 1992, one sample was collected at a trial site in Portugal where penconazole showed unexpectedly weak performance. "Baseline"- samples were taken either from ornamental vines that had never been treated, or from vineyards where biological control had been used, in various regions of the respective countries. Table 1 describes the origin of the samples.

TABLE 1. Description of the monitoring samples of *U. necator* collected in different European countries.

country	**baseline**		**active-monitoring**	
	variety	sampling period	*variety*	sampling period
France	*Cabernet S., Chasselat, Carignan, Merlot*	25.07.-03.08.93	*Carignan*	03.08.-04.08.93
Portugal	*Loureiro*	20.07.93	*Carignan, Fernandinho, Carignan*	02.08.92 21.07.93
Germany	*Riesling, Portugieser, M. Thurgau, Gutedel*	17.07.-02.08.93	*Trollinger, Kerner, Juwel*	18.07.-27.07.93
Italy	-	-	*Moscato, Cortese*	13.07.93
Switzerland	?	11.08.-22.08.93	*Gamay, Chasselas, Pinot noir, Silvaner*	26.07.93

Sensitivity tests

Sensitivity tests for all samples from France and Portugal were carried out by BIORIZON with a mycelium growth test on leafdiscs of the susceptible cultivar "*Cinsaut*". This method was originally established for triadimenol (Steva, 1992) and for the present studies it was adapted for penconazole. Based on the evaluation of the filament length after 72 hours at a range of fungicide concentrations (0-0.01-0.03-0.1-0.3-1-3-10 mg/l AI), this procedure gives a quantitative description of the sensitivity distribution of conidia within a given sample and an estimation of the MIC-value (minimum inhibitory concentration) can be made.

Tests for the samples from the other countries were carried out by CIBA. The sensitivity of *U. necator* was determined in a sporulation test on leaf discs of grapevine variety "*Portugieser*". This test was also adapted for penconazole from the method described by Steva (1992). Dose-response curves, based on the percentage of sporulating leaf area, were established for each sample at the same range of concentrations as mentioned above.The MIC was determined 12 days after inoculation. All samples were tested either directly or after one propagation. Tests at CIBA were repeated.

RESULTS

Baseline studies

Table 2 summarizes the results of the baseline studies. Sensitivity data are expressed as MIC- values of the tested populations.

TABLE 2. Sensitivity of *U. necator* to penconazole for samples collected from ornamental vines and vineyards where biological control had been used in Europe, 1993.

country	number of samples	range of MIC-values[1] (mg/l)
France	5	0.1-1
Portugal	6	0.1-1
Germany	7	0.3-3
Switzerland	3	1-3

[1] MIC for France and Portugal estimated by BIORIZON on *cv. Cinsaut* in a mycelium growth test and for Germany and Switzerland determined by CIBA on *cv. Portugieser* in a sporulation test.

Results both from CIBA and BIORIZON indicate only little variation in the sensitivity of powdery mildew samples collected within and across the European countries (range= x3). The tests revealed that wild-type populations of *U. necator* are generally controlled at 1 mg/l penconazole under the test conditions used by BIORIZON and at 3 mg/l penconazole in the sporulation test carried out by CIBA. Small differences in the amount of AI needed can be explained by the two varieties used as host plant tissue in the different test systems (*cv. Cinsaut* and *cv. Portugieser*).

Active monitoring

Samples from penconazole treated vineyards show a reduction in sensitivity compared to the baseline (Table 3). Based on the EC50-values evaluated for the populations collected in France and Portugal, a mean resistance factor of 15 was noted (data not shown). The MIC-values of DMI-treated samples were generally detected between 1 and 10 mg/l. Data for the disease levels shown in table 3 indicate that there was no correlation between the sensitivity results and product performance.

TABLE 3. Sensitivity of *U. necator* to penconazole for samples from DMI treated vineyards in Europe, 1993 (values in mg/l AI).

country	no. of samples	no. of DMI treatments[1]	% attack on bunches	MIC-value[2]
France	4	6-8 (trial)	0.1-1	1-3
	1	8(trial)	1	0.3-1
Portugal	1	2	1	0.3-1
	1	2	40	3-10
	1	3	25	1-3
	2	5	<1	0.3-1; 1-3
	1[4]	7 (trial)	7	1-3
	1(1992)[4]	7 (trial)[3]	27	1-3
Germany	1	1	?	1-3
	1	2	1	3-10
	1	4[3]	25	3-10
	2	5 (trial)[3]	15-20	3-10
Italy	2	4	2-5	3-10
Switzerland	1	1	30	1-3
	1	2	1	1-3
	1	2	50	3-10

[1] use rates according to local registration. [2] MIC for France and Portugal estimated by BIORIZON on *cv. Cinsaut* in a mycelium growth test and for Germany, Italy and Switzerland determined by CIBA on *cv. Portugieser* in a sporulation test. [3] samples collected under high disease pressure and with curative treatments.[4] same trial location as in 1993.

Fig. 1 demonstrates the sensitivity distribution of a "baseline" population in comparison to a sample collected from a trial plot under the selection pressure of penconazole. Samples were analyzed with the mycelium growth test described by Steva (1992). Although both samples were collected in the same region, a quantitative shift in sensitivity becomes obvious. The sensitivity of the conidia from the baseline population is comprised between 0.01-1 mg/l penconazole, whereas the population from the treated vineyard had a range between <0.01-3 mg/l.

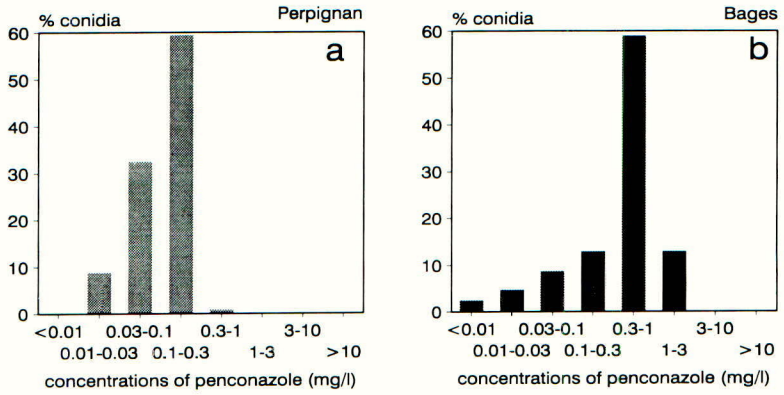

FIGURE 1. Sensitivity of *U. necator* to penconazole for populations collected in one region in France (1993) from a) a vine not previously treated ("baseline"), b) a trial plot treated 8 times with penconazole (0.2% attack on bunches).

The sensitivity distribution for samples collected in a specific trial plot in Portugal is shown in Fig. 2. In 1992 the level of attack on bunches reached 27% under a situation of high disease pressure, whereas performance was good in 1993 (7% attack), when the pressure was lower and the epidemic started later than the year before. It can be seen, that conidia in both years could be controlled with 3 mg/l penconazole in the mycelium growth test, and distributions did not differ significantly.

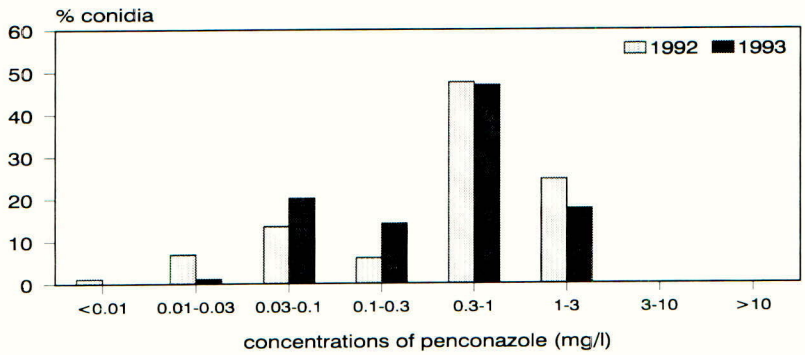

FIGURE 2. Sensitivity of *U. necator* to penconazole for 2 samples collected at the same trial site in Portugal, in a year with high (1992) and low (1993) disease pressure.

DISCUSSION

The fact that penconazole has been used for the control of *U. necator* for about a decade made it particularly difficult to find samples representing the wild-type sensitivity of the fungus to this compound. For a product already on the market this might not be a true "baseline" but it still provides useful information and should be established. Only the knowledge of the baseline sensitivity allows one to decide whether subsequently reported shifts in the pathogen sensitivity are true effects or are merely within limits of the normal variability of a population.

The "baseline"-monitoring for penconazole revealed relatively little variation in the sensitivity of *U. necator* between and within European countries. This is similar to earlier findings of Steva (1992) for triadimenol sensitivity in France and Portugal. These results now provide a basis to follow the grape powdery mildew sensitivity in future years, and to draw conclusions on any apparent significant shifts towards a decrease in sensitivity.

In this study, samples collected in vineyards where penconazole had been applied generally showed a decrease in sensitivity when compared to the "baseline". The sensitivity level of those samples collected under the selection pressure of the fungicide was reduced by a factor of 10-15. However, the number of samples tested, their origin with regard to the number of treatments and the use of the DMIs might be insufficient to draw a general conclusion about the sensitivity situation in penconazole-treated areas across Europe.

Another aspect of the influence of DMI treatments on the sensitivity of the pathogen can be seen from the data presented in table 3. Based on the sporulation test the conidia isolated from vineyards with only 1 DMI- treatment tended to show only slightly reduced sensitivity compared to those from trial plots with multiple DMI applications. This indicates an influence of the number of treatments on the sensitivity level and suggests that a limit on the number of DMI applications could help to prevent further shifts towards decreased sensitivity. However, this result should be confirmed in further trials.

As a general rule, no correlation could be shown between the sensitivity values and the level of disease control in the various vineyards. However, the two years of experience at a specific trial-site in Portugal (Fig. 2), where both the sensitivity and efficacy of penconazole were assessed, suggest that under situations of high disease pressure (1992) the safety margin for activity of the compound is no longer sufficient to provide good control at this level of reduced sensitivity, although in other cases excellent powdery mildew control was achieved.

Similar findings were published for triadimenol by Steva (1992), although he showed that the decrease in the sensitivity level for this compound was more pronounced than it could be shown for penconazole in this study. This is also concluded by Steva and Clerjeau (1990), who carried out experiments with the progeny of four single spore isolates. They grouped the DMIs into 3 classes with different sensitivity levels of *U. necator*. The isolates showed no reduced sensitivity to penconazole in comparison to triadimefon and triadimenol, where resistance factors of up to ~100 could be detected.

The present investigation demonstrates that the sensitivity of *U. necator* can decrease under selection pressure from penconazole. Therefore, it is of vital importance to strictly follow the use recommendations implemented by the Fungicide Resistance Action Committee - Sterol Biosynthesis Inhibitor working group (FRAC-SBI), in order to preserve the DMIs as an effective tool for vine growers to protect vineyards from powdery mildew.

These use recommendations are as follows (FRAC, 1993): DMIs should not be used exclusively season-long. The number of DMI sprays should be limited to a maximum of four per season, just before and after the flowering stages. It is important to use the DMIs only at the fully recommended rate in a protective, not curative manner. The use of mixtures or alternation with non-cross-resistant fungicides also might be a possible strategy. The recommended timing and volume of application must be adhered to.

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TESTING TOMATO POWDERY MILDEW (*ERYSIPHE* SP.) FOR FUNGICIDE RESISTANCE

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ABSTRACT

The sensitivity of mass population samples of tomato powdery mildew (*Erysiphe* sp.) collected from commercial crops to three fungicides was determined using detached leaves and young plants. The sensitivity of one population to fenarimol seemed different to that of a reference sensitive isolate. Possible reasons for some samples failing to establish on detached leaves were investigated. A sample inoculated on to young plants established well and bupirimate and fenarimol gave control at one tenth of the label recommended rates. Further tests are required to determine if poor disease control in fenarimol-treated crops is associated with the presence of mildew showing reduced sensitivity to fenarimol.

INTRODUCTION

Powdery mildew ((*Erysiphe* sp.) was first recorded on protected tomatoes in England in 1987 (Fletcher *et al.*, 1988) and since then has affected many crops annually throughout the country. It affects leaf, stem and calyx and causes severe leaf yellowing, reduced yield and premature plant death. Fungicides applied for mildew control include the benzimidazole fungicide benomyl (Benlate), the hydroxy-pyrimidine fungicide bupirimate (Nimrod), and the sterol biosynthesis inhibitor (SBI) fenarimol (Rubigan). Resistance to all three fungicide groups has been reported in cucumber powdery mildew (*Sphaerotheca fuliginea*) (Schroeder & Provvidenti, 1969; Bent *et al.*, 1971; Schepers, 1983). Fenarimol is commonly used on tomato crops for control of mildew because, unlike benomyl and bupirimate, it does not disrupt biological pest control. However, some growers have reported poor disease control following treatment with fenarimol and have attributed this to fungicide-resistant strains of the pathogen. Work was therefore undertaken to develop techniques which could be used to determine the sensitivity of tomato powdery mildew to fenarimol and other fungicides.

MATERIALS AND METHODS

Samples of leaves affected by mildew were obtained from commercial tomato nurseries and from ADAS Reading (92/1). Sample 92/1 had not been exposed to any fungicide for at least two years. Samples 92/PV and 93/LV were both obtained as mass populations samples from fenarimol-treated crops. Various methods were used to encourage spore production from old pustules. In 1992, pustules were brushed to remove old spores and the leaves incubated in a humid chamber. In 1993, pustules were sub-cultured on untreated detached leaves.

Resistance tests were carried out using detached leaves, cvs. Gardener's Delight or Pronto, or young plants, cv. Counter. Detached leaves were dipped in fungicide, allowed to dry and inoculated with mildew spores of the test sample using a cotton bud. Pot-grown plants were sprayed to run-off with the test fungicide or water, allowed to dry and inoculated by placing small leaf discs infected with mildew in contact with treated leaves. Fungicides were tested at label recommended rates (benomyl, 500 mg/l; bupirimate 500 mg/l; fenarimol 10.8 mg/l) and at three dilutions (1 in 2, 1 in 10 and 1 in 20). Inoculated detached leaves (5 leaves/treatment; 2 inoculation sites/leaf) were incubated over water in an illuminated incubator at 23°C. Inoculated plants (3 plants/treatment; 3 inoculated leaves/plant) were covered with a clear polythene bag for 48 h before standing them in an unheated glasshouse. Leaves were assessed for the number of inoculation sites at which mildew had developed and for abundance of mildew development (0-5 scale according to proportion of inoculated area affected). Factors which might affect development of mildew were investigated including cultivar, leaf age and a comparison of abaxial and adaxial leaf surfaces.

RESULTS

Mildew developed consistently on detached leaves when spores were transferred from young, freely-sporing pustules but not when older, discoloured pustules were used. Results for sample 92/1, tested in May 1992 on leaves dipped in bupirimate or fenarimol, and sample 93/LV, tested in April 1993 on leaves dipped in fenarimol, are shown in Table 1. Sample 92/1 was also tested against benomyl and no mildew developed at 25 mg/l, the lowest concentration tested. Sample 93/LV was relatively resistant to fenarimol and developed after 12 days on most leaves treated at one tenth (1.1 mg/l) of the recommended rate.

TABLE 1. Establishment of mildew after 6 and 12 days on detached tomato leaves treated with water or fungicide.

Treatment	Rate (µg/ml)	No sites with mildew (0-10) 92/1		93/LV		Mean disease score (0-5) 92/1		93/LV	
		6d	12d	6d	12d	6d	12d	6d	12d
Water		10	10	10	10	4.6	5.0	4.7	5.0
Uninoculated		0	0	0	0	0	0	0	0
Bupirimate	500	1	1	-	-	0.2	0.3	-	-
	250	2	3	-	-	0.3	0.6	-	-
	50	4	10	-	-	0.4	1.8	-	-
	25	5	10	-	-	0.6	2.4	-	-
Fenarimol	10.8	1	1	0	0	0.1	0.1	0	0
	5.4	0	1	0	0	0	0.2	0	0
	1.1	0	1	6	8	0	0.1	0.8	0.8
	0.5	7	9	10	10	1.9	2.5	3.7	3.7

The cultivar, age and surface of detached leaves were investigated as possible reasons for poor establishment. Mildew established more consistently on leaves of cv. Gardener's Delight (39/40 inoculation sites) than cv. Pronto (28/40 sites). The success of establishment was not affected by leaf surface (22/40 on abaxial; 23/40 on adaxial). There was slightly better establishment on young leaves of cv. Pronto (9/40) than on old leaves (3/40) of the same variety. Incubation of leaves in a damp chamber was unsuccessful in producing new pustules as leaves were rapidly rotted by grey mould (*Botrytis cinerea*) or bacterial soft rot. Transfer of spores from old pustules to untreated detached leaves was generally unsuccessful in producing new pustules.

Four weeks after inoculation of young plants with sample 92/PV, all but one of the water-treated leaves was infected with mildew. At this time both bupirimate and fenarimol were giving good control of the disease at one tenth of normal spray rates, but not at one twentieth. (Table 2).

TABLE 2. Establishment of mildew (92/PV) on young tomato plants treated with bupirimate or fenarimol.

Treatment	Rate (mg/l)	Number of inoculation sites (of 9) developing mildew		
		Weeks after inoculation		
		3	4	5
Water		6	8	8
Uninoculated		0	0	0
Bupirimate	500	0	0	3
	250	0	0	2
	50	0	0	3
	25	0	2	6
Fenarimol	10.8	0	0	0
	5.4	0	0	2
	1.1	0	0	4
	0.5	0	4	5

DISCUSSION

Development of a rapid, accurate and reproducible method for evaluating the response of tomato powdery mildew to fungicides is important because it would help to enable appropriate treatment to be selected. A detached leaf, or leaf disc technique has been used successfully for investigating *Sphaerotheca fuliginea* on cucumber (Schepers, 1983). The technique reported here for tomato mildew was relatively rapid (2-4 weeks) but was poorly reproducible. It is possible that a water film on leaves or a high light intensity (Cohen, 1993) may have affected establishment of mildew in these tests. Further work is required to define more precisely the conditions under which mildew spores will germinate and develop to produce sporing pustules on detached leaves.

When resistance to SBI fungicides occurs, it generally develops in small steps making it difficult to determine when poor control is due to fungicide resistance rather than to other factors. Reduced disease control of *S. fuliginea* by fenarimol was associated with an increase in the LD_{95} from 0.2 to 17 mg/l (Huggenberger *et al.*, 1984). In the tests described the sensitivity of the population 93/LV seems different to that of the reference sensitive isolate 92/1. However, further samples of mildew, collected from crops where different degrees of control have been observed, need to be tested to determine the levels of sensitivity that occur and to improve interpretation of tests on detached leaves. An examination of the range of sensitivities in populations using single pustule samples may be more revealing than studies on mass populations. The sensitivity of tomato powdery mildew to benomyl and bupirimate was similar to that reported by Fletcher *et al.*, (1988), who observed no mildew on tomato plants treated with benomyl at concentrations as low as 100 mg/l, and a small amount of mildew at all concentrations of bupirimate up to 370 µg/ml.

The speed of mildew development was slower on young plants (3 - 4 weeks) than on detached leaves (6 days), probably because the former tests were done at ambient temperature. It is suggested that the critical time for assessment in sensitivity tests is as soon as mildew is present at most inoculation sites on untreated leaves or plants.

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VARIATION IN SENSITIVITY TO FUNGICIDES AMONG UK ISOLATES OF *VENTURIA INAEQUALIS*

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ABSTRACT

Dodine and myclobutanil (a triazole fungicide) are commonly used in the UK for the control of *Venturia inaequalis*, causal agent of apple scab. A total of 35 samples of *V. inaequalis* from the UK, from both fungicide treated and untreated areas, (13 single spore isolates and 22 mass spore samples) has been used in a continuing investigation of variation for sensitivity to these fungicides. The *in vitro* LD50 values for response to dodine varied from 0.85 - 2.10 mg/l. In an *in vitro* assay based on germling growth the majority of samples were as sensitive to myclobutanil as a standard isolate obtained in 1949. However samples from a site where scab control was considered to be inadequate were found to have a response to myclobutanil equivalent to a reference isolate exhibiting reduced sensitivity.

INTRODUCTION

The high level of apple scab (*Venturia inaequalis*) control demanded of UK growers is only achievable by frequent fungicide treatment. Up to fifteen applications per season of a range of fungicides is not unusual. Dodine and triazole fungicides (eg. myclobutanil) are among those most frequently used. Variation for sensitivity to dodine and triazole fungicides, associated with loss of efficacy, has been reported in North America (Sholberg *et al.*, 1989; Szkolnik & Gilpatrick, 1973) and several European countries (Fiaccadori *et al.*, 1987; Stanis & Jones, 1985; Thind *et al.*, 1986). The objective of this study is to ascertain for the first time the extent of variation for response to dodine and myclobutanil among isolates of *V. inaequalis* from UK orchards.

MATERIALS AND METHODS

Dodine

Dodine sensitivity was assessed *in vitro* on potato dextrose agar amended with 0, 0.5, 1.0 or 2.0 mg dodine/l. Percentage germination was assessed for a population of >100 spores after 24 h (Sholberg *et al.*, 1989).

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Triazoles

a) *In vitro*

Sensitivity to myclobutanil was assessed by measuring the mean length of hyphal growth from 20 germlings after incubation for 60-65 h on water agar amended with 0.05 and 0.5 mg myclobutanil/l. Data are expressed as percentage reduction in mean hyphal length compared to growth on unamended agar.

b) *In vivo*

Apple seedlings (at 3-5 leaf stage) were inoculated with conidia of *V. inaequalis* 24 h after the plants had been treated with myclobutanil (0, 10, 20 or 40 mg/l; *c.* 0.4 ml/seedling). Numbers of seedlings with sporulating lesions were recorded 14 d after inoculation.

RESULTS AND CONCLUSIONS

The results of *in vitro* assays are presented in Tables 1 and 2 and of *in vivo* assays in Table 3. Reference isolates sensitive or with reduced sensitivity to dodine were not available for inclusion in these assays. Published reports indicate that a *Venturia inaequalis* population with an LD50 >0.7 mg/l for response to dodine can result in reduced field efficacy (McKay & MacNeill, 1979). The LD50 value for sensitivity to dodine of all isolates and mass-spore samples of *V. inaequalis* tested in this study was >0.7 mg/l and a factor of approximately x3 separated the most and least sensitive samples (Table 1). Four of five single spore isolates were from trees that had not been treated with dodine and one of these had a high LD50.

A conidial germling growth assay allowed reproducible assessment of variation in sensitivity to myclobutanil (as a representative of triazole fungicides). Variation in sensitivity to myclobutanil was observed among isolates and mass-spore samples of UK origin (Table 2). The least sensitive samples responded similarly to a reference reduced sensitive isolate obtained from a German orchard (Stanis & Jones, 1985) where the control achieved with triazole fungicides was reported to be inadequate. All orchard samples were from trees in orchards where triazole fungicides comprised part of a routine spray schedule. The assay results for the orchard samples indicated that those from three of the four could be classified as sensitive or intermediate but those from one orchard (site 4, Table 2) tended towards reduced sensitivity. Reduced sensitivity evident *in vitro* was reflected in an *in vivo* seedling assay (Table 3).

The significance of these observations with respect to field efficacy of both dodine and triazole fungicides in UK orchards remains to be determined.

TABLE 1. Range of response to dodine of mass spore samples and single spore isolates of *Venturia inaequalis* from the UK.

	LD50 (mg/l)	
	Range	Mean ± SE
Single spore isolates	1.12 - 1.85	1.35 ± 0.12
Mass spore samples		
a) Commercial orchards	1.09 - 2.10	1.62 ± 0.10
b) Untreated plots	0.85 - 1.59	1.23 ± 0.08

TABLE 2. Range of responses to myclobutanil of mass spore samples and single spore isolates of *Venturia inaequalis* from the UK.

	% reduction in hyphal length at 0.5 mg/l	
	Range	Mean ± SE
Single spore isolates		
Reference isolates		
E1 (sensitive)	60.3 - 80.0	73.7 ± 4.4
TR77 (reduced sensitive)	-18.9 - 48.8	19.9 ± 3.3
Other UK isolates (13)	24.3 - 78.9	61.1 ± 4.0
Mass spore samples		
Commercial orchard 1.	43.5 - 71.7	59.3 ± 4.3
2.	59.1 - 85.2	75.4 ± 5.3
3.	50.0 - 78.8	70.5 ± 5.9
4.	7.5 - 49.6	30.9 ± 5.0

TABLE 3. Response of two isolates of *V. inaequalis* to myclobutanil, in an *in vivo* assay, on fungicide treated apple seedlings.

	Myclobutanil (mg/l)							
	0		10		20		40	
	A*	B*	A	B	A	B	A	B
	% infected seedlings							
E1 (sensitive)	100	100	8	43	0	0	0	0
TR77 (reduced sensitive)	86	97	50	57	22	42	3	0

* A & B refer to two separate experiments

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