Inheritance of Resistance to Fenpropimorph and Terbinafine, Two Sterol Biosynthesis Inhibitors, in *Nectria haematococca*

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ABSTRACT

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The genetic control of resistance to two sterol biosynthesis inhibitors was investigated in the fungal pathogen *Nectria haematococca*. After UV light irradiation, resistant mutants were selected on either terbinafine, a squalene epoxidase inhibitor, or on fenpropimorph, an inhibitor of the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase. They were genetically characterized by random analysis of sexually produced ascospores. In seven induced mutants, terbinafine resistance resulted from mutations in a single gene (*Ter*1), leading to high resistance levels. All these strains exhibited a positive cross-resistance towards other squalene epoxidase inhibitors (naftifine, tolnaftate). The genetic analysis of five fenpropimorph-resistant mutants resulted in the identification of three independent genes. The *Fen*1 gene was recognized in four mutants. One highly resistant mutant carried mutations in two unlinked genes, *Fen*2 and *Fen*3, which showed an important additive effect. Depending on the gene or on the allelic mutation involved, the fenpropimorph concentrations causing a 50% reduction in the mycelial growth rate ranged from 2.2 to 25.3 μg/ml. The corresponding values for the wild type strains were 0.1–0.2 μg/ml. Single gene mutants exhibited a positive cross-resistance towards fenpropidin but not always towards tridemorph. In most *Ter*1 mutants and in some *Fen*1 mutants, resistance was not coupled with changes in characteristics such as growth rate, sporulation, and pathogenicity. The potential risk of resistance development is discussed.

Additional keywords: Allylamines, *Fusarium solani* f. sp. *pisi*, morpholines, mutagenesis.

Sterol biosynthesis inhibitors are broad-spectrum fungicides widely used in agriculture against fungal diseases such as powdery mildews, rusts, and apple and pear scabs (24). They make up a heterogeneous group of chemicals that act as site-specific inhibitors at different steps in the sterol biosynthesis pathway (4). Most of the commercial sterol biosynthesis inhibitors inhibit the sterol 14 α-methyl-demethylase. They contain a triazole (e.g., propiconazole, flusilazol, triadimenol, tebuconazole, myclobutanil), an imidazole (e.g., prochloraz), a pyrimidine (e.g., fenarimol), or a pyridine (e.g., buthiobate) ring (13). Some chemical analogs have also been developed as medical antimycotics (22). Another group of agricultural fungicides mainly developed on cereals, inhibits the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase. They are either morpholine (e.g., fenpropimorph, tridemorph) or piperidine (e.g., fenpropidin) derivatives (23,26). Several other toxicants such as allylamines (e.g., terbinafine, naftifine) or thiocarbamates (e.g., tolnaftate) inhibit squalene epoxidase (31). These are used or tested against human mycoses, and their in vitro toxicity against some phytopathogenic fungi suggests that they may have potential applications in agriculture (5,28,30).

As expected with any site-specific inhibitor, resistance phenomena could occur under field conditions. Currently, such problems are encountered with sterol demethylation inhibitors, which have been used extensively (25). The genetic analysis of both field isolates and laboratory induced mutants revealed that resistance to such fungicides could result from mutations in a single gene (19,33), in several genes (20,39), or may involve a polygenic complex system (18). Surprisingly, no case of resistance to morpholines or piperidines in practice has yet been reported, whereas such resistant mutants have been easily obtained under laboratory conditions in various fungi (3,9,12,27,29). However, to our knowledge, the mode of resistance inheritance in these mutants has not been investigated. Such genetic studies may be helpful in understanding the differential behavior of these fungicides in practice with respect to resistance, comparatively to sterol demethylation inhibitors. In the case of squalene epoxidase inhibitors, resistant mutants have only been described in *Ustilago maydis* (30). Information about the risk of developing resistance to such fungicides is needed in view of a potential agricultural use.

As a rule, several genes may control insensitivity to the same fungicide or group of fungicides, and the easiest way to recognize them is to use an organism whose perfect stage is produced readily and which gives large numbers of analyzable progeny within a few days. These advantages are offered by the heterothallic ascomycete *Nectria haematococca* Berk. and Br., the perfect form of *Fusarium solani* (38). Isolates of *N. haematococca* mating population VI (MP VI), pathogenic to pea, are sensitive to terbinafine (an allylamine derivative) and to fenpropimorph (a morpholine compound) and therefore appear particularly suitable to a genetic study of resistance to such fungicides through random ascospore analysis. It should be noticed that such a conventional genetic analysis has already been applied to the related fungus *N. haematococca* MP I and has led to the identification of genes involved in resistance to fungicides such as chlorinated nitrobenzenes (15,16), dodine (21), and the sterol demethylation inhibitors fenarimol (20) and triadimenol (19).

In this paper, we describe the isolation of mutants resistant to terbinafine or fenpropimorph and the genetic control of resistance in such mutants. We also report on the phenomenon of cross-resistance towards other sterol biosynthesis inhibitors and on the fitness characteristics and pathogenicity of the mutants.

MATERIALS AND METHODS

Fungal strains and culture conditions. Two strains of *N. haematococca* MP VI, S1 and S2, were used in this study as wild type strains. They carried opposite alleles, designated respectively MAT1-1 and MAT1-2 according to Van Etten and Kistler (38), at the mating type locus. They were kindly provided by Prof. H. D. Van Etten (Cornell University, Ithaca, NY) and are identical to the strains 6-36 and 6-94 previously described (37).

Resistant strains were obtained from S1 or S2 following UV-light irradiation of the conidia according to the method previously described (9). The mutants were selected on potato-dextrose agar (PDA) amended with either fenpropimorph at 10 μg/ml or terbinafine at 20 μg/ml. The treatment with UV doses of 1,500, 2,100, and 2,600 ergs/mm^2 gave 25, 10, and 5% conidia survival, respectively. Fenpropimorph (F)- and terbinafine (T)-resistant mutants were designated by a series of two numbers: the first one refers to the original strain (1 or 2 if derived from S1 or S2, respectively), followed by the mutant number. Two spontaneous resistant strains (F1-sp and F2-sp) were also isolated on culture medium containing 10 μg/ml of fenpropimorph.

The mycelial growth rate was determined by inoculating PDA agar plates with an inverted 4-mm-diameter mycelium plug cut from the outer edge of an actively growing colony and measuring the diameter of the developing colony daily for a week. Conidial production was evaluated after 10 days growth in the dark, according to De Falandre et al (9).

All the strains were grown on PDA at 26 C, and single conidia transfers were made once a month. For long term storage, the strains were maintained on PDA agar slants at 4 C.

Antifungal assays. The effect of fungicides on the mycelial growth rate of the various strains was studied according to the method of Leroux et al (28). The effective concentrations causing a 50% reduction in the growth rate (EC_{50}) were determined using the dose response curves. The ratio of EC_{50} resistant strain per EC_{50} sensitive strain gave an estimation of the resistance level (RL). The fungicides used (technical grades) were kindly supplied by the companies that discovered or produced them. They were added to molten PDA after sterilization as ethanolic solution, with the exception of tolnaftate, which was dissolved in dimethylsulfoxide (DMSO) because of its poor solubility in ethanol; the solvent amount was 0.5% (v/v) in treated and control medium. Triplicates of each experimental condition were made.

Genetic methods. Culture conditions favorable to perithecium formation in *N. haematococca* were chosen according to Dietert et al (11). Crosses were performed on V8 vegetable juice agar medium (34). Mycelium of compatible strains (i.e., strains of opposite mating types) were inoculated as pairs on V8 agar plates of 50 mm in diameter. After incubation in continuous dark at room temperature for 10 days to allow mycelial growth, sterile water was poured on the cultures. After 15 min, the excess water was eliminated and the fertilized cultures transferred to 23 C under continuous fluorescent light. Mature perithecia developed within 2–3 wk. Oozing ascospores were removed from the ostiole with a flamed needle and dispersed in sterile water on water agar plates (40 g of agar per liter). Single ascospores were isolated at random and transferred to PDA medium. Sensitivity of the progeny to fungicides was tested on PDA supplemented with fenpropimorph (10 μg/ml) or terbinafine (20 μg/ml), and their mating type was determined by crosses with the two tester strains S1 and S2.

Pathogenicity tests. Pea seeds (cultivar Arkel) were surface-sterilized with sodium hypochlorite (2%) for 3 min, washed with sterile water, and placed on water agar plates (4 g of agar per liter) at 20 C in the dark to allow germination. Inoculation was performed by dipping 4-day-old seedlings for 30 min in a spore suspension (3×10^7 spores per milliliter) of the strain to be tested. The control plants were treated with sterile water. The seedlings were then grown in pots (four seedlings per pot) containing vermiculite moistened with Hoagland's solution (17). Seedling assays were conducted in a controlled environment chamber at 25 C, 60% relative humidity with 18 h of illumination per 24 h. Twenty seedlings were used for each strain in each experiment.

Disease development on the pea plants was evaluated 20 days after inoculation according to the following numbering system: 0 = no symptoms; 1 = small brown lesions on stem; 2 = brown lesions on the stem and roots; 3 = plant dead. The mean value obtained from individual ratings reflects the disease severity caused by the strain and characterizes its pathogenicity.

To check the presence of the fungus in the inoculated plants, diseased fragments were collected, surface-treated with ethanol, rinsed with sterile water, and placed on PDA agar plates amended with streptomycin (100 μg/ml) and penicillin G (100 μg/ml). Colonies that developed were identified and tested for fungicide sensitivity.

Statistical analysis. Analyses were made with the Statistical Analysis System (SAS Institute, Inc., Cary, NC). As suggested by Swallow (35), the protected least significant difference (LSD) was used for mean separation of the growth rates of strains. For analysis of crosses, the segregation ratio of the markers was tested with a chi-square procedure. A nonparametric method, the Mann-Whitney test (6), was used to test the differences between disease severity ratings of strains.

RESULTS

Selection and characterization of resistant mutants. Mutants resistant to either terbinafine or fenpropimorph have been isolated after UV light-irradiation of conidia. As can be seen in Table 1, the number of resistant mutants for each of the tested fungicides increased with increasing UV doses. At a dose allowing a 5% conidia survival, fenpropimorph-resistant mutants were obtained with a frequency of about 5×10^{-6}, which is 10-fold higher than that observed for terbinafine-resistant mutants. This difference probably explains why only fenpropimorph-resistant mutants have been recovered without any mutagenic treatment. Resistant mutants displayed various patterns of growth and morphology. So, for further characterization, we chose mutants exhibiting different phenotypes among mutants whose resistance, as evaluated in preliminary tests, appeared to be high. We selected seven mutants resistant to terbinafine and seven mutants resistant to fenpropimorph (five induced and two spontaneous).

Selected resistant mutants were compared to the wild type strains with respect to phenotypic characteristics such as sporulation, growth rate, pigmentation, sex, and mating type (Table 2). The wild type strains showed a white aerial mycelium replaced by confluent blue-colored sporodochia with aging. They did not excrete any pigment into the culture medium and produced both microconidia and macroconidia; these latter were predominant under our experimental conditions. Several resistant strains displayed similar characteristics. Others differed in the presence of an abundant aerial mycelium, the absence of sporodochia, or the production of pigments diffusing in the culture medium. Growth rates of most terbinafine-resistant mutants were similar to those of wild type strains. Growth rates of fenpropimorph-resistant mutants were more variable. Some mutants showed a reduced growth rate (e.g., T2-7 and F1-38) (Table 2). With regard to conidial production, few qualitative and quantitative differences were observed between strains. Two mutants, T2-3 and F1-38, produced mainly microconidia and the latter in a larger amount. When resistant mutants were evaluated for their ability to cross with the tester isolates, two mutants, F2-2 and F2-sp, did not result in fertile crosses. Others, corresponding to those producing microconidia or excreting pigments in the culture medium, ap-

TABLE 1. Induction of resistance to terbinafine and fenpropimorph by UV treatment in *Nectria haematococca*

UV doses[w]	Conidia survival (%)	Number of resistant mutants[v]			
		Terbinafine		Fenpropimorph	
		I[x]	II[y]	I	II
None	100	None isolated		4	3
1,500	25	2	1	NT[z]	NT
2,100	10	NT	NT	28	12
2,600	5	7	5	41	56

[v] Number of resistant mutants per 10^6 surviving conidia; mutants were selected on potato-dextrose agar containing 20 μg/ml terbinafine or 10 μg/ml fenpropimorph.
[w] Irradiation doses with ultra-violet light (UV) are given in ergs per square millimeter.
[x] Mutants obtained from strain S1.
[y] Mutants obtained from strain S2.
[z] NT: not tested.

TABLE 2. Characteristics of the studied strains of *Nectria haematococca*

Strains[w]	Phenotype[v]			Growth rate[x]	Mating type[y]	Sex[z]
	Sporodochia	Mycelium	Pigment			
Group 1						
S1	+	−	−	9.6 ± 0.2 b	MAT1-1	Mal^+ Fem^+
F1-1	+	−	−	9.2 ± 0.2 c	MAT1-1	Mal^+ Fem^+
F1-38	−	+ wh	−	7.5 ± 0.2 e	MAT1-1	Mal^+ Fem^-
F1-sp	+	−	+ br	8.7 ± 0.2 d	MAT1-1	Mal^+ Fem^-
T1-1	+	−	−	8.7 ± 0.2 d	MAT1-1	Mal^+ Fem^+
T1-2	+	−	−	10.0 ± 0.2 a	MAT1-1	Mal^+ Fem^+
T1-10	+	−	−	9.4 ± 0.2 bc	MAT1-1	Mal^+ Fem^+
Group 2						
S2	+	−	−	10.4 ± 0.2 a′	MAT1-2	Mal^+ Fem^+
F2-1	+	−	−	9.7 ± 0.2 b′	MAT1-2	Mal^+ Fem^+
F2-2	+	−	+ br	8.7 ± 0.2 c′	ND	Mal^- Fem^-
F2-8	+	−	+ by	9.7 ± 0.2 b′	MAT1-2	Mal^+ Fem^-
F2-sp	+	−	+ br	8.6 ± 0.3 c′	ND	Mal^- Fem^-
T2-1	+	−	−	10.3 ± 0.2 a′	MAT1-2	Mal^+ Fem^+
T2-3	−	−	+ bo	10.2 ± 0.2 a′	MAT1-2	Mal^+ Fem^-
T2-7	+	+ gr	+ o	5.5 ± 0.4 d′	MAT1-2	Mal^+ Fem^-
T2-9	+	−	−	10.3 ± 0.2 a′	MAT1-2	Mal^+ Fem^+

[v] Phenotypes were characterized through the presence (+) or the absence (−) of sporodochia with macroconidia, the abundance (+) or not (−) of the aerial mycelium, which can be white (wh) or grey (gr), and the production (+) or not (−) of pigments, which colored the culture medium in brown-red (br), brown-yellow (by), brown-orange (bo), or orange (o).

[w] Fenpropimorph-resistant mutants (F) and terbinafine-resistant mutants (T) were derived from the two strains S1 and S2, which defined Group 1 and Group 2, respectively. All the resistant mutants were UV-induced, except two spontaneous mutants designated sp.

[x] Diametral growth rates (in millimeters per day) were determined on potato-dextrose agar. The means of at least 10 independent experiments, plus or minus half of the 95% t confidence interval, are given. Means followed by a common letter are not significantly different (LSD = 0.3 for Group 1 and for Group 2 strains; $P = 0.05$).

[y] Mating type is designated MAT1-1 and MAT1-2, or ND = not determined due to lack of fertility.

[z] Mal^+ means the ability, and Mal^- the inability to function as a male, Fem^+ the ability, and Fem^- the inability to function as a female, when crossed with S1 or S2.

TABLE 3. Inheritance of resistance in random ascospore progeny from crosses between mutant and wild type strains of *Nectria haematococca*

Crosses R × S[z]	Number of ascospores tested	Progeny phenotype[x]		P values associated with x^2 values for ratio[y]	
		Resistant	Sensitive	1R:1S	3R:1S
T2-1 × S1	147	67	80	0.28	
T2-3 × S1	130	69	61	0.48	
T2-7 × S1	108	59	49	0.33	
T2-9 × S1	83	39	44	0.58	
T1-1 × S2	134	63	71	0.49	
T1-2 × S2	136	66	70	0.70	
T1-10 × S2	58	28	30	0.79	
F2-1 × S1	70	36	34	0.81	
F2-8 × S1	84	44	40	0.66	
F1-1 × S2	111	52	59	0.51	
F1-sp × S2	141	76	65	0.35	
F1-38 × S2	183	137	46		0.96

[v] Fungicide sensitivity of ascospore progeny was determined on potato-dextrose agar, supplemented with terbinafine (20 μg/ml) or fenpropimorph (10 μg/ml).

[y] Model was considered to fit if $P \geq 0.05$.

[z] R = resistant; S = sensitive to fungicides.

peared unable to function as a female parent in a cross.

Genetics of resistance. Ascospore germination in crosses between the 12 fertile resistant mutants and the wild type tester strains was more than 70%. Random ascospore analysis from these crosses (Table 3) showed that in all but one (F1-38 × S2), resistance and sensitivity segregated in a 1:1 ratio, indicating single gene control of the resistant phenotype. In the latter cross, the progeny segregated according to a 3:1 ratio for resistance/sensitivity. Among the resistant progeny, two nonparental types (R1 and R2) could be distinguished from the parental types by their intermediate growth response on medium supplemented with fenpropimorph. When the R1 and R2 recombinants were crossed with the compatible wild type strain, the resistant and sensitive phenotypes were obtained in a ratio of 1:1 among the progeny. Such results indicate that resistance in the F1-38 mutant was conferred by mutations in two independent genes. This was confirmed by crossing R1 and R2 recombinants, which gave a progeny segregation similar to the segregation observed in the initial cross F1-38 × S2 (Table 4).

Crosses between compatible resistant strains carrying mutations in a single gene were done to determine the number of loci involved in the resistance phenomenon. Ascospore germination was again high (more than 70%) in crosses involving terbinafine-resistant mutants but weaker (less than 50–60%) in crosses involving fenpropimorph-resistant mutants, even when a resistant progeny served as the other parent. In each cross, at least 100 random ascospores were examined for fungicide sensitivity. The mutations were considered to be allelic if less than 1% of the progeny was fungicide-sensitive recombinants. No sensitive progeny were detected among the crosses between the seven selected terbinafine-resistant mutants, indicating that resistance resulted from a mutation occurring in a single gene, assigned *Ter*1. Twelve additional induced terbinafine-resistant mutants were crossed with a compatible *Ter*1-carrying strain. No sensitive progeny were recovered from these crosses in which about 50 random ascospores were analyzed. This indicates that these mutants also carried a mutation at the *Ter*1 locus, but we cannot rule out the presence of other mutations, because crosses with wild type strains were not done.

Crosses between the four monogenic fenpropimorph resistant mutants also indicated that a single gene, designated *Fen*1, was involved. When the double-mutant F1-38 was crossed with a compatible *Fen*1-carrying strain (F2-1), sensitive recombinants were recovered among the progeny (Table 4). This result indicates that the two genes determining fenpropimorph resistance in F1-38 were segregating independently from the *Fen*1 gene. These were designated *Fen*2 and *Fen*3. Crosses between terbinafine- and fenpropimorph-resistant strains indicated that the *Ter*1 gene segregated independently from the *Fen*1, *Fen*2, and *Fen*3 genes (Table 4).

In addition, the segregation of several other traits, such as mating type, reduced growth rate (F1-sp, T2-7), modified morphology (F1-38, T2-7), or excretion of pigments (F1-sp, F2-8, T2-3), was followed in crosses of mutants with wild type strains. Each of these characteristics was found to be controlled by a single gene.

The modified morphology cosegregated with the *Fen*2 gene in F1-38 and with the reduced growth rate in T2-7. No linkage relationship was detected between the three genes *Ter*1, *Fen*1, *Fen*3 and the other studied markers (Table 4).

Resistance pattern of mutant strains. The sensitivity of wild type and resistant strains towards terbinafine was assessed. EC_{50} values were 2.9 μg/ml and 2.1 μg/ml for S1 and S2, respectively, and greater than 300 μg/ml for mutants carrying the *Ter*1 gene. These resistant strains thus showed high resistance levels (RL > 100). A positive cross-resistance was observed towards two other squalene epoxidase inhibitors, naftifine and tolnaftate, although these fungicides exhibited only low activities against the wild type strains (Table 5). The terbinafine-resistant mutants were not resistant to sterol demethylation inhibitors nor to inhibitors of the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase (data not shown).

The EC_{50} values and resistance levels of fenpropimorph-resistant mutants towards inhibitors of the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase are presented in Table 6. Strains carrying the *Fen*1 gene showed high resistance levels towards fenpropimorph (20 < RL < 40). The double-mutant strain, F1-38, carrying the *Fen*2 and *Fen*3 genes, was more highly resistant towards fenpropimorph (RL > 100) than the single gene mutants *Fen*2 or *Fen*3, thus reflecting an additive effect between these two genes. Strains carrying mutations in any one of these three genes exhibited a positive cross-resistance towards fenpropidin (10 < RL < 40). Resistance levels towards tridemorph were low in *Fen*1 and in *Fen*2 strains (RL < 10), whereas *Fen*3 strains appeared sensitive to this fungicide. All single gene mutants were sensitive to various sterol demethylation inhibitors (propiconazole, flusilazol, tebuconazole, myclobutanil, prochloraz, fenarimol) and to terbinafine. The double-mutant *Fen*2*Fen*3 appeared slightly resistant (2 < RL < 5) towards some of these products (propiconazole, flusilazol, fenarimol, terbinafine) (data not shown).

Pathogenicity tests. Pathogenicity of wild type and resistant strains was assessed through the disease severity they showed on pea plants. Comparisons were made among strains belonging to the same group (Table 7). Strains carrying allelic mutations at the *Ter*1 locus were found to be as pathogenic as the wild type strains with the exception of T2-3 in which pathogenicity was reduced. Fenpropimorph-resistant mutants showed more variation in pathogenicity. Strains carrying allelic mutations at the *Fen*1 locus did not determine the same severity of disease. For example, pathogenicity appeared similar to that of wild type strains in F2-1 and F2-8 strains, moderately reduced in F1-sp, and more severely affected in F1-1. Strains in which resistance resulted from mutations at the two loci *Fen*2 and *Fen*3 (F1-38) or in which the genetic basis of resistance has not been yet identified (F2-2 and F2-sp) showed a strongly, respectively moderately reduced pathogenicity.

TABLE 4. Linkage relationships between some studied genes in *Nectria haematococca*

Crosses[w]	Number of ascospores tested	Progeny phenotype[x]				*P* values associated with x^2 values for ratio[y]		
						3:1	7:1	1:1:1:1
Fenpropimorph resistance		R	S					
R1 (*Fen*2) × R2 (*Fen*3)	160	125	35			0.36		
F2-1 (*Fen*1) × F1-38 (*Fen*2, *Fen*3)	183	158	25				0.63	
Terbinafine resistance (Ter^R) and fenpropimorph resistance (Fen^R)		Ter^R Fen^R	Ter^R	Fen^R	S			
T2-1 (*Ter*1) × F1-1 (*Fen*1)	110	24	31	29	26			0.79
T1-2 (*Ter*1) × R1 (*Fen*2)	136	29	33	37	37			0.73
T2-1 (*Ter*1) × R2 (*Fen*3)	131	30	34	32	35			0.93
Resistance (R) and mating type (mt)		R mt1	S mt2	R mt2	S mt1			
T1-2 (*Ter*1) × S2	60	14	13	16	17			0.88
F1-sp (*Fen*1) × S2	86	20	22	23	21			0.97
R2 (*Fen*3) × S2	60	15	16	13	16			0.94
Resistance (R) and pigmentation (p)		$R\ p^+$	$S\ p^-$	$R\ p^-$	$S\ p^+$			
T2-3 (*Ter*1) × S1	130	32	31	30	37			0.83
F1-sp (*Fen*1) × S2	128	37	35	35	21			0.16
F2-8 (*Fen*1) × S1	143	35	42	27	39			0.31
Resistance (R) and morphology (m)		$R\ m^+$	$S\ m^-$	$R\ m^-$	$S\ m^+$			
T2-7 (*Ter*1) × S1	95	20	24	20	31			0.33
R1 (*Fen*2) × S2	108	50	58	0	0			NS[z]
Resistance (R) and growth rate (g)		$R\ g^+$	$S\ g^-$	$R\ g^-$	$S\ g^+$			
F1-sp (*Fen*1) × S2	128	27	40	30	31			0.40

[w] Characteristics of strains involved in crosses are presented in Table 2. R1 and R2 are ascospore progeny from the F1-38 × S2 cross. Resistance genes of strains are mentioned in parentheses.

[x] R = resistant, and S = sensitive to fungicides; mt1 and mt2 = phenotypes corresponding respectively to MAT1-1 and MAT1-2; p^+ = excretion of pigments and p^- = absence of pigments in the culture medium; m^+ = modified morphology and m^- = wild type morphology; g^+ = reduced growth rate and g^- = wild type growth rate. Fungicide sensitivity of ascospore progeny was determined on potato-dextrose agar (PDA) supplemented with terbinafine (20 μg/ml) or fenpropimorph (10 μg/ml). Other characteristics were determined on PDA.

[y] Model was considered to fit if $P \geq 0.05$.

[z] NS: no segregation.

TABLE 5. Effect of squalene epoxidase inhibitors on diametral growth of wild type and terbinafine-resistant strains of *Nectria haematococca*

Fungicide application[y]	Growth rate (% control)[x]								
	S1	S2	T1-1	T1-2	T1-10	T2-1	T2-3	T2-7	T2-9
Terbinafine	10 ± 1	8 ± 1	72 ± 5	65 ± 5	67 ± 5	62 ± 6	64 ± 3	96 ± 5	62 ± 4
Naftifine	31 ± 3	26 ± 4	70 ± 5	67 ± 4	73 ± 5	65 ± 4	68 ± 4	84 ± 5	64 ± 5
Tolnaftate	69 ± 6	60 ± 5	92 ± 5	92 ± 1	96 ± 4	92 ± 3	95 ± 4	ND[z]	97 ± 5

[x] Values are means based on two to five independent experiments, plus or minus half of the *t* confidence interval.

[y] Fungicides were added to potato-dextrose agar medium at a concentration of 100 μg/ml; tolnaftate was dissolved in dimethylsulfoxide.

[z] ND: not determined.

TABLE 6. Sensitivity to some morpholine and piperidine derivatives in wild type and fenpropimorph-resistant strains of *Nectria haematococca*

Strain[u]	Mutations[v]	Fenpropimorph		Tridemorph		Fenpropidin	
		EC_{50}[w]	RL[x]	EC_{50}	RL	EC_{50}	RL
S1	None	0.2		5.1		1.7	
S2	None	0.1		3.6		1.3	
F1-1	*Fen*1	3.9	19.5	18.5	3.6	18.5	10.9
F2-1	*Fen*1	3.6	36.0	24.0	6.7	22.0	16.9
F2-8	*Fen*1	4.0	40.0	23.0	6.4	23.5	18.1
F1-sp	*Fen*1	5.6	28.0	21.2	4.1	40.2	23.6
F2-2	ND	4.9	49.0	18.5	5.1	35.0	26.9
F2-sp	ND	5.0	50.0	19.9	5.5	40.9	31.5
R1	*Fen*2	2.2	11.0[y], 22.0[z]	13.5	2.6[y], 3.7[z]	22.7	13.3[y], 17.5[z]
R2	*Fen*3	6.2	31.0[y], 62.0[z]	4.8	0.9[y], 1.3[z]	53.2	31.3[y], 49.9[z]
F1-38	*Fen*2*Fen*3	25.3	126.0	12.6	2.5	>100	>59

[u] R1 and R2 are ascospore progeny from the F1-38 × S2 cross.
[v] ND: not determined due to lack of fertility.
[w] EC_{50} value (μg/ml) is the concentration of fungicide causing a 50% reduction in the diametral growth rate on potato-dextrose agar.
[x] RL value is the resistance level obtained from the ratio of the EC_{50} value for the resistant strain relative to that for the wild type strain.
[y] Resistance levels of R1 and R2 strains, with S1 as the reference.
[z] Resistance levels of R1 and R2 strains, with S2 as the reference.

TABLE 7. Pathogenicity to pea of wild type and fungicide-resistant strains of *Nectria haematococca*

Strain[x]	Mutations[y]	Disease severity[z]
Group 1		
S1	None	2.7 a
F1-1	*Fen*1	1.1 c
F1-sp	*Fen*1	1.8 b
F1-38	*Fen*2*Fen*3	1.0 c
T1-1	*Ter*1	2.4 ab
T1-2	*Ter*1	2.8 a
T1-10	*Ter*1	2.8 a
Group 2		
S2	None	2.6 a′
F2-1	*Fen*1	2.5 a′
F2-8	*Fen*1	2.6 a′
F2-2	ND	1.7 b′
F2-sp	ND	1.9 b′
T2-1	*Ter*1	2.7 a′
T2-3	*Ter*1	1.7 b′
T2-9	*Ter*1	2.6 a′

[x] Group 1 and Group 2 are constituted by the wild type strains, S1 and S2, and the mutants derived from them, respectively.
[y] ND: not determined due to lack of fertility.
[z] Disease severity caused by each strain was rated on a 0–3 scale, with 3 being the rating for greatest disease severity. The means of the ratings of strains in which three to 12 pathogenicity tests were run are given. The control for these experiments had a rating of 0.1. Values followed by a common letter are not significantly different according to the Mann-Whitney test ($P = 0.05$).

DISCUSSION

Mutants of *N. haematococca* MP VI, resistant to two sterol biosynthesis inhibitors (terbinafine and fenpropimorph), could be readily isolated following UV mutagenesis. Depending on the strain and on the experimental conditions, mutation frequencies for resistance to fenpropimorph, an inhibitor of the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase, ranged from 1 to 6×10^{-5}. These frequencies did not differ from mutation rates obtained for resistance towards sterol demethylation inhibitors: $1\text{–}3 \times 10^{-5}$ for tebuconazole resistance in the same organism (1), and 2.4×10^{-5} for fenarimol resistance and 9×10^{-5} for triadimenol resistance in *N. haematococca* MP I (19). Similar results were also reported in *Penicillium caseicolum* (9) and *Monilinia fructicola* (29). On the other hand, mutation frequencies for resistance to terbinafine, a squalene epoxidase inhibitor, appeared about 10 times lower ($1\text{–}7 \times 10^{-6}$).

Resistance to terbinafine appeared to be determined, in the seven mutants analyzed, by mutations in a single gene *Ter*1, and there was presumptive evidence that in 12 other mutants mutations had also occurred at this locus, but some complementary crosses are needed to confirm this. Mutations in the *Ter*1 gene led to high levels of resistance towards terbinafine, the fungicide used for the selection of mutants, and a positive cross-resistance towards two other inhibitors of squalene epoxidase but not towards other sterol biosynthesis inhibitors. Most of the strains carrying a mutation at the *Ter*1 locus had no modification in their mycelial growth rate, sporulation, and pathogenicity. This situation is quite similar to that currently observed with benzimidazoles or phenylamides. Resistance to such fungicides was shown to be due to single gene mutations, which led to target site modifications and resulted in a reduced affinity for the fungicides (7). Likewise, a target site modification may be involved in terbinafine resistance, but such a mechanism of resistance remains to be proved for the *Ter*1 gene in *N. haematococca*. Through these results it appears that the development in agriculture of fungicides with a mode of action similar to that of terbinafine may present an important risk with respect to resistance. However, the characterization of *U. maydis* laboratory-induced mutants resistant to terbinafine led to different conclusions (30). As most of the 152 selected mutants exhibited a slow growth rate, a low to moderate risk was assessed for terbinafine resistance (30). Furthermore, some of these mutants showed a different pattern of cross-resistance towards sterol demethylation inhibitors and morpholines, when compared with *N. haematococca* mutants. Such differences may reflect the involvement of different mechanisms of resistance in each type of mutant. Further studies would specify this point.

Resistance to fenpropimorph appeared to be determined by a more complex system because at least three loci have been identified. Among the seven studied mutants, four strains carried mutations in the same gene *Fen*1. Allelic mutations at the *Fen*1 locus resulted in high levels of resistance towards fenpropimorph but with varying degrees. These mutations also had pleiotropic effects with respect to ascospore viability. The detection of *Fen*1 in most of the fertile induced mutants and in a spontaneous one, indicates that mutations in this gene may occur frequently. Two other genes, *Fen*2 and *Fen*3, were both found to be inherited in a highly resistant mutant. They proved to have an important additive effect on the resistance phenotype.

Two mutants were sterile in crosses and consequently the genetic basis of their resistance could not be elucidated. The stability of resistance in subculturing the mutants on nonselective conditions agreed with mutations in nuclear genes. In addition, the phenotypic expression of resistance of these strains towards fenpropimorph, fenpropidin, tridemorph, and towards other sterol biosynthesis inhibitors was similar to that determined by *Fen*1 mutations, suggesting that they carried mutations either in this gene or in another with similar characteristics.

Mutations in *Fen*1, *Fen*2, or *Fen*3 genes gave resistance levels between 10 and 60 towards fenpropimorph but also conferred

resistance towards fenpropidin with resistance levels between 10 and 40. However, considering tridemorph, another potential inhibitor of the sterol Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase, we observed only low resistance levels (RL < 10) in *Fen*1 and *Fen*2 strains and no resistance in *Fen*3 strains. A lack of cross-resistance or a low positive cross-resistance towards fungicides showing the same mode of action were similarly observed in tridemorph-resistant mutants of *P. caseicolum* towards fenpropimorph and fenpropidin (9). Such a phenomenon might be explained by a differential inhibition of the sterol Δ^{14}-reductase or the $\Delta^{8}\rightarrow\Delta^{7}$-isomerase, or both, depending on the fungicide involved. Indeed, fenpropimorph and fenpropidin have been shown to preferentially inhibit the reductase, whereas tridemorph was more active on the isomerase (2,8). Through these observations, it could be hypothesized that mutations in the *Fen*3 gene affect the sterol Δ^{14}-reductase, but this point has to be studied thoroughly at the enzymatic level. No cross-resistance towards other sterol biosynthesis inhibitors occurred in single gene mutants, whereas the double mutant *Fen*2*Fen*3 appeared slightly resistant towards some of them.

Although growth rate and pathogenicity could be more or less affected in some fenpropimorph-resistant strains, single gene mutants with high resistance levels and characteristics of growth, sporulation, and pathogenicity similar to those of wild type strains were nevertheless obtained. Thus, the occurrence of mutations similar to *Fen*1 in target species may pose a threat to disease control. However, in spite of several years use, no problem of resistance has been encountered to date with powdery mildews of cereals, the main target organisms of morpholine and piperidine fungicides. It is suggested that a lower competitive ability of resistant strains compared to sensitive strains in the absence of fungicide could explain this fact (13). Such competitiveness studies have to be done in *N. haematococca* to test this hypothesis.

Several induced mutants were found to carry double mutations, one being responsible for resistance, the other one affecting some other characteristics (i.e., morphology, growth rate, or pigmentation). This finding was also observed with mutant strains selected for tebuconazole resistance after UV mutagenesis in the same organism (1). Such results indicate that strains of *N. haematococca* MP VI could mutate easily at different loci and that mutations determining modified characteristics are not necessarily linked with mutations for resistance to sterol biosynthesis inhibitors, as was previously suggested (13). Consequently, in the absence of any genetic analysis, predictions on the resistant strains' fitness must be conducted very carefully. In several resistant strains, female sterility has been observed, associated with particular phenotypes, such as the excretion of pigments or the production of an abundant aerial mycelium along with absence of sporodochia. Similar observations were reported in both MP I (14,32) and MP VI (36) of *N. haematococca*.

The present study allowed us to identify single genes conferring high resistance levels to either terbinafine or fenpropimorph. Although recently described for triadimenol resistance in *N. haematococca* MP I (19), such a situation is quite different from what is commonly observed with sterol demethylation inhibitors. Levels of resistance towards such fungicides were mostly below 10, and higher levels could generally be obtained through the additive effect of two or more single genes (1,20,33,39). In some laboratory strains, the mechanism of resistance was identified as an increased fungicide efflux (1,10). In our case, the resistance mechanisms remain to be investigated. Such a study can now be initiated in genetically characterized strains.

LITERATURE CITED

1. Akallal, R. 1989. Résistance de *Fusarium solani* f. sp. *pisi* à des fongicides inhibant la C14-déméthylation des stérols. Thèse de Doctorat, Université Paris-Sud, Orsay, France.
2. Baloch, R. I., and Mercer, E. T. 1987. Inhibition of sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and Δ^{14}-reductase by fenpropimorph, tridemorph and fenpropidin in cell-free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry 26:663-668.
3. Barug, D., and Kerkenaar, A. 1984. Resistance in mutagen-induced mutants of *Ustilago maydis* to fungicides which inhibit ergosterol biosynthesis. Pestic. Sci. 15:78-84.
4. Burden, S. R., Cooke, D. T., and Carter, G. A. 1989. Inhibitors of sterol biosynthesis and growth in plants and fungi. Phytochemistry 28:1791-1804.
5. Carter, G. A., Kendall, S. J., Burden, R. S., James, C. S., and Clarck, T. 1989. The lipid compositions of two isolates of *Cladosporium cucumerinum* do not explain their differences in sensitivity to fungicides which inhibit sterol biosynthesis. Pestic. Sci. 26:181-192.
6. Conover, W. J. 1971. Practical Nonparametric Statistics. John Wiley & Sons, New York. 462 pp.
7. Davidse, L. C. 1987. Mechanisms of resistance in fungi to benzimidazoles, inhibitors of sterol biosynthesis and phenylamides. Pages 216-227 in: Combating Resistance to Xenobiotics. Biological and Chemical Approaches. M. G. Ford, D. W. Holloman, B. P. S. Khombay, and R. M. Sawicki, eds. Ellis Horwood Ltd., Chichester, England and VCH Verlagsgesellschaft mbH, Weinheim, Germany.
8. Debieu, D., Akallal, R., Lasseron, A., Malosse, C., and Leroux, P. 1989. Effect of sterol biosynthesis inhibitors in a filamentous fungus: *Fusarium solani* f. sp. *pisi*. CR Acad. Agric. Fr. 75:179-181.
9. De Falandre, A., Bouvier-Fourcade, I., Seng, J. M., and Leroux, P. 1987. Induction and characterization of *Penicillium caseicolum* mutants resistant to ergosterol biosynthesis inhibitors. Appl. Environ. Microbiol. 53:1500-1503.
10. De Waard, M. A., and Van Nistelrooy, J. G. M. 1980. An energy-dependent efflux mechanism for fenarimol in a wild-type and fenarimol-resistant mutants of *Aspergillus nidulans*. Pestic. Biochem. Physiol. 13:255-266.
11. Dietert, M. F., Van Etten, H. D., and Matthews, P. S. 1983. *Nectria haematococca* mating population VI. Cultural parameters affecting growth conidiation and perithecial formation. Can. J. Bot. 61:1178-1184.
12. Elliot, T. J., and Challen, M. P. 1985. Mushroom genetics and breeding. Glasshouse Crops Res. Inst. Annu. Rep. 1984:106-108.
13. Fuchs, A., and De Waard, M. A. 1982. Resistance to ergosterol biosynthesis inhibitors. I. Chemistry and phenomenological aspects. Pages 71-86 in: Fungicide Resistance in Crop Protection. J. Dekker and S. G. Georgopoulos, eds. PUDOC. Wageningen, The Netherlands.
14. Georgopoulos, S. G. 1963. Genetic markers and linkage relationship from tetrad data in *Hypomyces solani* f. sp. *cucurbitae*. Can. J. Bot. 41:649-659.
15. Georgopoulos, S. G. 1963. Tolerance to chlorinated nitrobenzenes in *Hypomyces solani* f. sp. *cucurbitae* and its mode of inheritance. Phytopathology 53:1086-1093.
16. Georgopoulos, S. G., and Panopoulos, N. J. 1966. The relative mutability of the *cnb* loci in *Hypomyces*. Can. J. Genet. Cytol. 8:347-349.
17. Hoagland, D. R., and Arnon, D. I. 1938. The water culture media for growing plants without soil. Univ. Calif., Agric. Exp. Stn., Circ. 34:39.
18. Hollomon, D. W., Butters, J., and Clarck, J. 1984. Genetic control of triadimenol resistance in barley powdery mildew. Proc. Br. Crop Prot. Conf. Pests and Dis. 2:477-482.
19. Kalamarakis, A. E., Demopoulos, V. P., Ziogas, B. N., and Georgopoulos, S. G. 1989. A highly mutable major gene for triadimenol resistance in *Nectria haematococca* var. *cucurbitae*. Neth. J. Plant Pathol. 95 (suppl. 1):109-120.
20. Kalamarakis, A. E., Ziogas, B. N., and Georgopoulos, S. G. 1986. Resistance to ergosterol biosynthesis inhibitors in *Nectria haematococca* var. *cucurbitae*. 3E.06.in: Abstr. Int. Congr. Pestic. Chem., IUPAC, 6th.
21. Kappas, A., and Georgopoulos, S. G. 1970. Genetic analysis of dodine resistance in *Nectria haematococca* (syn. *Hypomyces solani*). Genetics 66:617-622.
22. Kelly, S. L., Kenna, S., Bligh, H. F. J., Watson, P. F., Sotansfield, I., Ellis, S. W., and Kelly, D. E. 1990. Lanosterol to ergosterol—Enzymology inhibition and genetics. Pages 223-243 in: Biochemistry of Cell Walls and Membranes in Fungi. P. J. Kuhn, A. P. J. Trinci., M. J. Jung, M. W. Goosey, and L. G. Copping, eds. Springer-Verlag, Berlin.
23. Kerkenaar, A. 1987. Mechanism of action of morpholine fungicides. Pages 159-171 in: Modern Selective Fungicides. H. Lyr, ed. VEB Gustav Fischer Verlag, Jena and Longman Group, UK Ltd, London.
24. Kuck, K. H., and Scheinpflug, H. 1986. Biology of sterol-biosynthesis inhibiting fungicides. Pages 65-96 in: Chemistry of Plant Protection. Vol. 1. G. Hang and H. Hoffmann, eds. Springer-Verlag, Berlin.
25. Leroux, P. 1987. La resistance des champignons aux fongicides. Phytoma. 385:6-14.

26. Leroux, P., and Benveniste, P. 1985. Mode d'action des fongicides inhibiteurs des stérols. Pages 67-78 in: Fongicides et protection des plantes: 100 ans de progrès. Monograph n. 31. M. Smith, ed. Br. Crop Prot. Counc. Publ., Croydon, England.
27. Leroux, P., and Gredt, M. 1984. Resistance to fungicides which inhibit ergosterol biosynthesis in laboratory strains of *Botrytis cinerea* and *Ustilago maydis*. Pestic. Sci.15:85-89.
28. Leroux, P., Gredt, M., and Boeda, P. 1988. Resistance to inhibitors of sterol biosynthesis in field isolates or laboratory strains of the eyespot pathogen *Pseudocercosporella herpotrichoides*. Pestic. Sci. 23:119-129.
29. Nuninger-Ney, C., Schwinn, F. J., and Staub, T. 1989. In vitro selection of sterol biosynthesis inhibitor (SBI)-resistant mutants in *Monilinia fructicola* (Wint) Hone. Neth. J. Plant Pathol. 95 (suppl.1):137-150.
30. Orth, A. B., and Sisler, H. D. 1990. Mode of action of terbinafine in *Ustilago maydis* and characterization of resistant mutants. Pestic. Biochem. Physiol. 37:53-63.
31. Ryder, N. S. 1990. Squalene epoxidase enzymology and inhibition. Pages 189-203 in: Biochemistry of Cell Walls and Membranes in Fungi. P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey, and L. G. Copping, eds. Springer-Verlag, Berlin.
32. Snyder, W. C., Georgopoulos, S. G., Webster, S. K., and Smith, S. N. 1975. Sexuality and genetic behavior in the fungus *Hypomyces* (*Fusarium*) *solani* f. sp. *cucurbitae*. Hilgardia 43:161-185.
33. Stanis, V. F., and Jones, A. L. 1985. Reduced sensitivity to sterol-inhibiting fungicides in field isolates of *Venturia inaequalis*. Phytopathology 75:1098-1101.
34. Stevens, R. B. 1974. Mycology Guidebook. University of Washington Press, Seattle. 703 pp.
35. Swallow, W. H. 1984. Those over-worked and oft-misused mean separation procedures. Duncan's, LSD, etc. Plant Dis. 68:919-921.
36. Tegtmeier, K. K., and Van Etten, H. D. 1982. Genetic studies on selected traits of *Nectria haematococca*. Phytopathology 72:604-607.
37. Van Etten, H. D. 1978. Identification of additional habitats of *Nectria haematococca* mating population VI. Phytopathology 68:1552-1556.
38. Van Etten, H. D., and Kistler, H. C. 1988. *Nectria haematococca* mating populations I and VI. Pages 189-206 in: Advances in Plant Pathology. Vol. 6. Genetics of Plant Pathogenic Fungi. G. S. Sidhu, ed. Academic Press Inc., Orlando, FL.
39. Van Tuyl, J. M. 1977. Genetics of fungal resistance to systemic fungicides. Meded. Landbouwhogesch. Wageningen. 77:1-136.