Genetic Control of Resistance to Tridemorph in *Ustilago maydis*

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Mutants of *Ustilago maydis* with low resistance to tridemorph isolated in a mutation frequency of 7×10^{-6} after UV-irradiation and selection on media containing 25 μg ml^{-1} tridemorph. Genetic analysis with nine such mutant isolates resulted in the identification of two unlinked chromosomal loci, *U/tdm*-1 and *U/tdm*-2. The *U/tdm* mutations are responsible for low resistance levels to tridemorph (resistance factor, Rf, of 3 or 5 based on effective concentration causing a 50% reduction in the growth rate (EC_{50}) or minimal inhibitory concentration (MIC) values, respectively) and low to moderate level of resistance to fenpropimorph (Rf 10 or 16 based on MIC or EC_{50}, respectively) and fenpropidin (Rf 5 or 11 based on MIC or EC_{50}, respectively). Haploid strains carrying both *U/tdm* mutations exhibit higher levels of resistance to the above fungicides, indicating interallelic interaction between nonallelic genes. Crosses between mutants carrying the *U/tdm*-genes with compatible isolates carrying the *U/fpm*-1 or *U/fpm*-2 mutations, which were found in previous work to carry fenpropimorph resistance, yielded in all cases a large number of recombinants with wild-type sensitivity, indicating that the mutant genes involved were not allelic. Cross-resistance studies with the inhibitors of C-14 demethylase showed that the *U/tdm*-mutations were responsible for increased sensitivity to the triazoles triadimefon, triadimenol, propiconazole and flusilazole, and to the pyridine pyrifenox. Study of gene effect on the fitness of *U. maydis* showed that *U/tdm*-mutations appeared to be pleiotropic, having more or less adverse effects on growth rate in liquid culture and pathogenicity on young corn plants.

KEY WORDS: *Ustilago maydis*; fungicide resistance; tridemorph; morpholines; ergosterol biosynthesis inhibitors; genetic control of resistance.

INTRODUCTION

Tridemorph belongs to the important group of morpholine fungicides, the toxicity of which is dependent upon inhibition of sterol biosynthesis (10,26,30). It was introduced in 1969 for the control of cereal fungal diseases, particularly powdery mildews and yellow rust (*Puccinia striiformis*) (27,33). Moreover, it has been used extensively against Sigatoka disease (*Mycosphaerella musicola*). It is also active against a number of other pathogens such as powdery mildew of cucurbits, tobacco, legumes, apple, ornamentals and sugar beet, and postharvest diseases of citrus fruits (33).

Most investigations on the effects of tridemorph on fungal growth and morphology showed that tridemorph does not suppress the germination of fungal spores to any great extent but that it is highly effective in inhibiting mycelial growth (28,29). The first

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indication that tridemorph had an inhibitory effect on sterol biosynthesis was reported by Kato *et al.* (25). Later, Berg *et al.* (3) showed that in *Saprolegnia ferax*, an oomycete, tridemorph inhibits the nuclear double-bond transformations in the $\Delta^{8,7}$- sterol isomerase rather than Δ^{14}-sterol reductase; this was confirmed by Baloch *et al.* (2), Ziogas *et al.* (36) and Debieu *et al.* (12). The accumulation of only Δ^{8}-sterol, such as fecosterol, ergosta-8,22-dienol and ergosta-8-enol was found in a wide range of fungal species after tridemorph treatment (26,30).

The development of resistance in fungi to fungicides with a specific mechanism of action has become a serious problem in the control of plant diseases (5,15,35). Currently, such problems are encountered with sterol demethylation inhibitors (DMIs), which have been used extensively (9,17,22). Morpholine fungicides could be used successfully in controlling strains resistant to DMIs and, despite the longstanding use, there are no reports of considerable reduced effectiveness in the field (4,22,24). The two or three sites of action and the negative impact of resistant mutations on phytopathogenic fitness were considered responsible for the absence of resistance outbreaks in the field (2,24). Although laboratory resistance to morpholines has been reported for several fungi, problems with practical resistance have not appeared after more than 25 years of use (22,30). However, in the last few years there have been reports of a decrease in sensitivity of cereal powdery mildews to these fungicides in Europe (6-8,18,21,31,34).

Although considerable progress has been made in the research with morpholine fungicides in the last few years, little information is currently available on fungal resistance to these compounds and many problems have to be solved. Knowledge of the basis for resistance could be important in developing strategies for resistance management (35). In particular, much more research has to be carried out on the genetic basis and mechanism of resistance. Our previous study indicated that major gene mutations like the *U/fpm*-1A and *U/fpm*-2 may cause loss of the morpholine fenpropimorph effectiveness and reduce tridemorph sensitivity in the field to the pathogens with genetic variability similar to *Ustilago maydis* (32). To expand our knowledge in this field and, subsequently, to explore the resistance risk for the morpholine fungicides, a study of genetic control of resistance to tridemorph in the phytopathogenic basidiomycete *U. maydis* was initiated. Such genetic studies may be helpful in understanding the differential behavior, with respect to resistance, of this fungicide in comparison with other sterol biosynthesis inhibitors (SBIs). *U. maydis* is the causal agent of maize smut, it is heterothallic, and the perfect stage (teliospores) is produced readily. Moreover, it is sensitive to almost all SBIs and is particularly suitable for the study of resistance to such fungicides.

MATERIALS AND METHODS

Organism and culturing conditions The compatible wild-type strains No. 201 and No. 501 of *U. maydis* (DC.) Corda, isolated from infected field-growing corn plants, were used to obtain mutants resistant to tridemorph. All isolates were maintained on a simplified Ustilago complete agar medium (UCM) according to Holliday (23), at 10°C in the dark. Sporidia were obtained by growing the fungus in complete liquid medium at 29°C on a rotary shaker at 150 rev. min^{-1}.

Fungicides The SBIs used were: *(a)* morpholines and related fungicides: tridemorph, fenpropimorph and fenpropidin; *(b)* DMIs: triforine, fenarimol, pyrifenox, imazalil, triadimefon, triadimenol, clotrimazole, triflumizole, propiconazole and flusilazole; and *(c)*

the allylamine terbinafine, which is a squalene epoxidase inhibitor. All fungicides were technical grade and obtained from their respective manufacturers.

Measurement of fungitoxicity The fungitoxicity of SBIs was measured in agar and liquid medium. In the first case the colony-forming ability was determined by spreading cells on the surface of UCM containing various concentrations of the fungicide. The number of sporidia capable of producing colonies in control and treated media was recorded after 4 days. For toxicity measurements in liquid culture, the fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of the liquid medium of Coursen and Sisler (11). Actively growing sporidia (5×10^4 cells ml^{-1}) were used as initial inoculum and the cultures were incubated at 29°C on a rotary shaker (150 rpm) for 22–24 h. The effect of the toxicant on growth was determined by measuring optical density changes at 450 nm of sporidial suspensions, or by dry weight measurements. The fungicides were added to the sterilized growth medium from stock solutions in ethanol, prior to inoculation. The amount of solvent never exceeded 1% (v:v) in treated or control media. At least six concentrations with three replicas for each fungicide were used in order to obtain the respective fungitoxicity curves. The effective concentrations causing a 50% reduction in the growth rate (EC_{50}) were determined from the data after probit analysis. The ratio of EC_{50} or minimal inhibitory concentration (MIC) for a resistant strain, to the EC_{50} or MIC for a sensitive strain, gave an estimate of the resistance level (resistance factor, Rf).

Mutation induction A suspension of 10^7 ml^{-1} log phase sporidia was exposed, under continuous agitation, to ultraviolet irradiation (TUV Philips, 15W, 253.7 nm) for 3.5 min, which resulted in 90–95% lethality. After irradiation the sporidia were kept for 30 min in the dark to minimize photorepair of radiation damage, then plated on UCM agar medium containing tridemorph (25 μg ml^{-1}) and incubated at 29°C for 18 days to enable resistant colonies to appear. The selected-resistant isolates were maintained on agar slants containing tridemorph (10 μg ml^{-1}).

Genetic methods The techniques described by Holliday (23), with minor modifications, were used for crossing each mutant with compatible wild-type and other mutant strains in 10-day-old maize seedlings (*Zea mays* L. cv. B-73) and for random analysis of progeny. Each of these isolates was crossed first with a compatible wild type and then all possible R×R crosses were made. The recognition of progeny phenotype was based on the analysis of a large number of progeny for their sensitivity to tridemorph using a 26-pin replicator. A random sample of at least 100 progeny from each cross was tested for sensitivity to tridemorph at the MIC for the wild type (10 μg ml^{-1}), and at the non-inhibitory concentration (25 μg ml^{-1}) and the MIC (50 μg ml^{-1}) for the resistant parent. In order to avoid any progeny preference in the isolation procedure, all of the colonies which originated from single spores that appeared in a plate or part of the plate were collected.

Pathogenicity tests Virulence of tridemorph-resistant isolates was tested on 8–10-day-old maize seedlings grown in a growth room (14 h day^{-1} illumination at 28°C and 60% r.h.). Mixed sporidial suspensions (10^6 sporidia ml^{-1}) of each mutant with the compatible wild type and other mutants carrying the same or different genes for resistance to tridemorph were injected just above the first node of the seedlings, using a 1 ml syringe. The inoculated seedlings (four seedlings per 17-cm pot, three pots per cross) were placed in a humidity chamber (100% humidity at 28°C) for 48 h, after which they were returned to the growth room for ~20 days. Virulence was evaluated according to: *(a)* the number

of infected or dead plants; *(b)* the time taken for galls to appear and mature; and *(c)* the production of mature teliospores. Approximately 5–8 g dry weight of gall from each cross was ground in a mortar after addition of water, and used for the last measurement. The maturity of galls was verified by light microscopy of thin sections.

Statistical analysis Analyses were made with the Statistical Analysis System (JMP, SAS Institute, Inc., Cary, NC, USA). The growth rate and the EC_{50} value for each isolate and fungicide were calculated from the data subjected to probit analysis. Dunnett's multiple range test was used to assess the differences between pathogenicity ratings of strains. For analysis of crosses, the segregation ratio of progeny was tested with a chi-square procedure at P=0.05 level of significance.

RESULTS

Selection and characterization of resistant mutants Mutants resistant to tridemorph were isolated after UV-light irradiation of sporidia of *U. maydis*. Approximately 8.2×10^6 irradiated sporidia of the two compatible wild-type isolates No. 201 and No. 501, which survived the mutagenic treatment, were plated on UCM containing 25 μg tridemorph. From this selection medium 58 resistant colonies were obtained during the 18 days of incubation, indicating a mutation frequency of 7×10^{-6}. Most of the resistant isolates appeared between the 8th and 12th days of incubation. All resistant isolates were transferred to UCM slants and assigned isolation numbers. Depending on the parent sensitive isolate, 201 or 501, they were designated as TD-20.. or TD-50.., respectively. As there were no considerable differences in growth on control and tridemorph-containing medium, a random sample of nine such isolates, four derived from the wild-type No. 201 and five from No. 501, were maintained for further studies.

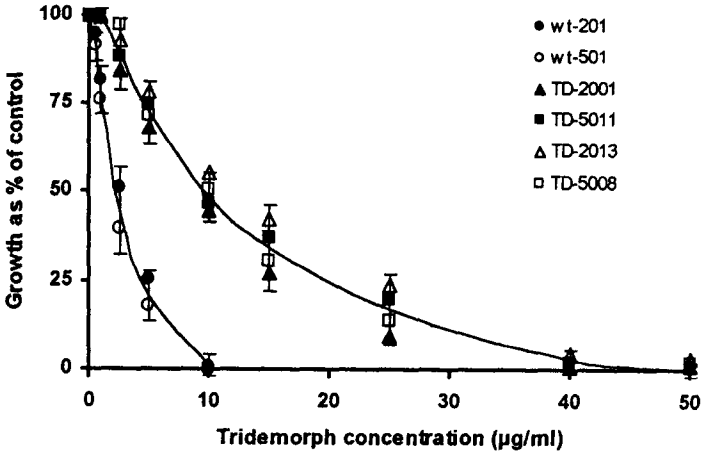

Fig. 1. Sensitivity of the wild-types (wt) and four representative tridemorph-resistant (TD) isolates of *Ustilago maydis* to tridemorph in liquid medium. Measurements were made after 24 h of incubation.

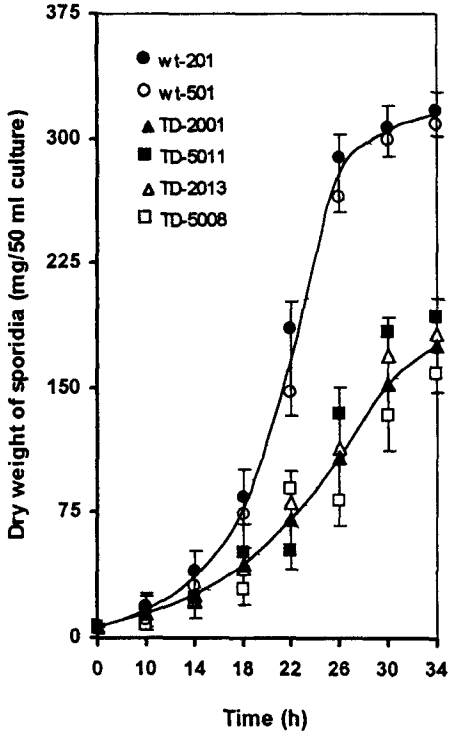

Fig. 2. Growth of the tridemorph-sensitive (wt) and four tridemorph-resistant (TD) isolates of *Ustilago maydis* in liquid medium without fungicide.

All mutant isolates were generally low-resistant to tridemorph, with Rf 5 based on MIC, and Rf 2-4 based on EC_{50} values. A dose-dependent decrease in growth was observed with the wild-type and mutant isolates (Fig. 1). Comparisons of growth rates in liquid culture, by measuring the optical density or dry weight increases, showed that the low-tridemorph-resistance was accompanied by a significant reduction (40–50%) in growth rate (Fig. 2). The doubling time was 353±16 min, *vs* 169±15 min for the wild-type strains.

Genetic analysis of tridemorph resistance

Crosses between wild-type and tridemorph-resistant isolates: As shown in Table 1, the ratio of resistant: sensitive (R:S) progeny was in all crosses exactly the same at both concentrations: the MIC for the sensitive parent (10 μg ml^{-1}), and the higher noninhibitory concentration for the resistant parent (25 μg ml^{-1}); and this segregation was not significantly different from a Mendelian 1:1 ratio ($x^2 = 3.841$) at the $P = 0.05$ level. Thus, each of these mutant isolates was the result of mutation of a single chromosomal gene for resistance to tridemorph. The progeny ratios in the present study were very close to the expected ratios for the segregation. Since great care was taken to avoid any progeny preference in the isolation procedure, we believe that the progeny segregation was a genuinely chance occurrence.

Crosses between tridemorph-resistant isolates: To determine the number of mutated chromosomal genes, ~100 progeny from each R×R cross were tested for resistance to 10 and 25 μg ml^{-1} tridemorph. The two mutants mated were assumed to be allelic if no

TABLE 1. Results of random analysis of progeny from crosses involving wild-type and tridemorph-resistant mutant isolates of *Ustilago maydis*

Cross[z]	Number of progeny tested	Ratio of resistant/sensitive progeny at the indicated tridemorph concentrations (μg ml^{-1})		x^2 for fungicide sensitivity[y]	Mutated loci
		10	25		
S×R					
201 × TD-5008	115	60/55	60/55	0.217a	
201 × TD-5006	86	45/41	45/41	0.186a	
201 × TD-5013	104	51/53	51/53	0.038a	
201 × TD-5011	96	50/46	50/46	0.166a	
201 × TD-5003	115	57/58	57/58	0.008a	
501 × TD-2002	96	45/51	45/51	0.375a	
501 × TD-2001	115	59/56	59/56	0.078a	
501 × TD-2013	74	39/35	39/35	0.216a	
501 × TD-2007	106	50/56	50/56	0.339a	
R × R					
TD-5008 × TD-2002	115	115/0	115/0	–	*U/tdm*-1
TD-5008 × TD-2001	97	97/0	97/0	–	*U/tdm*-1
TD-5008 × TD-2007	104	104/0	104/0	–	*U/tdm*-1
TD-5006 × TD-2002	115	115/0	115/0	–	*U/tdm*-1
TD-5003 × TD-2007	100	100/0	100/0	–	*U/tdm*-1
TD-5008 × TD-2013	74	56/18	56/18	0.018b	*U/tdm*-1 × *U/tdm*-2
TD-5006 × TD-2013	115	86/29	86/29	0.003b	*U/tdm*-1 × *U/tdm*-2
TD-5003 × TD-2013	115	84/31	84/31	0.235b	*U/tdm*-1 × *U/tdm*-2
TD-5013 × TD-2013	115	115/0	115/0	–	*U/tdm*-2
TD-5011 × TD-2013	115	115/0	115/0	–	*U/tdm*-2

[z] S = sensitive (wild type) isolates; R = tridemorph-resistant mutant isolates.
[y] Expected value of x^2 for a 1:1(a) or a 3:1(b) ratio at P=0.05 is < 3.841.

sensitive progeny were produced. Otherwise, they carried resistance genes at different loci. In this way, two chromosomal loci (*U/tdm*-1 and *U/tdm*-2) for resistance to tridemorph were identified (Table 1). Among the nine single-gene mutants studied, six carried the *U/tdm*-1 and three the *U/tdm*-2 mutation. In an attempt to recognize interallelic interactions when two nonallelic genes, *U/tdm*-1 and *U/tdm*-2, were present in the same haploid nucleus, progeny from crosses between nonallelic mutants were tested for sensitivity at three concentrations of tridemorph: the MIC for the wild-type (10 μg ml^{-1}); the noninhibitory (25 μg ml^{-1}) and the MIC (50 μg ml^{-1}) for the resistant parents. As shown by the examples given in Table 2, the segregation ratio was approximately 3R:1S for the first and second, and 1R:3S for the third of these concentrations. The 3:1 R/S ratio in all crosses at the concentrations of tridemorph to which both mutant isolates were resistant, shows that *U/tdm*-1 and *U/tdm*-2 are unlinked, segregating independently of each other. The 1:3 R/S ratio for the third concentration, to which both mutant isolates were sensitive, shows that the gene effect is additive. Therefore, tridemorph resistance in *U. maydis* is a quantitative trait and a unimodal distribution of fungal isolates regarding the tridemorph sensitivity is to be expected.

Crosses between isolates carrying the U/fpm and U/tdm mutations: Crosses between mutants carrying the *U/tdm*-genes with compatible isolates carrying the *U/fpm*-1 or *U/fpm*-2 mutations, were analyzed to determine whether the *U/tdm*-genes are allelic with one of

TABLE 2. Results of random analysis of progeny from crosses involving resistant isolates of *Ustilago maydis* which carried the *U/tdm* and the *U/fpm* genes

Cross	Mutations	Number of progeny tested	Ratio of resistant/sensitive progeny at the indicated tridemorph concentrations (μg ml^{-1})			Linkage between mutated genes	
			10	25	50	% Recombination[z]	x^2 values (3:1)[y]
U/tdm × U/tdm							
TD-5008 × TD-2013	*U/tdm*-1 × *U/tdm*-2	115	86/29	86/29	24/91	25.22	0.003
TD-2001 × TD-5011	*U/tdm*-1 × *U/tdm*-2	102	73/29	73/29	26/76	28.43	0.641
U/tdm × U/fpm							
TD-5003 × FP-2013	*U/tdm*-1 × *U/fpm*-1A	113	79/34	79/34	0/113	30.09	1.560
TD-5008 × FP-2020	*U/tdm*-1 × *U/fpm*-1B	115	85/30	85/30	0/115	26.09	0.072
TD-5006 × FP-2024	*U/tdm*-1 × *U/fpm*-2	98	70/28	70/28	0/98	28.57	0.667
TD-2013 × FP-5017	*U/tdm*-2 × *U/fpm*-1A	115	81/34	81/34	0/115	29.57	1.278
TD-2013 × FP-5003	*U/tdm*-2 × *U/fpm*-1B	98	72/26	72/26	0/98	26.53	0.122
TD-2013 × FP-5032	*U/tdm*-2 × *U/fpm*-2	86	60/26	60/26	0/86	30.23	1.256

[z] The recombination was calculated at the lower tridemorph concentration tested.
[y] Expected value of x^2 for a 3:1 ratio at P=0.05 is < 3.841.

TABLE 3. Expression of mutations for resistance to morpholine and related fungicides in *Ustilago maydis*

Fungicide	Resistance factor[z] based on					
	EC_{50}[y] (mean±SE[w])			MIC[x]		
	U/tdm-1	*U/tdm*-2	*U/tdm*-1,2	*U/tdm-1*	*U/tdm*-2	*U/tdm*-1,2
Tridemorph	3±0.34	3±1.17	4.4±0.28	5	5	7.5
Fenpropimorph	16±2.27	16±2.36	20±3.92	10	10	15.0
Fenpropidin	11±1.42	11±1.73	15±2.19	5	5	8.0

[z] Ratio of EC_{50} for mutant : EC_{50} for wild type, or of MIC for mutant : MIC for wild type.
[y] Effective concentration causing 50% reduction in growth rate.
[x] Minimal inhibitory concentration.
[w] Pooled standard error; six replications.

them. Approximately 100 random progeny from each of these crosses were tested for sensitivity to at least three concentrations of tridemorph, as detailed above. The MIC for the mutants (last concentration) was used in order to examine interallelic interaction when two nonallelic genes were present in the same haploid nucleus. As shown in Table 2, the segregation ratio was approximately 3R:1S at 10 μg ml^{-1} and 25 μg ml^{-1}, and all progeny were sensitive at 50 μg ml^{-1}. The large number of recombinants with wild-type sensitivity indicates that the mutant genes involved were not allelic. The absence of resistant progeny at the highest concentration indicates that there is no additive gene effect between *U/tdm* and *U/fpm* mutations.

Cross resistance As shown in Table 3, the identified mutations *U/tdm*-1 and *U/tdm*-2 decrease the sensitivity to the morpholine fenpropimorph (Rf 10 based on MIC and Rf 14–18 based on EC_{50} values) and the related piperidine fenpropidin (Rf 5 based on MIC and 9–13 based on EC_{50} values), which act at the same steps of sterol biosynthesis.

Studies of resistance to DMIs and terbinafine showed (Table 4) that the mutants carrying the *U/tdm*-1 or *U/tdm*-2 mutations present 2–5 times increased sensitivity to triadimefon, triadimenol, propiconazole, flusilazole and pyrifenox.

TABLE 4. Sensitivity to sterol demethylase inhibitors and to the allylamine terbinafine of wild type and tridemorph-resistant mutant isolates of *Ustilago maydis*

Fungicide	Wild type		Rf[z] based on			
			EC_{50}[y] (mean±SE[w])		MIC[x]	
	EC_{50} (mean±SE[y])	MIC	*U/tdm* - 1 / *U/tdm* - 2	*U/tdm*-1,2	*U/tdm* - 1 / *U/tdm*-2	*U/tdm* - 1,2
Triazoles						
Triadimefon	23.8±0.14	50	0.36±0.02[v]	0.34±0.01[v]	0.5[v]	0.5[v]
Triadimenol	2.5±0.19	25	0.48±0.01[v]	0.42±0.03[v]	0.2[v]	0.2[v]
Propiconazole	1.25±0.1	5	0.175±0.04[v]	0.178±0.06[v]	0.25[v]	0.25[v]
Flusilazole	0.18±0.02	1	0.36±0.07[v]	0.33±0.07[v]	0.2[v]	0.2[v]
Imidazoles						
Clotrimazole	1.75±0.2	10	1	1	1	1
Imazalil	2±0.04	5	1	1	1	1
Triflumizole	0.03±0.002	0.5	1	1	1	1
Pyrimidines						
Fenarimol	0.875±0.09	7.5	1	1	1	1
Pyridines						
Pyrifenox	0.025±0.003	0.25	0.46±0.07[v]	0.41±0.04[v]	0.4[v]	0.4[v]
Piperazines						
Triforine	18.5±0.4	125	1	1	1	1
Allylamines						
Terbinafine	1.5±0.14	10	1	1	1	1

[z] Resistance factor.
[y] Effective concentration causing 50% reduction in growth rate.
[x] Minimal inhibitory concentration.
[w] Pooled standard error; six replications.
[v] Negative cross resistance.

TABLE 5. Pathogenicity to corn seedlings of isolates of *Ustilago maydis* sensitive or resistant to tridemorph

Cross	Mutations	Number of infected seedlings[z]	Days after inoculation for formation of galls: young galls (mean±SE[x])	mature galls (mean±SE[x])	Teliospore production ($\times 10^6$)[y] (mean±SE[x])
201 × 501	wt × wt	12	7±0.48a[w]	15 ± 0.72a	12.6 ± 0.54a
201 × TD-5006	wt × *U/tdm*-1	6	14±1.73b	22±1.53b	5.2±1.12b
501 × TD-2013	wt × *U/tdm*-2	7	13±1.94b	24±2.15b	5.4±0.96b
501 × TD-1,2	wt × *U/tdm*-1,2	6	12±1.24b	23±2.25b	5.5±1.06b
TD-2002 × TD-5006	*U/tdm*-1 × *U/tdm*-1	7	14±1.63b	26±2.93b	3.2±0.92b
TD-5006 × TD-2013	*U/tdm*-1 × *U/tdm*-2	7	16±1.74b	27±2.18b	3.9±0.92b
TD-5006 × TD-1,2	*U/tdm*-1 × *U/tdm*-1,2	6	15±2.31b	28±1.53b	3.3±0.95b
TD-5011 × TD-2013	*U/tdm*-2 × *U/tdm*-2	8	16±1.72b	26±2.77b	3.4±0.84b
TD-2013 × TD-1,2	*U/tdm*-2 × *U/tdm*-1,2	5	14±2.12b	26±3.36b	2.9±0.85b

[z] 12 seedlings per cross were inoculated and the infected (or dead) seedlings were evaluated.
[y] Number of teliospores per gram dry weight of gall.
[x] Pooled standard error; 12 replications.
[w] Within columns, values followed by the same letter do not differ significantly according to Dunnett's multiple range test (P=0.05).

Pathogenicity The minor gene mutations *U/tdm*-1 and *U/tdm*-2, whether homozygous or heterozygous with their wild type (wt) allele, resulted in a 50% reduction in the number of infected seedlings, an increase of several days in the time taken for galls to form, and a reduction of 50–75% in teliospore production (Table 5). When both mutations were present in a dikaryon, a greater reduction in teliospore production was observed. Infection of seedlings by *U/tdm* × *U/fpm* crosses (results not shown) revealed a pathogenicity of the formed dikaryon equal to that of wt × wt or wt × *U/fpm*, which indicates an epistatic effect of major *U/fpm* genes on minor *U/tdm* genes.

DISCUSSION

Mutants of *U. maydis* resistant to tridemorph isolated at a frequency of 7×10^{-6}, following ultraviolet mutagenesis. All mutant isolates were classified into the same low-resistant phenotype. Laboratory mutants resistant to morpholine-type fungicides are easy to generate in several fungi, indicating that biochemical ways to overcome the fungitoxic effects of morpholines exist in fungal species (24). High mutation frequency (3×10^{-3}) for tridemorph resistance was found in *Penicillium caseicolum* (13). With the notable exception of *Aspergillus nidulans*, considerable high mutation frequencies ($1\text{-}30 \times 10^{-6}$) were found for fenpropimorph resistance in various fungal species such as *Nectria haematococca* (14,16), *P. caseicolum* (13), *Aspergillus niger* (20) and *U. maydis* (32). In the case of *A. nidulans*, UV-treatment of the conidia did not yield mutants resistant to fenpropimorph (20).

Genetic analysis of crosses among nine selected mutants resulted in the identification of two unlinked resistance genes, *U/tdm*-1 and *U/tdm*-2; among the nine mutants studied, six and three carried these two loci, respectively. The increase of resistance in morpholine and piperidine fungicides observed in haploid isolates carrying both nonallelic genes *U/tdm*-1 and *U/tdm*-2, indicates that resistance to tridemorph is under polygenic control and a gradual, unimodal shift towards lower sensitivity is possible. Crosses between mutants carrying the *U/tdm* mutations with compatible isolates carrying the *U/fpm*-1A or *U/fpm*-2 major genes, or the *U/fpm*-1B minor gene for resistance to fenpropimorph (32), revealed that the mutant genes involved were not allelic, and no increase in resistance resulted from combining the *U/tdm* with *U/fpm* mutations in one individual haploid nucleus.

Involvement of two or more genes for resistance to morpholine fungicides has also been found in a number of other pathogens, *viz.*, three genes in *N. haematococca* var. *cucurbitae* (16), three in *N. haematococca* var. *pisi* (14), two in *Erysiphe graminis* f.sp. *hordei* (7) and two genes located on linkage group II in *A. niger* (20). In all cases except for the *U/fpm*-1A and *U/fpm*-2 mutations for high resistance to fenpropimorph in *U. maydis* (32), the resistance level was low to moderate and there was an additive effect of gene action, indicating that the resistance to these fungicides is, mainly, a quantitative trait.

Generally, a positive cross-resistance occurs between fungicides belonging to the same group but not between toxicants with different modes of action. Cross-resistance studies of tridemorph with other morpholine-type fungicides showed that *U/tdm* mutations are responsible for a low to moderate level of resistance to fenpropimorph and had little effect on the response to fenpropidin. A positive cross-resistance – with various resistance factors – between morpholine fungicides was observed also in fenpropimorph-resistant mutants of *U. maydis* (32), *E. graminis* f.sp. *hordei* (7), *N. haematococca* var. *cucurbitae* (16), *N. haematococca* var. *pisi* (14), *P. caseicolum* (13) and *A. niger* (20). Study of cross-resistance

relationships between morpholine fungicides in *E. graminis* f.sp. *hordei* also showed that the resistant genes had comparatively little effect on response to tridemorph (7).

The low level of resistance which is encoded by *U/tdm* genes and the cross-resistance of tridemorph with fenpropimorph and fenpropidin, indicate that some factor other than target-site modification is the underlying biochemical mechanism of resistance. All three fungicides inhibit the reduction or isomerization of two double-bonds in the ergosterol biosynthesis pathway, but fenpropidin and fenpropimorph preferentially inhibit Δ^{14}-reductase, while tridemorph inhibits mainly the $\Delta^{8,7}$-isomerase (1,26,30,36). Recently, interference by fenpropimorph in the final part of sterol synthesis has been suggested also in *E. graminis* f.sp. *tritici* (19). A target site change at Δ^{8}-Δ^{7}-isomerase does not provide a reasonable explanation for the resistance to fenpropimorph and fenpropidin, and obviously another mechanism of resistance is encoded by the identified genes for resistance to the above fungicides. Such a study is now in progress with the genetically characterized strains.

Cross-resistance studies of tridemorph with the inhibitors of C-14 demethylase and squalene epoxidase revealed that the minor gene mutations (*U/tdm*-1 and *U/tdm*-2) increased the sensitivity to triazoles and to the pyridine pyrifenox; the minor gene *U/fpm*-1B mutants also showed increased sensitivity to the above DMIs (32). It is difficult at present to find a reasonable explanation for the negative cross-resistance between morpholines and these compounds.

In our study of gene effect on the phytopathogenic fitness of *U. maydis*, the minor gene mutations *U/tdm*-1 and *U/tdm*-2 in homozygous or heterozygous condition in dikaryotic mycelium reduced the phytopathogenic fitness-determining characteristics of mutant strains such as growth rate and pathogenicity by up to 50%. Similar results have been found in the same pathogen with the minor gene *U/fpm*-1B for resistance to fenpropimorph (32). De Falandre *et al.* (13), working with *P. caseicolum*, found two tridemorph-resistant phenotypes as regards level of resistance and the fitness of mutated strains. One phenotype was similar to the *U/tdm*-mutation in the present work (low resistance level and low fitness) whereas the other exhibited a high level of resistance without any negative impact on sporulation and growth rate.

Although tridemorph is a systemic fungicide with a specific action on the sterol biosynthesis pathway, it is considered at low risk for reduction in its effectiveness through development of resistance in target fungi. This conclusion is based mainly on the very few reported cases of reduction in sensitivity, despite many years of widespread use of morpholines in cereal and other crops (24). The findings of the present work, *viz.*, low mutation frequency, low level of resistance, and pleiotropic effects on phytopathogenic fitness of resistant strains, lend support to such a conclusion. A considerable difference between tridemorph and fenpropimorph regarding the resistance risk was found in *U. maydis*. Although fenpropimorph reacts at two or three sites in the sterol biosynthetic pathway (36), major gene mutations for resistance, with no loss of phytopathogenic fitness, were found in high frequency (32).

If a genetic variability similar to that in *U. maydis* is present also in pathogens such as powdery mildews which are controlled by morpholines, the development of resistance to tridemorph will be much slower than to fenpropimorph. In view of their findings that resistance genes in *E. graminis* f.sp. *hordei* for resistance to fenpropimorph and fenpropidin had little effect on response to tridemorph, Brown *et al.* (7) concluded that tridemorph belongs to a different cross-resistant group of fungicides from fenpropimorph

and fenpropidin. The results of our cross-resistance studies with *U/fpm* and *U/tdm* gene mutations, and unpublished data with fenpropidin- and piperalin-selected mutants and also on the mechanism of resistance to morpholine fungicides, do not support the above conclusion. In our opinion the mechanism of resistance which is encoded by the resistance genes is obviously more effective for the fungitoxicity of fenpropimorph and fenpropidin than for that of tridemorph.

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