European Journal of Plant Pathology **108:** 21–30, 2002.

Genetic control of resistance to the piperidine fungicide piperalin in *Ustilago maydis*

A.N. Markoglou and B.N. Ziogas*
Laboratory of Plant Pathology, Agricultural University of Athens, Votanikos, 188 55 Athens, Greece;
**Author for correspondence (Phone: +30 1 5294507; Fax: +30 1 3422062; E-mail: ziv@auadec.aua.gr)*

Accepted 20 September 2001

Key words: ergosterol biosynthesis inhibitors, fungicide resistance, piperidines, morpholines

Abstract

Mutants of *Ustilago maydis* resistant to the piperidine fungicide piperalin were isolated in a mutation frequency of 2.4×10^{-5} after UV-irradiation and selection on media containing 50 μg ml^{-1} piperalin. Genetic analysis with 15 such mutant isolates resulted in the identifications of two unlinked chromosomal loci, the *U/ppl*-1 locus with two allelic genes (*U/ppl*-1A and *U/ppl*-1B) and the *U/ppl*-2 locus. The *U/ppl*-2 and *U/ppl*-1A mutations are responsible for two levels of moderate and high resistance to piperalin (resistance factor, Rf: 54 and 135, respectively, based on effective concentration causing a 50% reduction in the growth rate, EC_{50}), while the *U/ppl*-1B mutation gives only a small reduction (approximately 8-fold) in piperalin sensitivity. Cross-resistance studies with other SBIs shows that the major gene (*U/ppl*-2 and *U/ppl*-1A) mutants are resistant to fenpropidin (Rf: 43 and 68), fenpropimorph (Rf: 261 and 283) and tridemorph (Rf: 9 and 10), but not to the inhibitors of C-14 demethylase (DMIs) and squalene epoxidase. The minor gene mutation *U/ppl*-1B codes a low-level of resistance (approximately 5–12-fold) to the above morpholine-type fungicides, but in contrast with the major gene mutations it increases 2–10 times the sensitivity to triazoles: triadimefon, triadimenol, propiconazole and flusilazole. Crosses between mutants carrying the *U/ppl*-genes with compatible isolates carrying the *U/fpd*, *U/fpm* or *U/tdm* mutations, which have been identified in previous genetic works for resistance to morpholine-type fungicides, yielded, with the exception of *U/ppl*-2 × *U/fpm*-2 cross, a large number of recombinants with wild-type sensitivity, indicating that the mutant genes involved in these crosses, were not allelic. An additive gene effect was observed only between nonallelic minor genes *U/ppl*-1B and *U/fpm*-1B or *U/tdm*-1,2. Studies of the fitness of piperalin-resistant isolates showed that the reduced sensitivity of major gene mutants was not associated with changes on the phytopathogenic fitness determining characteristics, such as growth in liquid culture and pathogenicity on young corn plants. Conversely, the minor gene mutation *U/ppl*-1B appeared to be pleiotropic, having significantly adverse effects on the phytopathogenic fitness.

Introduction

Piperalin belongs to the important group of morpholine-type fungicides which includes the morpholines (fenpropimorph, tridemorph, dodemorph, aldimorph and trimorphamide), and the piperidines (piperalin and fenpropidin) (Pommer, 1995). Although this fungicide has been known since 1960 (Taylor, 1967) and has been used for over 25 years for the control of powdery mildew on ornamentals, its specific mechanism of action was identified only recently. Biochemical studies indicated that the toxicity of piperalin is dependent upon the inhibition of $\Delta^{8,7}$-sterol isomerase rather than Δ^{14}-sterol reductase of sterol biosynthetic pathway (Schneegurt and Henry, 1992). This was confirmed by Debieu et al. (2000) with cell-free enzyme systems of *Microdochium nivale*. Inhibition of the above enzyme system leads to accumulation of fecosterol or abnormal Δ^{8}-sterols such as $\Delta^{8,22,24(28)}$-ergostatrienol, $\Delta^{8,22}$-ergostadienol and Δ^{8}-ergostenol and decrease of ergosterol.

The development of resistance in fungi to fungicides with specific mechanism of action is an increasing problem in the control of plant diseases. Currently, such

problems have been encountered with C-14 demethylase inhibitors (DMIs), particularly with azole fungicides, which have been extensively used in the field (De Waard, 1994). After the decrease of effectiveness of DMIs against powdery mildews, protection from these plant pathogens has relied almost exclusively on morpholine-type fungicides. So the development of practical resistance to piperalin and other morpholine-type fungicides would be of a great concern in the control of powdery mildews.

In order to increase our knowledge regarding the possibility of development of practical resistance to piperalin, genetic and pathogenicity studies were undertaken with laboratory mutants of the phytopathogenic basidiomycete *Ustilago maydis*. Isolation of laboratory mutants using specific inhibitors as selecting agents is very useful in understanding various aspects of resistance, including the genetic control of resistance, biochemical mechanisms, cross-resistance to other compounds and the impact of specific resistance mutations on phytopathogenic fitness. The specific objectives of the present study were the following: (a) to elucidate the genetic control of resistance to piperidine fungicide piperalin; (b) to examine the impact of mutations for piperalin resistance on the phytopathogenic fitness; (c) to elucidate the genetical basis of cross-resistance relations between morpholine-type fungicides; and (d) to examine interallelic interaction between nonallelic genes for resistance to morpholine and piperidine fungicides.

Materials and methods

The compatible wild-type (wt) isolates of *U. maydis*, which were used to obtain mutants resistant to piperalin, together with the conditions and methods of culturing, mutation induction, genetic analysis and pathogenicity testing, have been reported (Markoglou and Ziogas, 1999; 2000; 2001). The same papers give details on the fungicides used and on the methods by which their fungitoxicity were measured.

Results

Selection and characterization of resistant mutant isolates

Mutants of *U. maydis* resistant to piperalin were isolated at a mutation frequency of 2.4×10^{-5} after UV-light irradiation and selection on UCM agar medium containing 50 $\mu g\,ml^{-1}$ piperalin (pure technical grade, Dow Elanco, USA). Depending on the parent sensitive isolate 201 or 501, they were designated as PL-20 . . or PL-50 . . , respectively. Preliminary tests for their sensitivity to piperalin on UCM agar medium using a 26-pin replicator, resulted in the identification of two piperalin-resistant phenotypes (R_1 and R_2) regarding the level of resistance to piperalin. The R_1 phenotypic class was observed at a lower frequency (14% of piperalin-resistant mutants) appeared at the first 6–8 days of incubation, and included mutants with a high resistance to piperalin. The mutants of R_2 phenotypic class were moderate resistant to piperalin and appeared after 10–14 days of incubation. A random sample of 15 such mutant isolates, 7 from R_1 and 8 from R_2 resistant phenotype, were chosen for further studies. Fungitoxicity tests of piperalin in liquid culture with representative resistant strains showed a dose-dependent decrease of growth of wild-type and both classes of mutant isolates (Figure 1). An Rf of 135 and 54 based on EC_{50} values was calculated for R_1 or R_2 resistant phenotype, respectively.

Growth measurements in liquid culture without fungicide showed no apparent effect of piperalin resistance in the growth rate of mutant isolates. A doubling time of 169 ± 12 was calculated after probit analysis for both wild-type and mutant isolates (results not shown).

Genetic analysis of piperalin resistance

Crosses between wild-type and piperalin-resistant isolates

Each of the 15 piperalin-resistant isolates was crossed first with the compatible wild-type strain and approximately 100 random progeny from each of these $R \times S$ crosses were tested for sensitivity to piperalin at the following: (a) the MIC for the sensitive parent (5 $\mu g\,ml^{-1}$); and (b) the maximal noninhibitory concentration for the resistant parent (100 or 150 $\mu g\,ml^{-1}$; depending on the resistant phenotype R_2 or R_1, respectively). As shown in Table 1, with exception of 201 × PL-5020 cross, the ratio of resistant : sensitive (R/S) progeny was exactly the same at both concentrations and this segregation was not significantly different from a Mendelian 1 : 1 ratio ($x^2 = 3.841$) at the $P = 0.05$ level. This indicates that each of 14 mutant isolates was the result of mutation of a single chromosomal gene for resistance to piperalin.

Analysis of 113 progeny from 201 × PL-5020 cross gave results different from the ones described above,

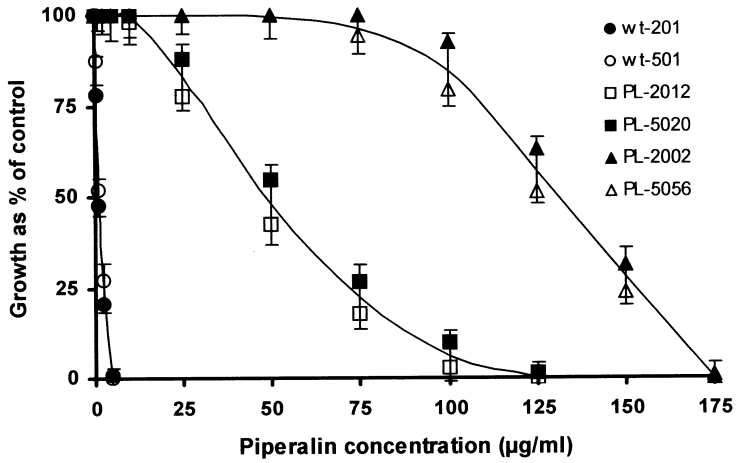

Figure 1. Sensitivity of a wild-type and four representative piperalin-resistant (PL) isolates of *U. maydis* to piperalin in liquid culture. Measurements were made after 24 h incubation.

Table 1. Results of random analysis of progeny from crosses between wild-type and piperalin-resistant mutant isolates of *U. maydis*

Cross	Number of progeny tested	Ratio of resistant/sensitive (R/S) progeny at the indicated piperalin concentrations (µg ml^{-1})					x^2 for fungicide sensitivity (1 : 1)[d]	Number of mutated loci
		5	25	50	100	150		
$S^a \times R_1{}^b$								
501 × PL-2033	72	40/32				40/32	0.889	1
501 × PL-2002	114	55/59				55/59	0.140	1
501 × PL-2058	84	39/45				39/45	0.429	1
201 × PL-5056	115	59/56				59/56	0.078	1
201 × PL-5001	110	62/48				62/48	1.782	1
201 × PL-5006	108	52/56				52/56	0.148	1
201 × PL-5072	106	50/56				50/56	0.339	1
$S \times R_2{}^c$								
501 × PL-2012	104	55/49			55/49		0.346	1
501 × PL-2036	78	38/40			38/40		0.051	1
501 × PL-2009	113	61/52			61/52		0.717	1
201 × PL-5017	63	35/28			35/28		0.778	1
201 × PL-5054	95	44/51			44/51		0.516	1
201 × PL-5041	74	38/36			38/36		0.054	1
201 × PL-5064	115	63/52			63/52		1.052	1
201 × PL-5020	113	86/27	86/27	59/54	59/54		30.805	2 (*U/ppl*-x,z)
S × PL-5020/progeny								
201 × PL-5020/6	103	49/54	49/54				0.243	1 (*U/ppl*-z)
201 × PL-5020/4	115	55/60	55/60	55/60	55/60		0.217	1 (*U/ppl*-x)
201 × PL-5020/7	112	82/30	82/30	54/58	54/58		24.143	2 (*U/ppl*-x,z)

[a]S: sensitive (wild-type) isolates. [b]R_1: highly piperalin-resistant mutant isolates. [c]R_2: moderately piperalin-resistant mutant isolates. [d]Expected value of x^2 for a 1 : 1 ratio at $P = 0.05$ is <3.841.

as the ratio of R/S progeny was 86 : 27 at the MIC for the wild-type parent (5 µg ml^{-1}) and 59 : 54 at 100 µg ml^{-1}, the maximal noninhibitory concentration for the resistant parent. By using intermediate piperalin concentrations (Table 1), it was found that of the 86 progeny isolates which were resistant at 5 µg ml^{-1}, approximately one-third grew on medium containing up to 25 µg ml^{-1} piperalin, and two-thirds up to 100 µg ml^{-1}, like the resistant parent PL-5020. The 3R : 1S ratio at the first concentration(s) of piperalin

shows that this mutant isolate, although obtained in a single-step selection, carries two genes (*U/ppl*-x and *U/ppl*-z) for resistance to piperalin, and these genes segregate independently of each other. Moreover, the 1R : 1S segregation ratio, which was observed at the piperalin concentrations of 50 or 100 μg ml^{-1}, indicates that a different level of resistance to piperalin is coded by the involved mutant genes.

To elucidate the above conclusion, appropriate progeny (PL-5020/6, PL-5020/4 and PL-5020/7) from this double mutant isolate were selected from media containing 25 and 100 μg ml^{-1} piperalin, and were crossed with the compatible wild-type strain. The progeny from each of these crosses were tested for sensitivity at various concentrations of piperalin. As shown by the results given in Table 1 the ratio of R/S progeny from wt × PL-5020/6 and wt × PL-5020/4 crosses was 1 : 1 at the MIC for the wild-type strain, indicating that each of these isolates carries a single chromosomal gene (*U/ppl*-z or *U/ppl*-x, respectively) for resistance to piperalin. On the contrary, the cross wt × PL-5020/7 gave a 3R : 1S progeny segregation indicating that the isolate PL-5020/7 carries both resistant genes, as the parent mutant PL-5020.

Crosses between piperalin-resistant isolates

To identify the mutated loci in R_1 and R_2 single-gene piperalin-resistant phenotypes, the allelism test was carried out in all possible (depending on mating type) $R \times R$ crosses, and the progeny from each cross were tested for resistance to 5, 100 and/or 150 μg ml^{-1} piperalin (Table 2). The two mutants mated were

Table 2. Results of random analysis of progeny from crosses involving piperalin-resistant mutant isolates of *U. maydis*

Cross	Number of progeny tested	Ratio of resistant/sensitive (R/S) progeny at the indicated piperalin concentrations (μg ml^{-1})					x^2 for fungicide sensitivity (3 : 1)[c]	Mutated loci
		5	50	100	125	150		
R_1[a] × R_1								
PL-2002 × PL-5056	115	115/0				115/0		*U/ppl*-1
PL-2002 × PL-5001	96	96/0				96/0		*U/ppl*-1
PL-2002 × PL-5006	87	87/0				87/0		*U/ppl*-1
PL-2002 × PL-5072	110	110/0				110/0		*U/ppl*-1
PL-2033 × PL-5056	103	103/0				103/0		*U/ppl*-1
PL-2058 × PL-5056	115	115/0				115/0		*U/ppl*-1
R_2[b] × R_2								
PL-2012 × PL-5017	98	98/0		98/0				*U/ppl*-2
PL-2012 × PL-5054	96	96/0		96/0				*U/ppl*-2
PL-2012 × PL-5041	114	114/0		114/0				*U/ppl*-2
PL-2012 × PL-5064	99	99/0		99/0				*U/ppl*-2
PL-2036 × PL-5017	115	115/0		115/0				*U/ppl*-2
PL-2009 × PL-5017	110	110/0		110/0				*U/ppl*-2
U/ppl-1 × *U/ppl*-2								
PL-2002 × PL-5017	115	89/26		89/26		60/55	0.351	*U/ppl*-1 × *U/ppl*-2
PL-2033 × PL-5054	100	72/28		72/28		48/52	0.480	*U/ppl*-1 × *U/ppl*-2
U/ppl-1 × *U/ppl*-x								
PL-2002 × PL-5020/4	110	82/28			59/51		0.012	*U/ppl*-1 × *U/ppl*-x
PL-2033 × PL-5020/4	114	83/31			55/59		0.292	*U/ppl*-1 × *U/ppl*-x
U/ppl-2 × *U/ppl*-x								
PL-2012 × PL-5020/4	108	108/0			0/108			*U/ppl*-2
PL-2036 × PL-5020/4	76	76/0			0/76			*U/ppl*-2
U/ppl-2 × *U/ppl*-z								
PL-2012 × PL-5020/6	64	46/18	29/35				0.333	*U/ppl*-2 × *U/ppl*-z
PL-2036 × PL-5020/6	110	76/34	57/53				2.048	*U/ppl*-2 × *U/ppl*-z
U/ppl-1 × *U/ppl*-z								
PL-2002 × PL-5020/6	88	88/0	47/41					*U/ppl*-1A × *U/ppl*-1B
PL-2033 × PL-5020/6	102	102/0	48/54					*U/ppl*-1A × *U/ppl*-1B

[a]R_1: highly piperalin-resistant mutant isolates. [b]R_2: moderately piperalin-resistant mutant isolates. [c]Expected value of x^2 for a 3 : 1 ratio at $P = 0.05$ is <3.841.

assumed to be allelic if no sensitive progeny were produced. Otherwise, they should carry resistance genes at different loci. In this way, two chromosomal loci (*U/ppl*-1 and *U/ppl*-2) for resistance to piperaline were identified. The large number of recombinants with wild-type sensitivity at the concentration of piperalin to which both mutant isolates are resistant, shows that the *U/ppl*-1 and *U/ppl*-2 are unlinked, segregating independently of each other. Among the 14 single-gene mutants studied, 7 carried the *U/ppl*-1 and 7 the *U/ppl*-2 mutation.

Crosses between mutants carrying the *U/ppl*-x or *U/ppl*-z mutations with appropriate compatible progeny carrying the *U/ppl*-1 or *U/ppl*-2 genes were analyzed to determine whether the *U/ppl*-x or *U/ppl*-z genes are allelic with one of the *U/ppl*-1 or *U/ppl*-2 genes (Table 2). Analysis of crosses between *U/ppl*-1 × *U/ppl*-x and *U/ppl*-2 × *U/ppl*-z yielded, in all cases, a large number of recombinants with wild-type sensitivity, indicating that the mutant genes involved were not allelic. The absence of sensitive recombinants between 184 progeny from *U/ppl*-2 and *U/ppl*-x crosses indicate that only one chromosomal gene was involved. Analysis of 190 progeny from crosses between *U/ppl*-1 and *U/ppl*-z mutants also yielded no recombinants with wild-type sensitivity at the concentration of piperalin to which both mutant isolates were resistant. However, the 1R : 1S progeny segregation which was observed at the concentration of piperalin at which only the *U/ppl*-1 mutant isolates are resistant, shows that the *U/ppl*-z is an allele mutation of the *U/ppl*-1 locus. The allele determining high resistance is designated as *U/ppl*-1A in the rest of the paper, and that determining low resistance as *U/ppl*-1B.

In an attempt to recognize interallelic interactions when the two nonallelic genes *U/ppl*-1A and *U/ppl*-2 or *U/ppl*-1B and *U/ppl*-2 were present in the same haploid nucleus, progeny from crosses between nonallelic mutants were tested for sensitivity at three concentrations of piperalin: (a) the MIC for the wild-type (5 μg ml^{-1}); (b) the MIC for the less resistant parent (50 or 125 μg ml^{-1}, depending on the resistant phenotype); and (c) the MIC for the more resistant parent (125 or 175 μg ml^{-1}). As shown by the examples given in Table 3, the segregation ratio was approximately 3R : 1S at the first, 1R : 1S at the second, and all progeny were sensitive at the third of piperalin concentrations. The absence of resistant progeny at the highest concentration (125 or 175 μg ml^{-1}) indicates that there is no additivity of gene effect between these nonallelic genes.

Crosses between isolates carried the U/ppl *and the* U/fpd, U/fpm *or* U/tdm *mutations*

Crosses between mutants carrying the *U/ppl*-genes with compatible isolates carrying the *U/fpd*, *U/fpm* or *U/tdm* mutations, which have been recognized in our previous genetic studies on resistance to fenpropidin, fenpropimorph and tridemorph, respectively, were analyzed to determine whether the *U/ppl*-genes are allelic with some of them (Table 3). Generally, a random sample of approximately 100 progeny from each of these crosses was tested for sensitivity to piperalin at the following: (a) the MIC for the wild-type strain (5 μg ml^{-1}), in order to examine if the mutant genes are mutations of the same locus; (b) the MIC for the less resistant parent (50 or 125 μg ml^{-1}), in order to elucidate if the involved mutant genes are alleles of the locus which codes a different level of resistance to piperalin; and (c) the MIC for the more resistant parent (50, 125 or 175 μg ml^{-1}). The last concentration was used in order to examine interallelic interaction when two nonallelic genes are present in the same haploid nucleus. As shown by the examples given in Table 3, with exception of *U/ppl*-2 × *U/fpm*-2 cross, the segregation ratio was approximately 3R : 1S at the first, 1R : 1S at the second, and 1R : 3S or all progeny were sensitive at the third of these concentrations. The large number of recombinants with wild-type sensitivity, in the crosses of mutant isolates carrying the *U/ppl*-1A or *U/ppl*-1B mutations, indicates that the *U/ppl*-1 gene is not allelic with one of the *U/fpd*-1, *U/fpm*-1, *U/fpm*-2, *U/tdm*-1 and *U/tdm*-2 genes. The 1 : 1 segregation ratio at the second concentration indicates that a different level of resistance is coded by the involved mutant genes. The absence of resistant progeny at the highest concentration indicates that there is no additivity of gene effect between nonallelic genes. An additivity of gene action was observed only between *U/ppl*-1B and *U/fpm*-1B, *U/tdm*-1 and *U/tdm*-2. The absence of recombinants with wild-type sensitivity in the *U/ppl*-2 × *U/fpm*-2 cross, and the 1 : 1 segregation ratio at the second concentration of piperalin, where only the *U/fpm*-2 parent strain is resistant, indicates that the *U/ppl*-2 and *U/fpm*-2 genes are allelic mutations of the same locus.

Cross-resistance

As shown in Table 4, the major gene mutations *U/ppl*-1A and *U/ppl*-2 are responsible for a moderate level of resistance to fenpropidin, a high resistance level to fenpropimorph and a low level of resistance to

Table 3. Results of random analysis of progeny from crosses involving resistant isolates of *U. maydis* which carried the *U/ppl*, *U/fpd*, *U/fpm* and *U/tdm* genes

Cross	Mutations	Number of progeny tested	Ratio of resistant/sensitive (R/S) progeny at the indicated piperalin concentrations (μg ml^{-1})				Linkage between mutated genes	
			5	50	125	175	% recombination[a]	x^2 values (3 : 1)[b]
U/ppl × *U/ppl*								
PL-2002 × PL-5017	*U/ppl*-1A × *U/ppl*-2	110	82/28		57/53	0/110	25.45	0.012
PL-2033 × PL-5054	*U/ppl*-1A × *U/ppl*-2	114	82/32		54/60	0/114	28.07	0.573
PL-5020/6 × PL-2012	*U/ppl*-1B × *U/ppl*-2	115	83/32	61/54	0/115		27.83	0.490
PL-5020/6 × PL-2036	*U/ppl*-1B × *U/ppl*-2	87	65/22	43/44	0/87		25.29	0.004
U/ppl × *U/fpd*								
PL-5056 × FD-2013	*U/ppl*-1A × *U/fpd*-1	115	84/31		55/60	0/115	29.96	0.235
PL-5020/6 × FD-2013	*U/ppl*-1B × *U/fpd*-1	84	62/22	45/39	0/84		26.19	0.063
PL-5054 × FD-2013	*U/ppl*-2 × *U/fpd*-1	109	81/28		0/109		25.69	0.028
U/ppl × *U/fpm*								
PL-5056 × FP-2013	*U/ppl*-1A × *U/fpm*-1A	112	81/31		52/60	0/112	27.68	0.429
PL-5056 × FP-2020	*U/ppl*-1A × *U/fpm*-1B	94	69/25	48/46		0/94	26.59	0.128
PL-5056 × FP-2024	*U/ppl*-1A × *U/fpm*-2	113	84/29			0/113	25.66	0.027
PL-5020/6 × FP-2013	*U/ppl*-1B × *U/fpm*-1A	96	72/24	45/51	0/96		25.00	0.000
PL-5020/6 × FP-2020	*U/ppl*-1B × *U/fpm*-1B	115	86/29	26/89			25.22	0.003
PL-5020/6 × FP-2024	*U/ppl*-1B × *U/fpm*-2	103	75/28	48/55		0/103	27.18	0.262
PL-5054 × FP-2013	*U/ppl*-2 × *U/fpm*-1A	115	84/31		0/115		26.96	0.235
PL-5054 × FP-2020	*U/ppl*-2 × *U/fpm*-1B	114	81/33	53/61	0/114		28.95	0.947
PL-5054 × FP-2024	*U/ppl*-2 × *U/fpm*-2	106	106/0		50/56	0/106	0.000	35.33
U/ppl × *U/tdm*								
PL-5056 × TD-2002	*U/ppl*-1A × *U/tdm*-1	110	79/31	51/59		0/110	28.18	0.594
PL-5056 × TD-2013	*U/ppl*-1A × *U/tdm*-2	98	71/27	52/46		0/98	27.55	0.341
PL-5020/6 × TD-2002	*U/ppl*-1B × *U/tdm*-1	111	79/32	26/85			28.83	0.868
PL-5020/6 × TD-2013	*U/ppl*-1B × *U/tdm*-2	100	74/26	21/79			26.00	0.053
PL-5054 × TD-2002	*U/ppl*-2 × *U/tdm*-1	114	82/32	65/49	0/114		28.07	0.573
PL-5054 × TD-2013	*U/ppl*-2 × *U/tdm*-2	67	49/18	31/36	0/67		26.87	0.124

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

Table 4. Expression of mutations for resistance to piperidine and relative fungicides in *U. maydis*

Fungicide	Resistance factor[a]					
	EC_{50}[b] (mean ± SE[d])			MIC[c]		
	U/ppl-1A	*U/ppl*-1B	*U/ppl*-2	*U/ppl*-1A	*U/ppl*-1B	*U/ppl*-2
Piperalin	135 ± 12.24	8.3 ± 1.28	54 ± 5.32	35	10	25
Fenpropidin	68 ± 7.24	10.8 ± 1.92	43 ± 5.18	20	5	15
Fenpropimorph	283 ± 14.32	12.2 ± 3.14	261 ± 9.13	100	10	75
Tridemorph	10.1 ± 1.14	4.6 ± 1.28	9.3 ± 1.67	5	5	5

[a]Ratio of EC_{50} for mutant : EC_{50} for wild-type or MIC for mutant : MIC for wild-type. [b]Effective concentration causing 50% reduction in growth rate. [c]Minimal inhibitory concentration. [d]Pooled standard error; six replications.

tridemorph, while the minor gene mutation *U/ppl*-1B reduced the sensitivity to all three fungicides by a similar low factor.

Fungitoxicity tests with ten C-14 DMIs and terbinafine, an inhibitor of squalene epoxidase, showed that the major gene mutations *U/ppl*-1A and *U/ppl*-2 do not affect the sensitivity to inhibitors of steps of ergosterol biosynthesis preceding the Δ^{14}-reductase. By contrast, mutants carrying the minor gene mutation *U/ppl*-1B present 2–10 times increased sensitivity to

triazoles: triadimefon, triadimenol, propiconazole and flusilazole (results not shown).

Pathogenicity tests

Table 5 shows the results of the study of possible pleiotropic effects of mutations for resistance to piperalin on phytopathogenic fitness of *U. maydis*. All seedlings were infected by the crosses wt × wt, major gene × wt, major gene × minor gene or major gene × major gene. The incubation periods for appearance and maturity of the galls were 7–9 days and 13–16 days, respectively, in all of these crosses. In all crosses involving major gene mutations in homozygous or heterozygous condition there was no significant

Table 5. Effect of *U/ppl* mutations for resistance to piperalin on pathogenicity of *U. maydis* to corn seedlings

Cross	Mutations	Number of infected seedlings[a]	Days after inoculation for formation of galls		Teliospore production ($\times 10^6$)[b] (mean ± SE[c])
			Young (mean ± SE[c])	Mature (mean ± SE[c])	
201 × 501	wt × wt	12	7 ± 0.48a[d]	15 ± 0.72a[d]	12.6 ± 0.54a[d]
U/ppl × wt					
PL-5056 × 201	*U/ppl*-1A[f] × wt	12	8 ± 0.54a	15 ± 1.12a	15.6 ± 1.65a
PL-5020/6 × 201	*U/ppl*-1B[g] × wt	6	12 ± 1.24b	24 ± 3.11b	4.6 ± 0.64b
PL-5054 × 201	*U/ppl*-2[f] × wt	12	8 ± 1.71a	15 ± 2.26a	12.9 ± 0.69a
U/ppl × U/ppl					
PL-2002 × PL-5056	*U/ppl*-1A × *U/ppl*-1A	11	7 ± 1.09a	14 ± 1.34a	13.2 ± 2.03a
PL-2002 × PL-5020/6	*U/ppl*-1A × *U/ppl*-1B	12	9 ± 1.13a	13 ± 2.83a	14.6 ± 2.62a
PL-5056 × PL-2036	*U/ppl*-1A × *U/ppl*-2	12	8 ± 1.03a	15 ± 1.91a	13.8 ± 1.19a
PL-5020/6 × PL-2036	*U/ppl*-1B × *U/ppl*-2	11	8 ± 1.27a	14 ± 1.68a	16.7 ± 3.39a
PL-5054 × PL-2036	*U/ppl*-2 × *U/ppl*-2	12	7 ± 0.23a	15 ± 1.22a	14.9 ± 1.74
U/ppl × U/fpm					
PL-5056 × FP-2013	*U/ppl*-1A × *U/fpm*-1A[f]	12	7 ± 0.82a	14 ± 2.04a	14.8 ± 1.48a
PL-5056 × FP-2024	*U/ppl*-1A × *U/fpm*-2[f]	11	8 ± 1.16a	15 ± 0.93a	15.3 ± 2.14a
PL-5056 × FP-2020	*U/ppl*-1A × *U/fpm*-1B[g]	10	9 ± 0.18a	16 ± 1.22a	13.8 ± 1.26a
PL-5020/6 × FP-2013	*U/ppl*-1B × *U/fpm*-1A	12	7 ± 0.54a	15 ± 1.23a	14.5 ± 2.11a
PL-5020/6 × FP-2024	*U/ppl*-1B × *U/fpm*-2	12	7 ± 1.12a	15 ± 0.68a	13.4 ± 1.07a
PL-5020/6 × FP-2020	*U/ppl*-1B × *U/fpm*-1B	5	14 ± 1.64b	26 ± 3.16b	3.8 ± 0.92b
PL-5054 × FP-2013	*U/ppl*-2 × *U/fpm*-1A	12	7 ± 0.32a	16 ± 2.41a	13.4 ± 3.02a
PL-5054 × FP-2024	*U/ppl*-2 × *U/fpm*-2	12	8 ± 1.26a	14 ± 1.65a	11.9 ± 1.24a
PL-5054 × FP-2020	*U/ppl*-2 × *U/fpm*-1B	11	7 ± 0.82a	13 ± 2.38 a	12.7 ± 2.13a
U/ppl × U/fpd					
PL-5056 × FD-2013	*U/ppl*-1A × *U/fpd*-1[f]	12	7 ± 0.66a	14 ± 2.13a	15.8 ± 2.36a
PL-5020/6 × FD-2013	*U/ppl*-1B × *U/fpd*-1	12	8 ± 0.71a	14 ± 1.13a	15.8 ± 2.03a
PL-5054 × FD-2013	*U/ppl*-2 × *U/fpd*-1	12	9 ± 1.69a	16 ± 2.19a	16.6 ± 3.23a
U/ppl × U/tdm					
PL-5056 × TD-2002	*U/ppl*-1A × *U/tdm*-1[g]	11	8 ± 1.19a	14 ± 0.78a	12.4 ± 1.39a
PL-5056 × TD-2013	*U/ppl*-1A × *U/tdm*-2[g]	10	9 ± 2.36a	15 ± 2.18a	14.2 ± 1.28a
PL-5056 × TD-1,2[e]	*U/ppl*-1A × *U/tdm*-1,2[g]	11	9 ± 1.45a	14 ± 1.33a	13.8 ± 0.67a
PL-5020/6 × TD-2002	*U/ppl*-1B × *U/tdm*-1	6	15 ± 2.21b	28 ± 2.53b	3.2 ± 0.26b
PL-5020/6 × TD-2013	*U/ppl*-1B × *U/tdm*-2	7	13 ± 1.59b	24 ± 3.17b	5.2 ± 1.07b
PL-5020/6 × TD-1,2[e]	*U/ppl*-1B × *U/tdm*-1,2	4	16 ± 2.27b	29 ± 2.79b	2.6 ± 0.86b
PL-5054 × TD-2002	*U/ppl*-2 × *U/tdm*-1	12	9 ± 2.13a	15 ± 2.21a	10.8 ± 1.63a
PL-5054 × TD-2013	*U/ppl*-2 × *U/tdm*-2	11	8 ± 0.12a	15 ± 0.26a	15.7 ± 2.76a
PL-5054 × TD-1,2[e]	*U/ppl*-2 × *U/tdm*-1,2	10	7 ± 1.38a	14 ± 1.92a	14.3 ± 1.13a

[a]12 seedlings per cross were inoculated and the infected (or dead) seedlings were evaluated. [b]Number of teliospores per g dry weight of gall. [c]Pooled standard error; 12 replications. [d]Within columns, values followed by the same letter do not differ significantly different according to Dunnett's multiple range test ($P = 0.05$). [e]The TD-1,2 isolate carries two unlinked chromosomal genes *U/tdm*-1 and *U/tdm*-2. [f]Major gene mutations. [g]Minor gene mutations.

increase in the incubation periods, and the production of teliospores was equal or even higher compared with the wt × wt cross. Conversely, the minor gene mutation *U/ppl*-1B, in heterozygous condition in dikaryotic mycelium with its wild-type allele or other minor gene mutations *U/fpm*-1B, *U/tdm*-1 or *U/tdm*-2, resulted a 50% reduction in the number of infected seedlings, an increase for several days in the time taken for galls to form, and a reduction, more than 50%, in teliospore production. These results indicate an epistatic effect of major genes *U/ppl*-1A and *U/ppl*-2 on the minor gene *U/ppl*-1B and also on previously identified minor genes *U/fpm*-1B, *U/tdm*-1 and *U/tdm*-2.

Discussion

Genetic analysis of crosses with 15 piperalin-resistant mutants of *U. maydis* resulted in the identification of two unlinked chromosomal resistance loci (Tables 1 and 2). The *U/ppl*-1 locus with two allelic mutations, *U/ppl*-1A and *U/ppl*-1B, which are responsible for high and low resistance to piperalin, respectively, and the *U/ppl*-2 locus which encodes a moderate resistance level to piperalin.

Genetic analysis of resistance to morpholine-type fungicides, from different laboratories, has also shown involvement of two or more nonallelic genes in a number of fungal species: three genes in *Nectria haematococca* var. *cucurbitae* (Demakopoulou et al., 1989), three genes in *N. haematococca* var. *pisi* (De Falandre et al., 1991), two genes located on linkage group II in *Aspergillus niger* (Engels et al., 1998) and five genes in *U. maydis* (Markoglou and Ziogas, 1999; 2000) were determined. Study of genetic control of resistance to morpholine-type fungicides in the barley powdery mildew pathogen, *Erysiphe graminis* f.sp. *hordei*, revealed that in some cases resistance to fenpropidin and fenpropimorph may be controlled by a single gene mutation or by two unlinked complementary resistance genes (Brown et al., 1996). However, except the major gene mutations in *U. maydis*, which were identified in our laboratory, in all other cases described above, the resistance level was low to moderate and there was an additive gene effect, indicating a quantitative trait for resistance to morpholine-type fungicides in these fungal species.

Cross-resistance studies of piperalin with other sterol biosynthesis inhibitors showed that the major gene mutations *U/ppl*-1A and *U/ppl*-2 are responsible for a moderate level of resistance to fenpropidin, high resistance level to fenpropimorph and had little effect on the response to tridemorph (Table 4). The minor gene mutation *U/ppl*-1B codes a low level of resistance to the above morpholine-type fungicides, but in contrast with the major gene mutations it increases 2–10 times the sensitivity to the triazoles: triadimefon, triadimenol, propiconazole and flusilazole. A positive cross-resistance, with various resistance factors, between morpholine-type fungicides was also observed in *U. maydis* (Markoglou and Ziogas, 1999; 2000; 2001), *E. graminis* f.sp. *hordei* (Brown and Evans, 1992; Brown et al., 1996), *E. graminis* f.sp. *tritici* (Burnett and Zziwa, 1997; De Waard et al., 1992), *N. haematococca* var. *cucurbitae* (Demakopoulou et al., 1989), *N. haematococca* var. *pisi* (De Falandre et al., 1991) and *A. niger* (Engels et al., 1998). Tridemorph-resistant mutants of *Penicillium caseicolum* were cross-resistant to piperalin and to fenpropimorph, but not to fenpropidin (De Falandre et al., 1987). Studies with a limited number of isolates of *E. graminis* f.sp. *tritici* have indicated that fenpropimorph was cross-resistant to fenpropidin with a low level or no cross-resistance to tridemorph (De Waard et al., 1992; Readshaw and Heaney, 1994). Cross-resistance relationships between morpholine fungicides in *E. graminis* f.sp. *hordei* revealed that genes for resistance to fenpropidin and fenpropimorph had comparatively little effect on the response to tridemorph (Brown et al., 1996).

The high level of resistance and the cross-resistance relations between piperalin, fenpropidin and fenpropimorph which are encoded by *U/ppl*-1A and *U/ppl*-2 genes, indicate that some way other than target-site modification is the underlying biochemical mechanism of resistance. All these fungicides inhibit the Δ^{14}-reduction and/or $\Delta^{8,7}$-isomerization in the ergosterol biosynthetic pathway. Fenpropidin and fenpropimorph inhibit both Δ^{14}-reductase and $\Delta^{8,7}$-isomerase, while piperalin and tridemorph mainly inhibit the $\Delta^{8,7}$-isomerase (Baloch and Mercer, 1987; Debieu et al., 2000; Schneegurt and Henry, 1992; Ziogas et al., 1991). Furthermore, an interference of piperalin, fenpropidin and fenpropimorph at the first (Schneegurt and Henry, 1992; Ziogas et al., 1991) or at the final part (Engels and De Waard, 1998) of sterol biosynthesis were also observed. A target site change at Δ^{8}–Δ^{7}-isomerase does not provide a reasonable explanation for the resistance to fenpropidin and fenpropimorph, and obviously another mechanism of resistance is coded by the identified genes for resistance to the above fungicides. A study of

the underlying biochemical mechanism of resistance is now in progress with the genetically characterized mutant isolates.

Reports of a decrease in sensitivity of cereal powdery mildews (*E. graminis*. f.sp. *tritici* and *E. graminis*. f.sp. *hordei*) to tridemorph, fenpropimorph and fenpropidin in Europe have appeared during the last two decades (Brown et al., 1991; Burnett and Zziwa, 1997; Hollomon, 1982; Robertson et al., 1990; Wolfe et al., 1987). Fortunately, and despite the disease associated risk, which is high because of the sort generation time and the abundance of sporulation in powdery mildews, there has been only a slight development of field resistance to morpholine-type fungicides (Brent and Hollomon, 1998; Hollomon, 1994). However, according to our knowledge, there are no reports of reduction in piperalin effectiveness despite many years of use against powdery mildew on ornamentals, particularly on rose. A possible explanation for the absence of major gene mutations such as *U/ppl*-1A and *U/ppl*-2 in powdery mildew population, is that the biochemical mechanism coded by this type of mutation may be not effective on filamentous fungi such as powdery mildews. Apparently, the findings of the present work as the high mutation frequency, the high level of resistance and the absence of pleiotropic effects on phytopathogenic fitness of mutant isolates indicate a considerable resistance risk for piperalin and other morpholine-type fungicides, at least in pathogens with similar to *U. maydis* genetic variability.

Acknowledgements

Financial support from the State Scholarship Foundation and the Greek Ministry of Development, General Secretariat for Research and Technology is gratefully acknowledged.

References

Baloch RI and Mercer EI (1987) Inhibition of sterol Δ^8–Δ^7-isomerase and Δ^{14}-reductase by fenpropimorph, tridemorph and fenpropidin in cell-free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry 26: 663–668

Brent KJ and Hollomon DW (1998) Fungicide resistance: The assesment of risk. In: Global Crop Protection Federation (Brussels). FRAC Monograph No 2

Brown JKM and Evans N (1992) Selection on responses of barley powdery mildew to morpholine and piperidine fungicides. Crop Protection 2: 449–457

Brown JKM, Slater SE and See KA (1991) Sensitivity of *Erysiphe graminis* f.sp. *hordei* to morpholine and piperidine fungicides. Crop Protection 10: 445–454

Brown JKM, Le Boulaire S and Evans N (1996) Genetics of responses to morpholine-type fungicides and of avirulences in *Erysiphe graminis* f.sp. *hordei*. European Journal of Plant Pathology 102: 479–490

Burnett FJ and Zziwa MCN (1997) Effect of application rate on the sensitivity of *Erysiphe graminis* f.sp. *tritici* to fenpropimorph. Pesticide Science 51: 335–340

Debieu D, Bach J, Arnold A, Brousset S, Gredt M, Taton M, Rahier A, Malosse C and Leroux P (2000) Inhibition of ergosterol biosynthesis by morpholine, piperidine, and spiroketalamine fungicides in *Microdochium nivale*: effect on sterol composition and sterol $\Delta^{8,7}$-isomerase activity. Pesticide Biochemistry and Physiology 67: 85–94

De Falandre A, Bouvier-Fourcade I, Seng JM and Leroux P (1987) Induction and characterization of *Penicillium caseicolum* mutants resistant to ergosterol biosynthesis inhibitors. Applied and Environmental Microbiology 53: 1500–1503

De Falandre A, Daboussi MJ and Leroux P (1991) Inheritance of resistance to fenpropimorph and terbinafine, two sterol biosynthesis inhibitors, in *Nectria haematococca*. Phytopathology 81: 1432–1438

Demakopoulou MG, Ziogas BN and Georgopoulos SG (1989) Evidence for polygenic control of fenpropimorph resistance in laboratory mutants of *Nectria haematococca* var. *cucurbitae*. ISPP Chemical Control Newsletter 12: 34–35

De Waard MA (1994) Resistance to fungicides which inhibit sterol 14α-demethylation, an historical perspective. In: Heaney S, Slawson D, Hollomon DW, Smith M, Russell PE and Parry DW (eds) Fungicide Resistance. BCPC Monograph No 60 (pp 3–10) British Crop Protection Council Farnham, Surrey, UK

De Waard MA, Banga M and Ellis SW (1992) Characterization of the sensitivity of *Erysiphe graminis* f.sp. *tritici* to morpholines. Pesticide Science 34: 374–376

Engels AJG and De Waard MA (1998) Sterol composition of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph. Pesticide Science 52: 258

Engels AJG, Holub EF, Swart K and De Waard MA (1998) Genetic analysis of resistance to fenpropimorph in *Aspergillus niger*. Current Genetics 33: 145–150

Hollomon DW (1982) The effects of tridemorph on barley powdery mildew: its mode of action and cross-sensitivity relationships. Phytopathologische Zeitschrift 105: 279–287

Hollomon DW (1994) Field performance of morpholines. In: Heaney S, Slawson D, Hollomon DW, Smith M, Russell PE and Parry DW (eds) Fungicide Resistance. BCPC Monograph No 60 (pp 323–324) British Crop Protection Council Farnham, Surrey, UK

Markoglou AN and Ziogas BN (1999) Genetic control of resistance to fenpropimorph in *Ustilago maydis*. Plant Pathology 48: 521–530

Markoglou AN and Ziogas BN (2000) Genetic control of resistance to tridemorph in *Ustilago maydis*. Phytoparasitica 28: 349–360

Markoglou AN and Ziogas BN (2001) Genetic control of resistance to the piperidine fungicide fenpropidin in *Ustilago maydis*. Journal of Phytopathology 149: 551–559

Pommer EH (1995) Morpholine fungicides and related compounds. In: Lyr H (ed) Modern Selective Fungicides, 2nd edn (pp 163–183) Gustav Fischer Verlag, New York

Readshaw AE and Heaney SP (1994) Fenpropimorph sensitivity in *Erysiphe graminis* f.sp. *tritici*; survey of Northern France 1991–1993. In: Heaney S, Slawson D, Hollomon DW, Smith M, Russell PE and Parry DW (eds) Fungicide Resistance. BCPC Monograph No 60 (pp 297–302) British Crop Protection Council Farnham, Surrey, UK

Robertson S, Gilmour J, Newman D and Lennard JH (1990) Sensitivity of barley powdery mildew isolates to morpholine fungicides. Proceedings of 1988 Brighton Crop Protection Conference, Pest and Diseases 3: 1159–1162

Schneegurt MA and Henry M (1992) Effects of piperalin and fenpropimorph on sterol biosynthesis in *Ustilago maydis*. Pesticide Biochemistry and Physiology 43: 45–52

Taylor HM (1967) U.S. Patent 3322618 to Eli Lilly & Co

Wolfe MS, Slater SE and Minchin PN (1987) Mildew of barley. UK Cereal Pathogen Virulence Survey: 1986 Annual Report, 26–33

Ziogas BN, Oesterhelt G, Masner P, Steel CC and Furter R (1991) Fenpropimorph: a three site inhibitor of ergosterol biosynthesis in *Nectria haematococca* var. *cucurbitae*. Pesticide Biochemistry and Physiology 39: 74–83