Inheritance of Tolerance to Carboxin and Benomyl in Ustilago hordei

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ABSTRACT

In *Ustilago hordei,* carboxin tolerance is controlled by polygenes, resulting in transgressive segregation for level of tolerance. Tolerance appeared to be monogenically or polygenically controlled, depending on the evaluation method: a single fungicide concentration revealed a 1:1 ratio, while a series of concentrations exposed transgressive segregation. Analysis of tolerance to benomyl was complicated by gamete inviability in crosses involving the benomyl-tolerant strain. On fungicide-free medium teliospores produced promycelia, but only 14% formed sporidia. The benomyl-tolerant strain had disturbances in meiosis with no effect on mitosis. Variation among offspring in their response to benomyl indicated polygenic control of benomyl tolerance. Teliospore germination in crosses involving carboxin-tolerant strains was normal, but on carboxin medium was lower than on complete media. Sporidia grew equally well on both media.

In a mixed inoculation experiment, the benomyl-tolerant strain had a stronger sexual affinity for a compatible wild type than the carboxin-tolerant one. The benomyl-tolerant strain, competed successfully and eliminated the carboxin-tolerant strain, but was self-eliminating at teliospore germination. Biotype survival clearly depends on successful transmission through the sexual stage.

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Additional key words: defects in meiosis, procedure-dependent genetic ratios.

The increasing use of systemic fungicides in the control of plant diseases had created the hazard that tolerant strains might develop (5). Genetic control of tolerance to inhibitory and toxic compounds was reviewed by Georgopoulos and Zaracovitis (8). However, from the voluminous literature, relatively few papers deal with fungicides. Most investigators reported that tolerance to inhibitory compounds is monogenically controlled (i.e., 10, 11), whereas others concluded that tolerance is polygenically controlled and/or cytoplasmatically inherited (3, 12, 13, 15). Tolerance of a fungus to two fungicides could be monogenic for one fungicide and polygenic or cytoplasmic for the other (12, 13). The genetic control of tolerance in *Ustilago maydis* to 25 μM carboxin was claimed to be monogenic (7). Whether tolerance is monogenically, polygenically, or cytoplasmically controlled has an important implication in the endurance of such types in the population and might depend on the evaluation method as will be discussed further on.

In our studies, *U. hordei* was used to study the genetic control of tolerance.

MATERIALS AND METHODS.—*Media.*—Complete medium (CM) was prepared according to Vogel (14).

Fungicides.—The following compounds were used:

(i) Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) formulated as dust, 75% active ingredient (a.i.), produced by Uniroyal Chemical, Division of Uniroyal, Inc.

(ii) Benomyl [Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate], formulated as dust 50% a.i., produced by E.I. du Pont de Nemours & Co. (Inc.).

Strains.—Fungicide-sensitive strains were obtained from the collection of C.O. Person, University of British Columbia, Vancouver, B.C., Canada. The strains used are listed in Table 1.

Teliospores.—Teliospores were produced by inoculating barley seeds with appropriate mixtures of strains of *U. hordei* (1). Teliospore germination was tested by plating on CM with and without 1 μg/ml carboxin or 10 μg/ml benomyl, or both, and incubating at 20 C up to 6 days.

Sporidia.—These were obtained in two ways: (i) Erlenmeyer flasks (250-ml), containing 20 ml CM were inoculated with teliospores at a concentration of 10^5/ml and incubated for 18-20 hours at 20 C in a shaker bath. The stage of development of germinating teliospores was determined by calculating the average number of sporidia (basidiospores) produced per teliospore. Sporidia were harvested when this amounted to four to six sporidia per teliospore. Sporidia were plated on CM, and the colonies developed were transferred to master plates for replication on the differential media. (ii) Teliospores were plated on CM, and allowed to germinate until the appearance of the first generation of sporidia. The sporidia were separated from the promycelium by means of a micromanipulator (Leitz, Wetzlar). The strains grown from the isolated sporidia were examined for tolerance to fungicides by the replica plating technique.

RESULTS.—*Effect of fungicides on teliospore germination.*—Teliospores obtained from four different crosses were germinated on CM, CM + carboxin (1 μg/ml), CM + benomyl (10 μg/ml) and CM + carboxin (1 μg/ml) + benomyl (10 μg/ml). In the results (Table 2), a distinction is made between a germination of a promycelium and further production of basidiospores. All figures, for each cross, are relative to promycelium germination in this cross on CM. Germination was maximal on CM 32 hours after plating; it was 65%, 90%, 70%, and 50% for the four crosses in the order given in Table 2. Since the data is expressed relative to the control and germination on the poisoned media was maximal 18 hours after plating, it seems from the table as if percent germination decreased with time increase. The viability of teliospores in each cross might be different due to differences in the conditions under which they have been produced. Despite this difference, the marked decrease in the production of basidiospores by promycelia is evident in the crosses involving the benomyl-tolerant strain.

Promycelia of teliospores from these crosses are longer than normal on any medium tested. The greatly reduced sporidial formation in crosses involving the benomyl-tolerant strain was evident on both CM and CM + benomyl media. The infertile promycelia started to degenerate 18 hours after plating. On CM + carboxin, germination was delayed compared to CM, and three crosses produced only short promycelia without basidiospores. Only the cross *car*-1 × 3 produced basidiospores on CM + carboxin. Teliospore germination on CM + carboxin was lower than on CM. This may be explained by incomplete dominance of

TABLE 1. The *Ustilago hordei* strains used in our study of the inheritance of tolerance to carboxin and benomyl

Strain	Symbol	Mutant[a]	Mating type	Traits
1[b]	I_4^+	I_4^+	A	wild type
2[b]	*arg*$^-$	x-52	a	arginine-less
3[b]	*ad arg leuc nic*$^-$	u-40		adenine-less
		x-52		arginine-less
		u-38	a	leucine-less
		x-95		nicotinic acid-less
car-1	*car* I_4^+		A	wild type tolerant to 25 μg/ml carboxin, derived from No. 1
car-2	*car arg*$^-$		a	arginine-less tolerant to 50 μg/ml carboxin, derived from No. 2
ben-1	*ben* I_4^+		A	wild type tolerant to 2,000 μg/ml benomyl, derived from No. 1

[a]As listed in the original collection of C.O. Person, Univ. of B.C., Vancouver, B.C., Canada.
[b]These sensitive strains were tolerant to .2 μg/ml carboxin and .5 μg/ml benomyl.

TABLE 2. Effect of carboxin (1 μg/ml) and benomyl (10 μg/ml) on the percentage of teliospores of crosses of *Ustilago hordei* producing promycelium and sporidia on complete media after differing times of incubation. In each test 100 teliospores were counted

	Incubation time (hours)															
	18								32							
Teliospores resulting from the following cross[a]	CM[b]		C[c]		B[d]		CB[e]		CM		C		B		CB	
	P[f]	PS[g]	P	PS	P	PS	P	PS	P	PS	P	PS	P	PS	P	PS
1 × 3	100	100	0		120	0	0			100	12	0	93	0	0	
car-1 × 3	100	100	63	0	88	0	0			100	44	0	78	0	0	
ben-1 × 3	100	0	3	0	88	0	0		100	14	18	0	88	1	0	
car-1 × *ben*-1 × 3	100	0	0		160	0	0		100	32	22	0	160	2	0	

	Incubation time (hours)															
	72								144							
Teliospores resulting from the following cross	CM		C		B		CB		CM		C		B		CB	
	P	PS	P	PS	P	PS	P	PS	P	PS	P	PS	P	PS	P	PS
1 × 3		100	42	0	93	0	0			100	42	0	93	0	0	
car-1 × 3		100	51	51	78	0	0			100	51	51	78	0	0	
ben-1 × 3	100	14	63	0	88	1	0		100	14	63	0	88	2	0	
car-1 × *ben*-1 × 3	100	32	96	0	160	2	0		100	32	100	0	160	2	0	

[a]For details on strains see Table 1.
[b]CM = complete media.
[c]Complete media plus 1 μg/ml carboxin.
[d]Complete media plus 10 μg/ml benomyl.
[e]Complete media plus 1 μg/ml carboxin and 10 μg/ml benomyl.
[f]Percentage of teliospores which produced promycelium.
[g]Percentage of teliospores which produced promycelium and sporidia.

carboxin tolerance, which would result in a lower ED_{50}. On media containing both carboxin and benomyl no germination of teliospores from any cross took place. Since in each cross at least some promycelia germinated on benomyl and carboxin separately, inhibition of germination on CM + benomyl + carboxin was probably a synergistic effect. The last cross in Table 2 involved the three strains *car*-1, *ben*-1, and 3. Teliospores of this composite cross germinated on benomyl medium like teliospores from the cross *ben*-1 × 3 (Table 2), and on carboxin medium like teliospores of the sensitive × sensitive cross (Tables 2 and 3). Therefore, it seems that the dikaryon *ben*-1 × 3 predominated in the cross. It is not clear, however, why a higher proportion of teliospores from this cross germinated normally on CM compared to the other cross involving *ben*-1.

The effect of carboxin on sporidial propagation.—For the genetic analysis of carboxin tolerance, random sporidia were sampled from each cross involving a carboxin-tolerant mutant. Cultures established from the sporidia were replica-plated on CM + carboxin (1 μg/ml) for the determination of genetic ratios. The results summarized in Table 3 show that one gene confers carboxin tolerance in *car*-1, and another in *car*-2, and that the two genes are independent.

The mutant *car*-2 was tolerant to 50 μg/ml carboxin, while *car*-1 was tolerant to only 25 μg/ml carboxin. If this difference is due to two independent genes, one in each strain, with no synergism between the genes, then the ratio among offspring of the cross *car*-1 × *car*-2 would be 2:1:1 (2 tolerant of 50 μg/ml: 1 tolerant of 25 μg/ml: 1 sensitive). With synergism, the expected ratio would be 1:1:1:1 (1 tolerant of > 50 μg/ml: 1 tolerant of 50 μg/ml: 1 tolerant of 25 μg/ml: 1 sensitive). We established 273 cultures of random sporidia from the cross *car*-1 × *car*-2 and also 216 cultures from random sporidia of the cross

TABLE 3. Ratios of carboxin tolerant to carboxin sensitive cultures among offspring from different crosses of *Ustilago hordei*. Results of tests with 1 μg/ml carboxin

Cross[a]		Progenies tested (no.)	Tolerant (%)	Sensitive (%)	Ratio	*P*
1 × 3			0	100	0:1	
car-1 × 3	1[b]	216	46	54	1:1	0.30 - 0.50
	2[b]	201	54	46	1:1	0.30 - 0.50
	3[b]	279	52	48	1:1	0.50 - 0.70
car-2 × *ben*-1		61	46	54	1:1	0.30 - 0.50
car-2 × *car*-1		273	69	31	3:1	0.30 - 0.50
car-1 × *ben*-1 × 3		201	0	100	0:1	

[a]Details on strains see Table 1.
[b]Different batches from the same cross.

TABLE 4. Frequencies of carboxin tolerant and sensitive offspring of 2 crosses of *Ustilago hordei* with carboxin tolerant strains. Results of tests on different concentrations of carboxin using replica plating

Carboxin concn (μg/ml)	*car*-1 × 3[a]				*car*-1 × *car*-2[a]			
	Sporidia from tetrads 72 cultures		Random sporidia 216 cultures		Sporidia from tetrads 24 cultures		Random sporidia 273 cultures	
	Tolerant (%)	Sensitive (%)	Tolerant (%)	Sensitive (%)	Tolerant (%)	Sensitive (%)	Tolerant (%)	Sensitive (%)
0.1	100	0	93	7	100	0	100	0
0.2[b]								
0.5	79	21			79	21	70	30
1	72	28	48	52			70	30
2.5	53	47	34	66	66	34	70	30
5	38	62	26	74	62	38	24	76
10	23	77	12	88	54	46	22	78
15			7	93	45	55	21	79
20	11	89	6	94				
25[c]								
30	4.2	95.8	6	94	37	63	20	80
40	0	100	0	100				
50[d]					37	63	9	91
100					12	88	6	94
150					4	96	4	96
200					0	0	0	0

[a]For details of the strains see Table 1.
[b]The level of tolerance of wild type.
[c]The level of tolerance of *car*-1.
[d]The level of tolerance of *car*-2.

car-1 × 3. These cultures were replica-plated on a series of carboxin concentrations. Attempts were made to analyze ordered tetrads from both crosses. Few teliospores from these crosses produced a set of four basidiospores, and not all of the sporidia that were isolated grew to form colonies. Twenty-four cultures established from first-generation sporidia of the promycelia from the cross *car*-1 × *car*-2, and 72 cultures from first generation sporidia of the cross *car*-1 × 3, were tested on different concentrations of carboxin. Their responses are summarized in Table 4. On 1 μg/ml carboxin, genetic ratios clearly indicated a different gene for tolerance in each strain. At higher concentrations the change from ratios of 1:1 or 3:1 to a 0:1 ratio should follow a single short step of increase in concentration. The situation revealed here is different. Segregation was clearly transgressive, indicating a polygenic (rather than a monogenic) control of tolerance. The occurrence of colonies sensitive to very low concentrations of carboxin rules out the possibility of cytoplasmically inherited tolerance. The results show that, while parental levels of tolerance occur in only a relatively small fraction of the offspring, some are tolerant to higher levels. Thus, selection pressure by this chemical can be met by the appearance of better-adapted new strains. Another feature should be noted. When random sporidia are isolated from teliospores, the fittest are more likely to be isolated. However, when sporidia are picked straight from the promycelia the chances of obtaining less-fit individuals are increased. The incidence of progeny tolerant to higher concentrations of carboxin was greater among cultures from tetrads, than among cultures from randomly selected sporidia from the same cross. This indicated that the tolerant forms were generally less competitive in the population, as their frequency decreased after basidiospore production.

The effects of benomyl on the propagation of offspring sporidia.—Germination of teliospores from crosses with the benomyl-tolerant strain, *ben*-1, was shown to be disturbed even on a fungicide-free medium. If the proportion of teliospores that produce sporidia is low, genetic ratios mean little. Progeny from the cross *ben*-1 × 3 tested on 2,000 μg/ml benomyl yielded 96% tolerant and 4% sensitive offspring. The cross *ben*-1 × *car*-2 yielded 64% tolerant and 36% sensitive offspring. Thirty-one percent were tolerant of both benomyl and carboxin. The mixed cross *car*-1 × *ben*-1 × 3 yielded 88% benomyl-tolerant offspring. No carboxin-tolerant offspring were recovered, indicating that the benomyl-tolerant strain dominated in the mixed cross.

It is difficult to base genetic analysis on disturbed meiotic events. Nevertheless, tests of offspring on different concentrations of benomyl gave some clues. Some of the offspring from *ben*-1 × *car*-2 that were benomyl tolerant, tolerated only 100 μg/ml benomyl, others only 1,000 μg/ml while the parental strain tolerated 2,000 μg/ml. This, together with the occurrence of sensitive cultures, would indicate that benomyl tolerance is quantitatively inherited and not cytoplasmically determined.

DISCUSSION.—Our data show that experiments to establish the genetic control of tolerance may reach different conclusions, depending on the method of evaluation. According to Georgopoulos et al. (7) the tolerance of *U. maydis* to carboxin is monogenically controlled. By testing progenies on 1 μg/ml carboxin we showed that two tolerant mutants had different single genes for tolerance. When we extended the tests over a range of fungicide concentrations, tolerance appeared to be polygenically controlled, and transgressive segregation resulted in forms tolerant of higher concentrations of toxicant.

The benomyl-tolerant strain *ben*-1 grows by budding on a standard medium, whether or not benomyl is present. But, when crossed with other strains, teliospore germination is hampered. Most teliospores produced promycelia but no sporidia either on benomyl-containing or on benomyl-free medium. Since benomyl is known to affect DNA synthesis (2) and cell division (9), it would be expected to affect teliospore germination. The defect in meiosis, shown by our mutant, seems to be different from the defects in meiotically blocked mutants of *Sordaria,* for example (6). In *Sordaria,* genetic complementation circumvents the disturbances, but in our case it does not.

The situation with carboxin is different. Despite the fact that tolerance is dominant, teliospore germination in the presence of carboxin is affected. With carboxin, only half of the teliospores germinate, and all of those produce sporidia; while with benomyl most of them germinate, but only a few produce sporidia. Both cases show that a mutation for tolerance in one phase of the life cycle does not necessarily mean that the new mutant can go through to the next generation.

The establishment of tolerance in the population depends on the speed of selection, the fitness of the new mutants, and the rate of decrease, when the selection pressure is removed. Some workers (1, 4, 16) who have studied the relative survival of mutants were frustrated to find that mutants were better competitors in mixtures. For any mutant of a pathogen, it is imperative to find out not only how it survives in a developing phase, but how it survives through an overseasoning phase. Some of our mutants were quite successful in an asexual mixture (1); however, the sexual stage was an efficient barrier to the transfer of toxicant-tolerant mutants to the next generation.

The benomyl-tolerant mutant is of interest since it shows a kind of "suicide effect": it dominated the mixed cross with the carboxin mutant, and thus prevented the transfer of the carboxin tolerance to the next generation. However, the benomyl-tolerant mutant was defective in meiosis, and thus its chances for transfer to the next generation were markedly reduced.

The genetic control of tolerance could have an impact on the establishment of tolerance in the population. Monogenic tolerance would be more likely to be selected than polygenic tolerance, and both would be more easily selected than cytoplasmic tolerance. The establishment of tolerance may easily take place under the severe selection pressure imposed by the use of a fungicide. A more critical feature is the disappearance of tolerance from the population when the selection pressure is removed. Here monogenic tolerance is most likely to disappear, and cytoplasmic tolerance the least. Therefore, it is anticipated that carboxin tolerance, polygenically controlled, would be difficult to lose once it became established in the population.

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