Genetic Studies on Tolerance of Carboxin and Benomyl at the Asexual Phase of Ustilago hordei

Y. Ben-yephet, Y. Henis, and A. Dinoor

Graduate Assistant, Associate Professor, and Senior Lecturer in Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel. Based in part on a Ph.D. thesis by the senior author.

First two authors, Fellows of Conselho Nacional de Pequisas, Instituto Biologico, Sao Paulo 01000, Brazil, S. A.; and Boyce Thompson Institute, Yonkers, New York 10701, U. S. A. (R.C.S.), respectively.

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ABSTRACT

Mutants of *Ustilago hordei* tolerant to 2,000 ppm benomyl [methyl 1-(butylcarbamoyl--2-benzimidazole-carbamate] and to 5, 25, and 50 ppm carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) were induced by ultraviolet (UV) irradiation. Tolerance was stable even after 50 transfers on fungicide-free medium. No tolerant mutants (spontaneous or by training) were recovered from cultures not treated with a mutagen. No cross-tolerance showed up between benomyl and carboxin. Carboxin-tolerant mutants tolerated oxycarboxin. The tolerant mutants were somewhat less competitive than the sensitive strains in mixed cultures, but did not disappear. Tolerance was dominant in forced-dikaryons in agar cultures and in inoculated host plants. The center of forced-dikaryon colonies was slimy and gave rise to slimy and mycelial colonies when transferred. Mycelial colonies produced dikaryotic and (sometimes) diploid sporidia while slimy colonies gave rise to haploid and dikaryotic sporidia. Dikaryotic sporidia reacted with both mating types but the diploid sporidia were neutral. It is assumed that sexual affinity in diploids does not allow for any further matings. Dikaryotic colonies gave rise to somatic recombinants.

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Additional key words: forced-dikaryon, somatic recombination.

Tolerance of fungi to fungicides is a menace which has become of much more concern since the introduction of systemic fungicides. Tolerance to fungicides was encounted in the laboratory and in the field by different workers (1, 3, 8) and was claimed to be controlled by nuclear genes and/or the cytoplasm (8).

Ustilago species are very convenient tools for studying the genetical and physiological aspects of tolerance to fungicides (10). Previous studies on the mode of action of oxathiin and the physiological aspects of oxathiin tolerance (15, 17) were carried out either without a comparison between sensitive and tolerant strains or with a sensitive strain of one species and a tolerant strain of another species.

In our studies we compared related sensitive and tolerant strains, an approach also taken by Georgopoulos et al. (7). This paper reports genetic studies on related sensitive and tolerant smut strains, as a basis for physiological studies on fungicide tolerance.

MATERIALS AND METHODS. – *Strains.* – Wild type and auxotrophic mutants were obtained from C. O. Person, Vancouver, Canada. The following strains were irradiated with UV for recovery of tolerant mutants: Wild type I_4^+, and the auxotrophs X 52 *arg*$^-$ and V191 *pan*$^+$ with requirements for arginine pantothenic acid, respectively, and a multiple auxotroph (*ad arg leuc nic*$^-$) with requirements for adenine, arginine, leucine, and nicotinic acid. The superscript symbols (+) and (−) indicate mating types.

Media. – Complete (CM), minimal (MM), and differential media were prepared according to Vogel (16).

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.); (ii) benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] dust (50% a.i.); (iii) oxycarboxin (5, 6-dihydro-2-methyl-1,4-oxathiin-3-carbox anilide-4,4-dioxide) dust (75% a.i.).

Production of tolerant mutants. – Smut cultures were grown on solid CM for 18-20 hr, then transferred to liquid CM (10 ml in 100-ml Erlenmeyer flasks) at a final concn of 75 X 10^6 cell/ml, and incubated on a shaker for 3 hr. The cell suspension was then transferred to a 5-cm diam open petri dish and irradiated for 3 min (95% kill) by a far-ultraviolet (far-UV) source (Mineralight U.V.S., San Gabriel, Calif.) at a wavelength of 254 nm from a distance of 11 cm at an intensity of 14 erg/mm^2/sec. During irradiation, the suspension was constantly mixed with a magnetic stirrer. A sample (0.1 ml) of the irradiated sporidia was placed on a 0.5-ppm carboxin-CM medium and on 10-ppm benomyl-CM medium. Tolerant mutants showed up after 10-14 days. Sporidia were spread on fresh plates. (ca. 100 sporidia/plate) to give rise to colonies, mostly originating from single sporidia. The stability of tolerance was tested by regularly checking the cultures on fungicide-supplemented media after each of 50-repeated transfers on CM without fungicide.

Determination of spontaneous mutation. – the rate of spontaneous mutation was tested with smut samples from the field and with sporidia from a sensitive *arg*$^-$ strain. Teliospores from five different infected spikes were used. The teliospores were contaminated to a certain extent with yeasts and bacteria, preventing efficient evaluation of mutants in liquid media with the Most Probable Number (MPN) method (12). External disinfection of teliospores by $CuSO_4$ (2) and other chemicals affected the viability of the smut teliospores. Rate of spontaneous mutation was therefore determined by planting out 10^6 teliospores, from each of the five spikes, 10^5 per plate, on a CM with 0.5 ppm carboxin. The same technique was used to determine spontaneous mutation in 1.57 X 10^8 sporidia of *arg*$^-$ strain and in sporidia from smut samples collected from the field.

Specific techniques. – (i) Forced-dikaryons (5) were used to test dominance or recessiveness of traits and also for some composite inoculations. (ii) Crosses were performed between compatible strains inoculated to the seed (11) of sensitive varieties. (iii) Mating type was tested by the method of Dinoor and Person (5). (iv) In vivo tolerance was tested with plants grown from inoculated seed and irrigated with the appropriate fungicides 30 days after planting.

Staining techniques. – An alcoholic toluidine blue stain (6) was used to determine the number and size of sporidial nuclei.

RESULTS. – *Tolerant mutants.* – Tolerant mutants were recovered from each of the four strains used. A strain tolerant to 50 ppm carboxin was obtained from *arg*$^-$ (*car arg* −). Strains tolerant to 25 ppm and 1 ppm carboxin were obtained from I_4^+

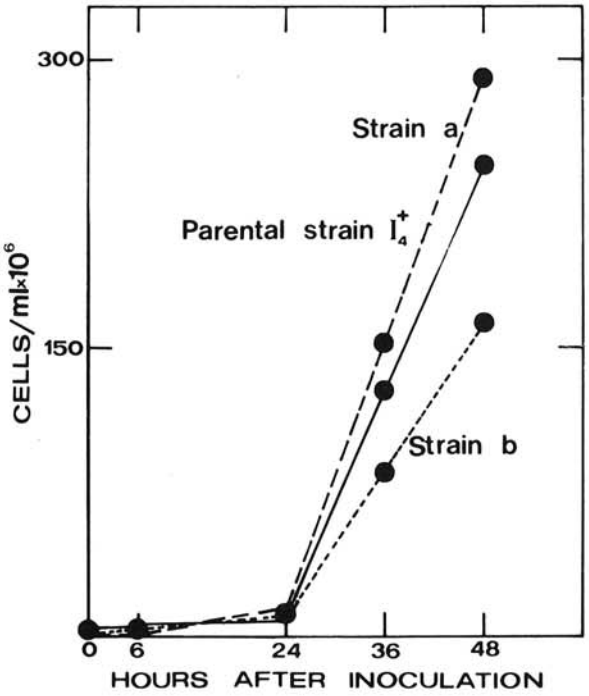

Fig. 1. Growth of carboxin-tolerant and sensitive strains of *Ustilago hordei* on complete medium (CM).

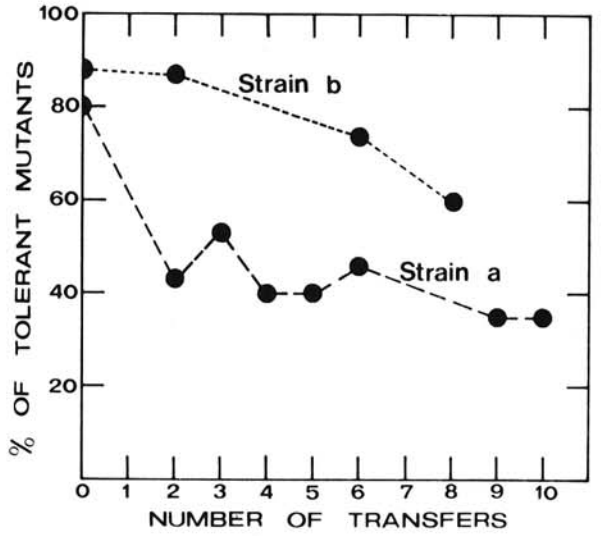

Fig. 2. The competitive ability of the tolerant strains of *Ustilago hordei*, relative to the parental sensitive strain. The strains were grown in a mixture on complete medium (CM) and transferred every 48 h.

(*car* I_4^+), whereas from the other two strains *pan*$^+$ and *ad arg leuc nic*$^-$, strains tolerant to 5 ppm carboxin were obtained (*car pan*$^+$, *car ad, arg, leuc nic*$^-$). Tolerance to benomyl was equal in mutants from all the strains and amounted to 2,000 ppm (*ben arg*$^-$, *ben pan*$^+$, *ben ad arg leuc nic*$^-$ *ben* I_4^+.

Rate of spontaneous mutation. – No tolerant mutant was recovered in any test for spontaneous mutations. A possible exception was found when carboxin-tolerant sporidia were spread on a benomyl medium and a single colony tolerant to both fungicides developed.

Training to tolerance. – Whatman antibiotic assay disks (13 mm in diam) were soaked in a solution of 1% methanol containing 1,000 ppm carboxin or benomyl and placed on solid CM. Three loopfuls of sporidial masses collected from cultures on solid CM, were streaked radially from the edge of the disks towards the edge of the petri plate. Colonies which developed nearest to the source of fungicide were transferred to new plates with fungicide in the center. After 10 transfers, there was no change in sensitivity to either fungicide.

Cross-tolerance.—No cross-tolerance was found in either carboxin-tolerant or benomyl-tolerant mutants to the other fungicide. All the carboxin-tolerant mutants were also tolerant to oxycarboxin at a much higher level. Thus tolerance to 20 and 50 ppm of carboxin was associated with tolerance to 100 and 250 ppm oxycarboxin, respectively. Benomyl-tolerant mutants were sensitive to oxycarboxin.

Vitality of the tolerant mutants.—It is generally believed and hoped that strains tolerant to fungicides will be less vigorous than sensitive strains and that their competitive ability when the fungicide is absent will be low. We compared two of our mutants (strains *a* and *b* tolerant to carboxin) to the sensitive parental strain (I_4^+) from which they were derived. First, we determined the rate of growth of each one of them separately. While strain *a* grew faster, strain *b* grew more slowly than the sensitive parental strain (Fig. 1). Our next tests were undertaken to compare the relative competitive ability of each of the tolerant strains with the sensitive strain in a fungicide-free medium. The results (Fig. 2) show that the percentage of tolerant strains in a mixed population with the sensitive strain declined. Although the percentage of *a* declined faster than that of *b*, neither disappeared from the mixed population during the 10 transfers studied. These results show that tolerant strains are not necessarily much less competitive than sensitive strains and that there is no correlation between rate of growth separately and in mixtures.

Tests with forced-dikaryons.—The forced-dikaryon technique was used to find out whether tolerance to fungicides was dominant or recessive.

The results are presented in Table 1. It is shown unequivocally that tolerance to either carboxin or benomyl is dominant and a dikaryon composed of at least one tolerant strain can also tolerate the same amount of fungicide.

The properties of the forced-dikaryons.—A colony of a forced-dikaryon forms a slimy center composed mainly of sporidia. These sporidia could be either haploids segregating from a decomposing dikaryon in a relatively rich or enriched medium (5) or they could be, dikaryotic or diploid sporidia.

We took samples from the slimy centers of different dikaryotic colonies and plated them out on CM master plates. These plates were later replicated on the following media: (i) MM medium; (ii) MM, supplemented with all combinations of the nutritional requirements of the parental strains; (iii) CM, with either carboxin or benomyl in a concn which inhibited the sensitive strains but not the tolerant ones; (iv) CM, with either plus (+) or minus (−) standard strains to determine their mating types. The results shown in Table 2 are representative of extensive data from different dikaryons carrying carboxin tolerance or benomyl tolerance, or both. All the data point to the same conclusions.

(A) The slimy center of a forced-dikaryon consists of several different cell types. There are many dikaryotic sporidia present, which give rise to prototrophic colonies that react with both mating types. The monokaryotic components of the forced-dikaryon were also present in the slimy center. These cell types were verified by staining representative samples. Prototrophic colonies which reacted with both mating type testers showed two nuclei in each sporidium, whereas parental colonies of single mating type were monokaryotic.

(B) In some forced-dikaryons the proportion of dikaryotic sporidia is very low, in others, very high. The proportions of parental types recovered is also variable. In most cases the *pan* parent strain is prevalent and the quadruple mutant is in the minority or even missing.

TABLE 1. Growth of forced-dikaryons on different media

Components of the dikaryons	CM[b]	MM[c]	CM *car*[a]	MM *car*[a]	CM *ben*[a]	MM *ben*[a]	CM *car*[a] *ben*[a]	MM *car*[a] *ben*[a]
Car[d] *arg*– × *pan*$^+$	+	+	+	+[f]	–[g]	–	–	–
Car ad arg leuc nic– × *pan*$^+$	+	+	+	+	–	–	–	–
Ben[e] *arg*$^-$ × *pan*$^+$	+	+	–	–	+	+	–	–
Ben ad, arg, leuc, nic$^-$ × *pan*$^+$	+	+	–	–	+	+	–	–
Car pan$^+$ × *ben arg*$^-$	+	+	+	+	+	+	+	+
Car arg$^-$ × *ben* $I_4{}^+$	+	+	+	+	+	+	+	+
Car ad arg leuc nic$^-$ × *car pan*$^+$	+	+	+	+	–	–	–	–

[a] The dose of fungicide in the medium was the amount tolerated by the tolerant component of the dikaryon.
[b] CM = Complete Medium.
[c] MM = Minimal Medium.
[d] *Car* = Carboxin.
[e] *Ben* = Benomyl.
[f] + = Growth.
[g] – = No growth.

(C) Prototrophic sporidia of the dikaryon *ben arg*$^-$ × *pan*$^+$ were of two types, one prevalent type was the regular dikaryotic sporidia found in all other dikaryons, whereas the other rare prototrophic type which comprised 3.6% of total population did not react with any of the mating types. It seemed to us that the rare type is a diploid rather than a dikaryon. When stained, each sporidium of this type showed a single nucleus which was a bit larger (1.6 μ in diam) than a nucleus of a haploid sporidium (1.25 μ in diam).

(D) Somatic recombinants were not recovered for forced-dikaryons except for a single case derived from the dikaryon *ben ad arg leuc nic*$^-$ × *pan*$^+$. In this case no dikaryotic sporidia were recovered. From the 60 colonies tested, 18 were quadruple mutants tolerant to benomyl and 24 were *pan* (parental types). Eighteen colonies were of the recombinant type *ben pan*$^+$.

(E) The colonies developing from single sporidia produced by the dikaryons were either slimy, mycelial, or intermediate. The results obtained by replica plating on different media showed that mycelial or intermediate colonies were always dikaryotic, while the slimy colonies were either mono- or dikaryotic.

(F) In further tests, the stability of the dikaryotic state in vitro was tested with a mycelial colony and a sporidial colony which were prototrophic and originated from dikaryotic cells as determined by staining. Both colonies were derived from the dikaryon *car arg*$^-$ × *pan*$^+$. The stability of the dikaryon was tested after 10 transfers on CM medium. A master plate was prepared from colonies of the tenth transfer. These colonies were replica-plated on MM supplemented with panothenic acid or arginine or both and on CM$^+$carboxin. Their mating type was also determined. Of 42 colonies

TABLE 2. The characteristics of colonies derived from the slimy center of a forced dikaryon

The dikaryon and its origin	Exp. no.	Total no. of colonies tested	No. of prototrophic colonies of double mating type: T[a]	No. of prototrophic colonies of double mating type: S[b]	No. of *pan* colonies of mating type$^+$: T	No. of *pan* colonies of mating type$^+$: S	No. of *ad, arg, leuc, nic* colonies of mating type$^-$: T	No. of *ad, arg, leuc, nic* colonies of mating type$^-$: S
Car ad arg leuc nic$^-$ × *pan*$^+$	1	76	49			26	1	
from Minimal Medium (MM)	2	41	3			29	9	
	3	39	26			13	0	
Car ad arg leuc nic$^-$ × *pan*$^+$	1	65	4			42	23	
from MM + carboxin	2	42	3			1	38	
	3	42	33			7	2	
Ad arg leuc nic$^-$ × *pan*$^+$	1	63		60		3		
from MM	2	42		1		39		
	3	42		30		12		

[a] T = Tolerant.
[b] S = Sensitive.

TABLE 3. Development of *Ustilago hordei* in barley cultivars ('Odessa' and 'Vantage') inoculated as seed and treated with benomyl and carboxin

		Treatment with fungicides									
						Irrigation of 1-month-old seedlings with					
		Control (no treatment)		Dusting of seed[d]		Carboxin		Benomyl		Carboxin and benomyl	
Inoculum	Cultivar	Tot.[b]	Inf.[c]	Tot.	Inf.	Tot.	Inf.	Tot.	Inf.	Tot.	Inf.
Arg− × I_4^+	Od[a]	140	16	70	0			70	0	70	0
	V[a]			70	0	140	0	70	0	70	0
Car arg− × *car* I_4^+	Od	140	5			70	0				
	V	70	0	210	0	140	1				
Car arg− × *ben* I_4^+	Od	140	0	140	0					140	2
	V	70	3	70	0					70	2

[a] Od = Odessa, V = Vantage.
[b] Tot. = Total number of plants.
[c] Inf. = Infected plants.
[d] Seeds inoculated with *arg*− × I_4^+ or with *car arg*− × *ben* I_4^+ were dusted with carboxin and benomyl, whereas those inoculated with *car arg*− × *car* I_4^+ were dusted with carboxin only.

from the mycelial colony, 40 were prototrophic, tolerant of carboxin, and reacted with both mating types. Two colonies were recombinants: one was prototrophic, reacted with both mating types but was sensitive to carboxin; and the other was prototrophic, of plus mating type, and tolerant of carboxin. This state results from somatic recombination at dikaryotic phase. All 42 colonies from the sporidial colony were prototrophic, tolerant of carboxin, and reacted with both mating types. These results indicate that once forced, the dikaryotic state is very stable in subsequent transfers even when forcing is relaxed.

The manifestation of tolerance in plants treated with fungicides. – Sensitive and tolerant strains were used to inoculate barley plants of the cultivars 'Odessa' and 'Vantage.' The following pairs of cultures were used: *arg*− × I_4^+, *car arg*− × I_4^+, and *car arg*− × *ben* I_4^+. Some of the inoculated seeds were dusted with either carboxin or benomyl (0.05% of the commercial product); other lots were planted and irrigated 1 month later with either carboxin 1,250 μg/14 plants (one pot), or benomyl 5,000 μg/14 plants (one pot), or a mixture of both 1,250 μg + 5,000 μg/14 plants (one pot). Irrigation with fungicides was used, since mixing the seed with the fungicides interfered with artificial inoculation. The results are presented in Table 3.

The degree of infection was generally quite low since the strains used were of moderate aggressiveness (14). Both carboxin and benomyl were efficient in controlling covered smut of barley which had no tolerance to fungicides. On the other hand, tolerant strains succeeded in developing even in treated plants. Even though infectivity was low, it seems that the tolerant strains are less aggressive on barley compared with the sensitive strains. Dusting of inoculated seed inhibited the development of any smut. When infection was not inhibited at the early stages, the development of the sensitive smut could later be stopped by fungicide treatment, while the development of tolerant strains was not inhibited. Thus, our results from the in vitro studies are confirmed in vivo: tolerance to either carboxin or benomyl is dominant.

DISCUSSION.—*Ustilago* species are very suitable tools for studying the genetic and physiological aspects of fungicide tolerance. Tolerant mutants can readily be induced by mutagens, but it seems to us that frequency of spontaneous mutation to tolerance is quite low (below 10^{-6} for teliospores and below 1.57×10^8 for sporidia) and slow adaptation is nonexistent. Despite the demonstration of tolerance of smut in the laboratory there is probably no immediate threat of tolerance developing in the field.

Oxycarboxin and carboxin are related compounds, with a similar spectrum of biological activity. Carboxin is more efficient against smuts. It should be stressed that all our carboxin-tolerant mutants were also oxycarboxin-tolerant and the level of tolerance was always higher for oxycarboxin than for carboxin. In none of the cases tested was there cross-tolerance between benomyl and carboxin.

The hope that tolerant mutants are less fit to survive and are doomed to extinction when the selection pressure of the fungicide is removed, does not seem to hold true in our case. Our tolerant mutants are indeed less competitive but do not seem to disappear from laboratory populations.

The forced-dikaryon method (5) is very convenient for determining whether a given trait is dominant or recessive. Thus, using this method, carboxin and benomyl tolerance was found to be dominant in *U. hordei*. The forced-dikaryon, once formed from known single sporidia, seemed to be quite a stable rather than a transient state. The genetic complementation enables two defective

strains to function normally and establish a vital colony. In the center of this colony, new cells were produced of three different types: haploids, dikaryons, and diploids. The production of dikaryotic and diploid cells enables the fungus to maintain the genetically complemented combination, and it does so even on CM where a dikaryon is known to dissociate to its components (4). The dikaryotic and diploid colonies have a tendency to produce mycelium but also propagate as sporidia. The stable dikaryotic and diploid states can give rise to somatic recombination, which was indeed found in our experiments. In contrast with findings in *U. maydis* (13) our diploid cultures were neutral in mating type tests, they did not react with either strains (+) or strains (−), whereas the dikaryotic cultures reacted with both. We assume that the sexual affinity of the paired complements in the diploid nucleus is stronger than that of the paired nuclei in a dikaryon, and any adjacent haploid nucleus cannot pull out its complement from a fused state such as the diploid. It is interesting to note that the diploid state was found in a dikaryon carrying tolerance to benomyl and on a benomyl medium. The effect of benomyl on DNA synthesis and cell division is probably responsible for several cytological effects such as haploidization in *Aspergillus* (9). Our results indicate that benomyl apparently affects diploidization in *U. hordei.* The dikaryons containing the *pan* mutant, upon dissociation, yielded haploid sporidia, the majority of which were *pan.* The explanation for these findings can be one of the following: (i) The dikaryon is producing extra amounts of pantothenic acid which is readily excreted into the medium and enables *pan* sporidia to grow. (ii) The *pan* nucleus divides faster than its partner nucleus and therefore gives rise to more *pan* sporidia.

The manifestation of tolerance of smut to fungicides in smut-inoculated and fungicide-treated plants confirms our in vitro studies and demonstrates the potential dangers of fungicide tolerance. This dominant trait can be readily established in the population, especially in fungicide-treated plants and can also give rise to recombinant offspring carrying multiple tolerance to different fungicides.

LITERATURE CITED

1. ASHIDA, J. 1965. Adaption of fungi to metal toxicants. Annu. Rev. Phytopathol. 3:153-174.
2. DAY, P. R., and S. L. ANAGNOSTAKIS. 1971. Meiotic products from natural infections of Ustilago maydis. Phytopathology 61:1020-1021.
3. DEKKER, J. 1969. Acquired resistance to fungicides. World Rev. Pest Contr. 8:79-85.
4. DICKINSON, S. 1927. Experiments on the physiology and genetics of the smut fungi.-Hyphal fusion. Proc. R. Soc., Lond. Ser. B, Biol. Sci. 101:126-136.
5. DINOOR, A., and C. PERSON. 1969. Genetic complementation in Ustilago hordei. Can. J. Bot. 47:9-14.
6. FURTADO, J. S. 1970. Alcoholic toluidine blue: A rapid method for staining nuclei in unfixed mycetozoa and fungi. Mycologia 62:406-407.
7. GEORGOPOULOS, S. G., E. ALEXANDRI, and M. CHRYSAI. 1972. Genetic evidence for action of oxathiin and thiazole derivatives on the succinic dehydrogenase system of Ustilago maydis mitochondria. J. Bacteriol. 110:809-817.
8. GEORGOPOULOS, S. G., and C. ZARACOVITIS. 1967. Tolerance of fungi to organic fungicides. Annu. Rev. Phytopathol. 5:109-130.
9. HASTIE, A. C. 1970. Benlate-induced instability of Aspergillus diploids. Nature (Lond) 226:771.
10. HOLLIDAY, R. 1961. The genetics of Ustilago maydis. Genet. Res. Camb. 2:204-230.
11. HOLTON, C. S. 1936. Inheritance of chlamydospore characteristics in oat smut fungi. J. Agric. Res. 52:535-540.
12. POCHON, J. 1954. Manual technique d'analyse microbiologique de sol. p. 35, Masson et Cie, Paris.
13. PUHALLA, J. E. 1968. Compatibility reactions on solid medium and interstrain inhibition in Ustilago maydis. Genetics 60:461-474.
14. THOMAS, P. L., and C. PERSON. 1965. Genetic control of low virulence in Ustilago. Can. J. Genet. Cytol. 7:583-588.
15. ULRICH, J. T., and D. E. MATHRE. 1972. Mode of action of oxathiin systemic fungicides. V. Effect on electron transport system of Ustilago maydis and Saccharomyces cerevisiae. J. Bacteriol. 110:628-632.
16. VOGEL, H. J. 1956. A convenient growth medium for Neurospora (Medium N). Microbiol. Genet. Bull. 13:42-43.
17. WHITE, G. A. 1971. A potent effect of 1,4-oxathiin systemic fungicides on succinate oxidation by particulate preparation from Ustilago maydis. Biochem. Biophys. Res. Commun. 44:1212-1219.