A Comparison of the Modes of Action of Three Benzimidazoles

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ABSTRACT

ED_{50} values representing the toxicity to *Fusarium oxysporum* f. sp. *melonis* of benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate], thiabendazole [2-(4′-thiazolyl)-benzimidazole], and furidazole [2-2(furyl)-benzimidazole] were in the approximate ratio of 1:4:13. Toxicity of all three compounds was enhanced as the pH was raised to neutrality or above. Under the selection pressure of benomyl, naturally occurring benomyl-tolerant mutants occurred at the rate of 1 in 8.6×10^7 spores screened. In ultraviolet-irradiated populations, tolerance to benomyl occurred at the rate of 1 in 4.6×10^5 spores surviving irradiation. Benomyl- and thiabendazole-tolerant mutants exhibited cross-tolerance with all three benzimidazoles, but the furidazole-tolerant mutant was as sensitive as the wild-type *Fusarium* to benomyl and thiabendazole. Reduction in fungitoxicity of the benzimidazoles occurred in the presence of purines and certain other compounds involved in nucleic acid synthesis. On the basis of the demonstration of cross-tolerance among benzimidazole mutants, and from evidence that the fungicides act as antimetabolites, it is concluded that furidazole exhibits a mode of action that is common to all three compounds, but benomyl and thiabendazole have an additional mechanism. Phytopathology 61:816-819.

Additional key words: fungicide tolerance.

The three benzimidazole fungicides, benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate], thiabendazole [2-(4′-thiazolyl)-benzimidazole], and furidazole [2-2-(furyl)-benzimidazole] have been shown to move systemically within plants (6, 7, 12). By virtue of this property they can be expected to be useful in the control of vascular wilt fungi. Their effectiveness, however, will depend on such things as the locus and the nature of the fungitoxic activity within the plant. In studies on wilt of tomato, caused by *Fusarium oxysporum* f. sp. *lycopersici,* Biehn & Dimond (2) have demonstrated that benomyl accumulates at the tips and margins of the leaves and that the efficacy of disease control is reduced over a period of time. There is also evidence to suggest that benomyl is fungistatic rather than fungicidal (9). Under circumstances in which the concentration of the fungicide in the plant drops to a near-lethal or sublethal level, one of two situations could arise: (i) the static yet viable fungus population could resume its pathogenic activity; (ii) selection pressure from the fungicide could act on the fungus population to favor tolerant mutants. While the former situation is the probable explanation for the loss of control noted by Biehn & Dimond (2), the possibility of mutation to tolerance in other circumstances cannot be ruled out. Indeed, strains of *Sphaerotheca fuligena* resistant to benomyl have been reported by Schroeder & Provvidenti (11) in field trials with powdery mildew of cucumber.

In aqueous solution, benomyl loses the butylcarbamoyl group from position one to form methyl 2-benzimidazolecarbamate (MBC) (4). Edgington et al. (5) have pointed out that MBC and the other two benzimidazoles, thiabendazole and furidazole, are structurally similar except for the substitution on position two. Georgopoulos (8) has shown that for chlorinated nitrobenzenes, structural similarity is coincident with similarity in the mechanism of fungicidal action. He demonstrated that tolerance in mutants of *Hypomyces solani* f. sp. *cucurbitae* was expressed not only to the chemical used in selecting the mutant but to each of the other compounds within the structurally related group. The question arises, then, as to whether cross-tolerance would be exhibited by mutants which had developed tolerance to any one of the group of the benzimidazole fungicides. Furthermore, since the structural moiety which relates benomyl, thiabendazole, and furidazole as fungicides also relates them as a group to the purines, the possibility of the benzimidazoles functioning as antimetabolites should be examined. In this regard, Staron et al. (14) reported that thiabendazole toxicity is antagonized by guanine, and Sisler (13) proposed interference with nucleic acid synthesis as a possible mechanism of action for benomyl and thiabendazole.

Our purpose in the present study was 3-fold: to evaluate the genetic potential of *Fusarium oxysporum* f. sp. *melonis* to develop tolerance to the benzimidazoles, particularly benomyl; to test independently selected mutants for cross-tolerance among the benzimidazoles so that mechanisms of fungitoxic action might be determined; and to assess the antimetabolic activity of the three fungicides on the basis of their ability to interfere with nucleic acid metabolism.

MATERIALS AND METHODS.—*Evaluation of fungitoxicity.*—A pathogenic isolate of the melon wilt fungus, *Fusarium oxysporum* f. sp. *melonis* (Leach & Currence) Snyd. & Hans. was used as the test organism. Stock solutions of the fungicides were prepared in ethylene glycol monoethyl ether (Cellosolve) and diluted 200-fold in potato-sucrose agar (PSA) adjusted to pH 6.0. The dosage response for each of these fungicides was determined by transferring 7-mm mycelial discs from stock cultures to PSA containing fungicide in concentra-

tions ranging from 5 to 80 μM. The colony diam was recorded after 6 days' incubation at room temperature.

Selection of natural mutants.—A spore suspension was incorporated into plates of PSA (pH 6) supplemented with 10 μM benomyl to give 1×10^5 spores/ml medium. This concentration of benomyl was 1.5 times the normally lethal dose. Seven days later, survivors (presumptive mutants) were picked off and subsequently maintained for 6 weeks through three successive transfers on PSA containing no fungicide. The rate of mutation to tolerance was based on that proportion of the total population of spores which remained tolerant to 10 μM benomyl after the selection pressure of the fungicide had been removed.

Selection of induced mutants.—Spores were suspended in saline to give a concentration of 2×10^6 spores/ml and exposed to ultraviolet irradiation until ca. 95% of the population had been killed (1). Aliquots of the spore suspension were then incorporated into PSA supplemented with either benomyl (10 μM), thiabendazole (30 μM), or furidazole (80 μM), and the survivors rescued after 10 days' incubation. The respective mutation rates were based on that proportion of the population which survived irradiation and retained tolerance after three successive transfers on PSA containing no fungicide.

Influence of pH on fungitoxicity.—Minimal medium (MM) was prepared with 6 g $NaNO_3$, 2 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 1 g KH_2PO_4, 0.02 g $FeSO_4 \cdot 7H_2O$, 20 g sucrose, and 15 g Bacto agar in 1 liter distilled water. After autoclaving, the pH of the medium was adjusted with HCl or NaOH to 4.0, 5.5, 6.8, 7.6, or 8.6. A fixed concentration of the test fungicide (ca. equivalent to the ED_{50} value at pH 6) was then incorporated into MM, and the influence of pH determined on the basis of the diameter of colonies arising from mycelial discs.

Relief of fungitoxicity.—Stock solutions of adenine, guanine, hypoxanthine, xanthine, aspartic acid, and biotin were prepared in sterile water and incorporated into MM at concentrations predetermined to have no effect on mycelial growth. The ability of each of the above compounds to interfere with the fungitoxic action of the benzimidazoles was assessed on the basis of colony development from mycelial discs at the ED_{50} levels of the respective fungicides.

RESULTS.—*Fungicide sensitivity.*—In PSA at pH 6.0 the ED_{50} values representing the toxicity to *F. oxysporum* f. sp. *melonis* of benomyl, thiabendazole, and furidazole were in the approximate ratio of 1:4:13 (Fig. 1).

Development of tolerance.—When large populations of spores were subjected to the selection pressure of 10 μM benomyl, a number of spores (1 in 1.7×10^7) survived the initial exposure to the chemical and developed into vigorous colonies; however, they failed to retain their tolerance to benomyl after being subcultured for a brief period in the absence of the selection agent. In addition to these unstable isolates, survivors with stable benomyl tolerance occurred spontaneously at the rate of 1 in every 8.6×10^7 spores screened. In ultraviolet-irradiated populations, induction of stable tolerance to benomyl occurred at the rate of 1 in 4.6×10^5 spores surviving the irradiation treatment (Table 1).

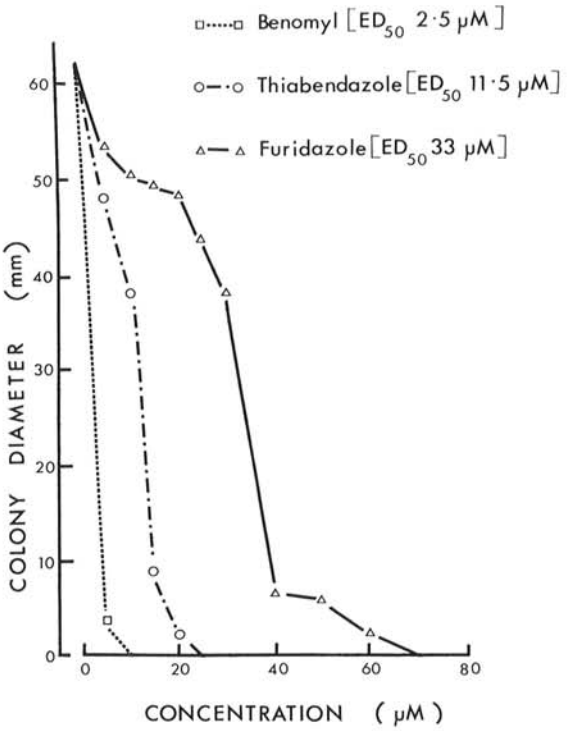

Fig. 1. The toxicity of benomyl, thiabendazole, and furidazole to mycelial growth of *Fusarium oxysporum* f. sp. *melonis*.

Cross-tolerance.—As a result of ultraviolet irradiation, mutants tolerant to each of the three benzimidazoles were obtained. The sensitivity of the respective benomyl- and thiabendazole-tolerant mutants was such that both of these strains grew normally in the presence of either 10 μM benomyl, 30 μM thiabendazole, or 80 μM furidazole. In contrast, the furidazole-tolerant mutant which grew normally in the presence of 80 μM furidazole was as sensitive as the wild-type isolate to both benomyl and thiabendazole.

Influence of pH.—No significant effects of pH upon growth from mycelial discs of the wild type isolate occurred within the pH range tested. However, when the individual fungicides were added to the culture medium,

TABLE 1. The development of ultraviolet (UV)-induced tolerance in *Fusarium oxysporum* f. sp. *melonis* under the selection pressure of 10 μM benomyl

Test series	No. UV-treated spores screened for tolerance	No. survivors (presumptive mutants)	No. mutants with stable tolerance
1	2.28×10^6	5	5
2	2.43×10^6	3	3
3	3.30×10^6	4	4
4	1.44×10^6	7	7
5	1.26×10^6	4	4
Totals	10.71×10^6	23	23[a]

[a] Mutation rate = 1 in 4.6×10^5.

TABLE 2. The effect of pH on the growth of *Fusarium oxysporum* f. sp. *melonis* on minimal medium containing benomyl, thiabendazole, or furidazole

pH	Average colony diameter in mm			
	Control	Benomyl (3 μM)	Thiabendazole (10 μM)	Furidazole (15 μM)
4.0	64.4	17.0	10.6	24.2
4.7	63.1	16.6	10.2	24.6
5.5	63.3	13.6	9.2	25.0
6.8	65.3	12.4	9.1	24.1
7.6	64.2	6.7	4.2	17.1
8.6	65.3	2.4	0	16.7

the sensitivity of the fungus to benomyl, thiabendazole, and furidazole at pH 8.6 was increased by ca. 86%, 100%, and 31%, respectively, over that recorded at pH 4.0 (Table 2).

Relief of toxicity.—The fungitoxicity of benomyl was partially relieved by biotin and hypoxanthine at pH 4.5 and by each of adenine, guanine, hypoxanthine, xanthine, biotin, and aspartic acid at pH 6.0 and 8.5. All of the metabolites interfered with thiabendazole activity at pH 6.0 and 8.5, but only biotin was effective at pH 4.5. None of the metabolites modified the activity of furidazole at pH 4.5; however, adenine, biotin, and aspartic acid partially relieved toxicity at pH 6.0, and all of the compounds in the series were effective at pH 8.5 (Table 3).

DISCUSSION.—The mode of entry of the benzimidazoles into fungal cells is not known, but if it is assumed that they enter by diffusion, then movement through membranes would be a function of the lipoid solubility of the molecule which is pH-dependent (3). At pH 4.5, where fungitoxicity was found to be relatively low, the majority of the fungicide molecules will possess an ionized benzimidazole moiety; however, at pH 8.6, the benzimidazole will be primarily undissociated, the compounds would then permeate the membrane more readily, and enhanced fungitoxicity, as we observed, would result.

It is evident that *F. oxysporum* f. sp. *melonis* has the genetic potential to develop tolerance to the benzimidazoles not only as a result of the direct mutagenic effects of ultraviolet light but also as a spontaneous event in a nonirradiated population. Although no special significance is attached to this phenomenon as it may occur in the melon wilt organism, it is tempting to speculate on the possibility of such tolerance developing under field conditions should the benzimidazoles become generally recommended for the control of vascular wilt fungi. Presumably, even isolates with adaptive or transient tolerance to a particular fungicide, and which would revert to the sensitive condition once selection pressure had been removed, might constitute a threat so long as the fungicide continued to be used in the protective program.

TABLE 3. The effect of metabolites on the toxicity of benomyl, thiabendazole, and furidazole to *Fusarium oxysporum* f. sp. *melonis* growing on minimal medium at various pH values.

Metabolite added	Relief of fungitoxicity[a]								
	Benomyl			Thiabendazole			Furidazole		
pH	4.5	6.0	8.5	4.5	6.0	8.5	4.5	6.0	8.5
Adenine (2 μM)	−	+	+	−	+	+	−	+	+
Guanine (2 μM)	−	+	+	−	+	+	−	−	+
Hypoxanthine (1 μM)	+	+	+	−	+	+	−	−	+
Xanthine (1 μM)	−	+	+	−	+	+	−	−	+
Aspartic acid (1 μM)	−	+	+	−	+	+	−	+	+
Biotin (2 μM)	+	+	+	+	+	+	−	+	+

[a] The increase (+) or lack of increase (−) in colony diameter as compared to growth in the presence of the fungicide alone.

Stable fungicide-tolerant mutants are useful tools for demonstrating the mode of action of fungicides (10). Our ultraviolet-induced mutants obtained under the selection pressure of either benomyl or thiabendazole were tolerant to all three of the benzimidazoles under test, and were indistinguishable on this basis. However, because cross-tolerance was not exhibited by the furidazole mutant, it appears that the modes of action of the three fungicides are not identical. Since these fungicides are structurally similar except for the substitution on position two of the benzimidazole moiety, it is presumed that fungitoxic action is affected by the nature of the substitution in this position. Furidazole exhibits a mode of action that is common to all three chemicals, but benomyl and thiabendazole have an additional mechanism.

The reduction in the fungitoxicity of the benzimidazoles in the presence of certain compounds involved in purine metabolism suggests that the fungicides act as antimetabolites. Interference with metabolism probably takes place both when the fungicides compete with free purines and with preformed nucleotides. Competition with the free purines, adenine and guanine, and their deaminated derivatives, hypoxanthine and xanthine, would occur during the uptake and conversion of these compounds to nucleotides. Competition might also occur in reactions involving preformed nucleotides. Our data on the antagonism of the fungitoxic action of benomyl, thiabendazole, and furidazole by aspartic acid and biotin indicate that the three fungicides act similarly and that inhibition is at the nucleotide level. Aspartic acid is directly involved in the synthesis of inosinic acid and in the formation of adenylic acid from inosinic acid, and thus may compete with the fungicides by enhancing synthesis of adenylic and inosinic acid. Since biotin is involved in aspartic acid synthesis, competition by biotin would be indirect. The differential antagonism exhibited by adenine, guanine, hypoxanthine, and xanthine to the three benzimidazoles, particularly at pH 6, can be explained on the basis of the degree to which the individual fungicides react with specific nucleotide phosphorlyases and pyrophosphorlyases (15). These observations, indicative of two modes of action among the benzimidazoles, support our earlier conclusions on mechanisms of action drawn from the cross-tolerance tests.

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