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Negatively correlated cross-resistance to dodine in fenarimol-resistant isolates of various fungi

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Abstract

The majority of fenarimol-resistant laboratory isolates of *Aspergillus nidulans, Cladosporium cucumerinum, Penicillium expansum, P. italicum,* and *Ustilago maydis* tested *in vitro*, displayed a moderate degree of negatively correlated cross-resistance to dodine. A limited number of isolates also possessed an increased sensitivity to guazatine. Colonies of fenarimol-resistant isolates of *A. nidulans* with increased sensitivity to dodine showed sector formation on dodine-amended agar. Subcultures from these sectors appeared to have the wild-type sensitivity to dodine and fenarimol. The results indicate that fenarimol resistance and increased sensitivity to dodine are closely linked. The potential practical significance of the results is discussed.

Additional keywords: guazatine, mixtures of fungicides, alternating use of fungicides, *Aspergillus nidulans, Cladosporium cucumerinum, Penicillium expansum, Penicillium italicum, Ustilago maydis.*

Introduction

The ergosterol biosynthesis inhibitors (EBI's) constitute an important group of systemic fungicides. They generally have a site-specific mechanism of action on ergosterol biosynthesis, which implies the risk of development of fungal resistance to these toxicants. Indeed laboratory resistance in various fungi to EBI's has been observed (cf. Fuchs and De Waard, 1982). In contrast, field resistance resulting in loss of disease control has not been reported. This discrepancy is explained by a decreased virulence of EBI-resistant mutants or a relatively low degree of resistance. However, the risk of development of field resistance remains a real threat, since upon increased selection pressure in space and time selection of mutants with a normal virulence and a relatively high degree of resistance may occur. The latter development can be due to a gradual accumulation of different genes for resistance in the course of time (De Waard et al., 1982). Therefore, careful use of EBI's remains important in order to reduce the likelihood of development of resistance; all means to obtain this goal should be considered (Dekker, 1982). One of these means is to make use of fungicides to which EBI-resistant mutants possess negatively correlated cross-resistance (De Waard and Van Nistelrooy, 1982). The present study describes negatively correlated cross-resistance of EBI-resistant isolates of various fungi to dodine and guazatine.

Materials and methods

Fungal strains. The following fungal species and strains were used: a. *Aspergillus nidulans:* wild-type 003 and fenarimol-resistant mutants J146, M193 and R264 (De Waard and Gieskes, 1977); b. *Cladosporium cucumerinum:* wild-type W, triarimol-resistant isolate Ta-22 and fenarimol-resistant isolate W1-0 (Fuchs and Viets-Verweij, 1975; Brown and Hall, 1979); c. *Penicillium expansum:* wild-type W and fenarimol-resistant isolates F12, UV5-1, UV30-1 and NG60-1; the resistant strains were isolated in our laboratory by Dr. M.L. Gullino (University of Turin, Italy); d. *Penicillium italicum:* wild-type W5 and fenarimol-resistant isolates A10-9, B10-4, C10-18, D100-4, E300-3, and E300-5 (De Waard et al., 1982); e. *Ustilago maydis:* wild-type W and fenarimol-resistant isolates F21, F22, and F23 (Barug and Kerkenaar, 1979).

Media. All fungal isolates were maintained on malt agar without fungicides , except for the fenarimol-resistant *U. maydis* strains which were cultured on malt agar amended with 500 μg fenarimol ml^{-1}. A basal synthetic glucose nitrate medium pH 6 was used for radial growth tests with *A. nidulans* (De Waard and Gieskes, 1977).

Chemicals. Fenarimol (technically pure) was a generous gift from Lilly Research Centre Ltd. (Erl Wood Manor, England). Dodine (technically pure) was supplied by AAgrunol (Groningen, the Netherlands) and guazatine, formulated as Panoctine 35 EC, by Ligtermoet Chemie B.V. (Roosendaal, the Netherlands).

Agar-diffusion test. Filter paper discs (diameter 13 mm) were dipped in methanolic solutions of fenarimol (3 mM), dodine (100 mM) or guazatine (100 mM) for 1 min and dried under sterile conditions. Petri dishes (diameter 14 cm) were filled with 20 ml malt agar and seeded with 0.25 ml of conidial or cell suspensions (10^7 conidia or cells ml^{-1}) of the various fungal isolates. The agar surface was dried under sterile conditions. Then, three filter discs impregnated with the same fungicide, were placed on the agar surface at equal distances from each other (in duplicate). The petri dishes were incubated at 22 °C or, in the case of *A. nidulans,* at 37 °C. The width of the inhibition zones of fungal growth around discs was measured after 3 days of incubation.

Radial growth test. Malt agar or basal nutrient agar was amended with fenarimol or dodine at various concentrations and poured into 9-cm diameter petri dishes. Inverted 5-mm agar discs with young mycelium of the various fungal isolates were placed on the agar surface (in duplicate, 3 discs per petri dish). The diameter of the colonies was measured after 3 *(A. nidulans, P. italicum),* 4 *(P. expansum),* or 5 *(C. cucumerinum)* days of incubation and corrected for the diameter of the agar discs.

Results

Toxicity tests. Toxicity of fenarimol, dodine and guazatine to fenarimol-sensitive and resistant isolates of various fungi was tested first in agar-diffusion tests. The results (Table 1) show that inhibition zones around fenarimol-treated discs on agar seeded with fenarimol-resistant isolates always were significantly smaller than those of the

Table 1. Toxicity of fenarimol, dodine and guazatine to growth of fenarimol-sensitive and -resistant isolates of various fungi in agar-diffusion tests.

Fungus	Isolate	Inhibition zone (mm) around discs[1]		
		fenarimol	dodine	guazatine
Aspergillus nidulans	003[2]	12.8	7.2	54.2
	J146	2.0[3]	9.8[3]	61.5[3]
	M193	5.8[3]	9.3[3]	57.5[3]
	R264	0	7.3	55.3
Cladosporium cucumerinum	W[2]	41.8	5.2	45.2
	Ta-22	23.7[3]	6.8[3]	48.8[3]
	W1-0	0	10.5[3]	54.0[3]
Penicillium expansum	W[2]	14.7	1.5	10.5
	F12	7.0[3]	3.8[3]	10.3
	UV5-1	6.2[3]	4.0[3]	11.7
	UV30-1	4.8[3]	4.3[3]	10.2
	NG60-1	5.2[3]	4.5[3]	11.2
Penicillium italicum	W5[2]	20.3	7.8	27.2
	A10-9	6.7[3]	12.3[3]	27.5
	B10-4	8.0[3]	11.0[3]	27.2
	C10-18	11.3[3]	6.3	26.2
	D100-4	6.3[3]	12.7[3]	N.D.[4]
	F300-3	1.3[3]	12.0[3]	25.5
	E300-5	2.7[3]	12.0[3]	30.5
Ustilago maydis	W[2]	14.5	6.5	24.5
	F21	0	10.0[3]	30.7[3]
	F22	0	8.2[3]	26.8
	F23	0	9.8[3]	32.5[3]

[1] Discs treated with methanolic solutions of fenarimol (3 mM), dodine (100 mM) or guazatine (100 mM).
[2] Wild-type isolates.
[3] Significantly different from wild-type isolate at P = 0.01 (Student test).
[4] N.D. = not determined.

Tabel 1. Toxiciteit van fenarimol, dodine en guazatine voor de groei van fenarimol-gevoelige en resistante isolaten van verschillende schimmels in agar-diffusietoetsen.

corresponding wild-type isolates. This observation confirms that all isolates were fenarimol-resistant.

With the majority of isolates tested, inhibition zones around dodine-treated discs on agar seeded with fenarimol-resistant isolates were larger compared to those of the corresponding wild-type isolates. This was most evident with isolates of *C. cucumerinum* and *P. italicum* which both had a relatively high degree of fenarimol resistance. Except for isolate R264 of *A. nidulans* and isolate C10-18 of *P. italicum*, all fenarimol-resistant isolates had an increased sensitivity to dodine (Table 1).

Table 2. Toxicity of fenarimol and dodine to growth of fenarimol-sensitive and -resistant isolates of various fungi in radial growth tests.

Fungus	Isolate	Fenarimol		Dodine	
		EC_{50}[1]	EC_{100}[1]	EC_{50}	EC_{100}
Aspergillus nidulans	003	1.2	10	15	100
	J146	10	100	10	30
	M193	9	100	9.5	30
	R264	11.5	100	12	30
Cladosporium cucumerinum	W	0.3	3	52	>300
	Ta-22	0.9	10	13	100
	W1-0	33	>100	5.1	100
Penicillium expansum	W	4.9	30	>300[2]	
	F12	10.5	100	>300[2]	
	UV5-1	11	>300	>300[2]	
	UV30-1	39	>300	100-300[2]	
	NG60-1	30	>300	300[2]	
Penicillium italicum	W5	0.7	10	16	>300
	A10-9	8.0	>300	4.4	300
	C10-18	3.3	>300	16	>300
	E300-5	19	>300	3.8	300

[1] EC_{50} and EC_{100} expressed in $\mu g\ ml^{-1}$.
[2] Figures indicate EC_{25} instead of EC_{50}.

Tabel 2. Toxiciteit van fenarimol en dodine voor de groei van fenarimol-gevoelige en resistente isolaten van verschillende schimmels in radiale groeitoetsen.

Guazatine was also tested because it is structurally related to dodine. Results similar to those obtained with dodine, were found with *C. cucumerinum* and a limited number of other isolates tested (Table 1).

In order to present more quantitative data on fenarimol and dodine toxicity, the sensitivity of all isolates, except those of *U. maydis,* was tested in radial growth tests. EC_{50} and EC_{100} values derived from dosage response curves are presented in Table 2. The data confirm the results obtained with agar-diffusion tests.

Subcultures from sectors in colonies of A. nidulans on dodine-amended agar. In radial growth tests isolate J146 of *A. nidulans* always showed sector formation in colonies on agar amended with 10, 30 or 100 μg dodine ml^{-1}. Sectors from agar with 10 μg dodine ml^{-1} were subcultured twice on dodine-amended agar and thereafter on fungicide-free agar. Then, toxicity of fenarimol and dodine was tested in agar-diffusion and radial growth tests (Table 3). The results indicate that the isolates, especially J146 D10-1, showed a wild-type sensitivity to both fenarimol and dodine (Table 3).

Table 3. Toxicity of fenarimol and dodine to growth of *Aspergillus nidulans* 003 (wild-type), J146 (fenarimol-resistant) and J146 D10-1 or J146 D10-2 (subcultures of J146 on dodine-amended agar) in agar-diffusion and radial growth tests.

Isolate	Agar-diffusion test[1]		Radial growth test[1]			
	inhibition zone (mm) around discs[2]		fenarimol		dodine	
	fenarimol	dodine	EC_{50}[5]	EC_{100}	EC_{50}	EC_{100}
003	12.8	7.2	1.2	10[6]	15	100
J146	2.0[3]	9.8[3]	10	100	10	30
J146 DIO-1	13.5[4]	7.7[4]	1.1	30[6]	16	100
J146 D10-2	8.8[4]	7.3[4]	3.3	30[6]	13	100

[1] Agar-diffusion and radial growth test assessed after 2 and 3 days of incubation, respectively.
[2] Discs treated with methanolic solutions of fenarimol (3 mM) or dodine (100 mM).
[3] Significantly different from wild-type isolate 003, J146 D10-1 and J146 D10-2 at P = 0.01 (Student test).
[4] Not significantly different from wild-type isolate 003 at P = 0.01 (Student test).
[5] EC_{50} and EC_{100} expressed in $\mu g\ ml^{-1}$.
[6] EC_{100} after 4 days of incubation 30 $\mu g\ ml^{-1}$.

Tabel 3. Toxiciteit van fenarimol en dodine voor de groei van Aspergillus nidulans 003 (wild-type), J146 (fenarimol-resistent) en J146 D10-1 of J146 D10-2 (subcultures van J146 op dodine-bevattende agar) in agar-diffusie en radiale groeistoetsen.

Discussion

Toxicity tests showed that the majority of fenarimol-resistant isolates tested had an increased sensitivity to dodine. Exceptions were *A. nidulans* isolate R264 and *P. italicum* isolate C10-18. These exceptions make it uncertain whether fenarimol resistance is absolutely coupled with increased dodine sensitivity. The recombinant strain R264 with three genes for resistant to imazalil was isolated from the progeny of several crosses for which mutants of different wild-type isolates were used (Van Tuyl, 1977). Wild-type 003 may, therefore, not be regarded as a reliable control for R264. The higher radial growth rate of R264 compared with that of 003 also points into this direction (De Waard and Gieskes, 1977). *P. italicum* C10-18, the other exceptional isolate, happened to have the lowest degree of fenarimol resistance of all *P. italicum* isolates tested. In view of these considerations one might argue that there is a close correlation between fenarimol resistance and increased dodine sensitivity and that both characteristics may be expressed by the same genetic factor. In consequence, the increased sensitivity to dodine can be described as negatively correlated cross-resistance. The isolation of strain J146 D10-1 from sectors of J146 on dodine-amended agar (Table 3) supports this explanation. This isolate may be regarded as a revertant with a back-mutation to wild-type sensitivity to both fungicides.

Negatively correlated cross-resistance has previously been reported for chemicals with a similar mechanism of action such as different benzimidazoles or carboxamides (Van Tuyl et al., 1974; White and Thorn, 1980). In these instances the phenomenon could only be observed in a limited number of mutants. Negatively correlated cross-resistance of EBI-resistant isolates of *P. italicum* to the EBI fungicide fenpropimorph appeared to be more common (De Waard and Van Nistelrooy, 1982). In contrast, the negatively correlated cross-resistance described in this paper relates to different types of fungicides. It is, as indicated above, present in the majority of resistant isolates of various fungi. Therefore, this observation may have significance for practice for the following reasons.

a. The observed negatively correlated cross-resistance described will not only be valid for fenarimol but also for other EBI's since cross-resistance to different EBI's is generally positively correlated (De Waard and Fuchs, 1982).

b. Negatively correlated cross-resistance to EBI's and dodine may not be limited to the latter chemical since dodine-related chemicals may show the same phenomonen. To some extent this has already been demonstrated for guazatine (Table 1). In view of these observations it seems of importance to search for other dodine-related chemicals which have the same or a better effect. Chemicals with a guanidine moiety may be of significance in this respect.

c. Mixtures of EBI's and dodine or a dodine-like chemical, or alternating use of such chemicals, may be of importance to reduce the likelihood of development of resistance to EBI's since development of EBI-resistant field isolates may be inhibited by the second chemical. This seems especially attractive when the second chemical also has a disease-eradicating action by its own. This is the case with *Venturia inaequalis* of which control in apple may be achieved with dodine.

d. The negatively correlated cross-resistance to EBI's and dodine-like chemicals might be reciprocal. If so, EBI's would be suitable fungicides to eradicate dodine-resistant pathogens. For instance, field resistance to dodine in *V. inaequalis* has already been observed in 1969 after 9-10 years of intensive use (Szkolnik and Gilpatrick, 1969). These isolates should be tested for a change in EBI sensitivity.

In all cases the practical significance of the results described is ultimately determined by the degree of negatively correlated cross-resistance of the isolates on their respective hosts plants and on the frequency in which field isolates without such a correlation occur. Experiments to assess this will be carried out.

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Samenvatting

Negatief gecorreleerde kruisresistentie tegen dodine in fenarimol-resistente isolaten van verschillende schimmels

Het merendeel van de fenarimol-resistente laboratorium-isolaten van *Aspergillus nidulans, Cladosporium cucumerinum, Penicillium expansum, P. italicum* en

Ustilago maydis vertoonden *in vitro* een matige graad van negatief gecorreleerde kruisresistentie tegen dodine. Een beperkt aantal isolaten bezat ook een verhoogde gevoeligheid voor guazatine. Kolonies van fenarimol-resistente isolaten van *A. nidulans* met een verhoogde gevoeligheid voor dodine vertoonden op dodine-bevattende agar sectorvorming. Subcultures van deze sectoren bleken even gevoelig te zijn voor dodine en fenarimol als de wild-stam. De resultaten wijzen er op dat fenarimol-resistentie en verhoogde gevoeligheid voor dodine nauw aan elkaar gekoppeld zijn. De potentiële praktische betekenis van de resultaten wordt besproken.

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