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Laboratory resistance to fungicides which inhibit ergosterol biosynthesis in Penicillium italicum

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Abstract

Laboratory isolates of *Penicillium italicum* with varying levels of resistance to fenarimol were obtained via mass selection of conidia on fenarimol-amended PDA. All fenarimol-resistant isolates showed cross-resistance to other fungicides which inhibit ergosterol biosynthesis (bitertanol, etaconazole, fenapanil, and imazalil), but not to fenpropimorph. In contrast, all isolates with a relatively high degree of resistance to fenarimol, exhibited increased sensitivity to fenpropimorph (negatively correlated cross-resistance). The varying degrees of resistance to ergosterol biosynthesis inhibitors (EBI's) suggest that different mutations for resistance are involved. Isolates with a high degree of resistance were selected from conidial populations of isolates with a low resistance level. This indicates that in such strains different mutations for resistance are present simultaneously.

Some *in vitro* growth parameters of resistant isolates slightly differed from those of the wild type. Virulence of most resistant isolates on oranges was visually normal and in competition experiments with mixed inocula of wild-type and resistant isolates, the latter could still be isolated after five successive infection cycles on fungicide-free oranges. Nevertheless, the proportion of resistant conidia in the successive inocula gradually decreased.

Decay of oranges inoculated with EBI-resistant isolates could still be controlled by a curative dip treatment with imazalil at dosage rates recommended in practice (500 μg ml^{-1}). However, with the highly resistant isolates, decay control was not complete at half this dosage, indicating only a marginal control at the full dosage rate.

On the basis of the results described it is assumed that at a high selection pressure of EBI's in practice, gradual accumulation of different mutations for resistance, together with selection of normal fitness may eventually lead to loss of control of *Penicillium* decay. Therefore, desease control strategies with a low selection pressure of EBI's are advisable.

Additional keywords: cross resistance, ergosterol biosynthesis inhibitors, bitertanol, etaconazole, fenapanil, fenarimol, fenpropimorph, imazalil.

Introduction

Ergosterol biosynthesis inhibitors (EBI's) seem to become one of the most prominent groups of systemic fungicides in the near future. They include structurally unrelated groups of chemicals which can be classified as imidazoles, morpholines, piperazines, pyridines, pyrimidines and triazoles (Fuchs and De Waard, 1982). The C-14 demethylation step in the ergosterol biosynthetic pathway is regarded to be the primary site of action of most EBI's (cf. Ragsdale, 1977). Only morpholines specifically interfere with other sites of this pathway (Kato et al., 1980; Kerkenaar et al., 1981). Such a specific fungitoxic action implies the risk that fungi are able to develop resistance to these

chemicals. Indeed, *in vitro* EBI-resistant mutants of various fungi can readily be isolated (cf. De Waard and Fuchs, 1982). The resistance level of mutants of *Cladosporium cucumerinum* and *Aspergillus nidulans* to EBI's is relatively low and inversely proportional to virulence or saprophytic fitness, respectively (Fuchs and Viets-Verweij, 1975; Fuchs et al., 1977; De Waard and Gieskes, 1977). Such a relation may have consequences for the likelihood of development of resistance to EBI's under practical conditions (Fuchs and Drandarevski, 1976). Up till now prolonged practical use of a number of EBI's have not led to failure of disease control. In spite of these promising prospects careful use of EBI's remains important since the observed relation between the level of resistance and the degree of pathogenicity may not be generally true. Furthermore, it is probable that upon increased selection pressure in space and time, selection of mutants of pathogens with the highest relative fitness takes place; in addition, accumulation of different mutations for resistance may occur, resulting in high levels of resistance and reduced disease control.

The aim of this study is to test under laboratory conditions the validity of some of these possibilities. *Penicillium italicum,* the causal agent of blue mold decay of citrus fruit, was chosen as the test organism.

Material and methods

Fungal strains. Oranges originating from various countries (Greece, Spain, USA) and showing blue mold disease symptoms were collected in local stores in 1978. From all oranges *P. italicum* strains with the same sensitivity to fenarimol or imazalil were isolated. Up to 1978 in none of these countries EBI's have ever been used for control of fungal diseases in oranges. Therefore, the strains were regarded to possess wild-type sensitivity to these fungicides. Strain W5 isolated from a Spanish orange was used in all experiments described. The strain was maintained on PDA. Mass production of conidia was obtained by culturing the fungus in Petri dishes with PDA supplemented with 5% (v/v) fresh orange juice.

Medium. In most experiments PDA from Merck (Darmstadt, Fed. Rep. Germany) was used; in the experiments on cross-resistance, however, home-made PDA containing potato extract of 200 g peeled potatoes, 20 g glucose, and 15 g agar per liter water.

Chemicals. Unless otherwise stated the fungicides used were technically pure products. Bitertanol (biloxazol) was kindly provided by Bayer A.G. (Leverkusen, Fed. Rep. Germany); etaconazole (CGA 64251) by Ciba-Geigy A.G. (Basle, Switzerland); fenapanil (phenapronil) by Rohm and Haas Company (Spring House, PA, USA); fenarimol by Lilly Research Centre Limited (Surrey, England); fenpropimorph by Dr R. Maag A.G. (Dielsdorf, Switzerland); imazalil by Janssen Pharmaceutica (Beerse, Belgium). Fungaflor (20% EC imazalil) was generously supplied by Duphar B.V. ('s-Graveland, the Netherlands) and Rubigan (12% EC fenarimol) by Eli Lilly Nederland (Utrecht, the Netherlands). Fungicides were mixed with PDA in appropriate amounts by adding 1000× concentrated stock solutions in methanol. Stock solutions of fenpropimorph were made in water after mixing the fungicide with three parts (w/w) of lactic acid. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Fluka A.G. (Buchs, Switzerland).

Selection of EBI-resistant isolates. Mass selection of *P. italicum* conidia for resistance to EBI's was carried out by mixing conidia in PDA (10^7 conidia ml^{-1}) amended with fenarimol (10 and 30 μg ml^{-1}) or imazalil (1 and 3 μg ml^{-1}) in plastic Petri dishes. In preliminary experiments 10 μg fenarimol ml^{-1} and 1 μg imazalil ml^{-1} were found to be the minimal inhibitory concentration (MIC) of the fungicides under the test conditions used. The dishes were incubated at 24 °C and scored for growth of colonies for a period of up to 2 weeks. Colonies were subcultured twice on PDA with fenarimol (10 μg ml^{-1}) or imazalil (1 μg ml^{-1}) and thereafter maintained on fungicide-free PDA.

Another way to obtain EBI-resistant isolates was based on mass selection of conidia treated with UV irradiation or with MNNG. UV irradiation of conidial suspensions (1.5 ml per 5-cm-diameter Petri dish) was carried out with a Hanovia UV lamp at 254 nm at a distance of 2 cm for 5 or 30 min. Conidia were treated with MNNG by incubation in 0.005 M phosphate buffer (pH 6.9) containing 50 μg MNNG ml^{-1} for 5 or 30 min. Thereafter, conidia were spun down in a centrifuge, washed twice and resuspended in sterile water. The mutagen-treated conidia were mixed with PDA (10^7 conidia ml^{-1}) amended with fenarimol (300 μg ml^{-1}) or imazalil (1 and 3 μg ml^{-1}). Isolates were obtained as described above for untreated conidia.

Determination of in vitro growth parameters. Conidial germination and germ tube growth experiments were carried out on PDA with 5% orange juice in Petri dishes inoculated with 10^5 conidia on the agar surface. Germination percentages were determined after 18 h of incubation at 24 °C by counting germination of 100 conidia. At the same time length of 100 germ tubes selected at random was measured. Radial growth of colonies developing on the same medium from 10-μl droplets each containing 10^5 conidia, was measured in triplicate after 22 and 88 h of incubation at 24 °C. Conidial suspensions from 88-h-old colonies were made by treating colonies in 10 ml 0.01% Tween-20 in an ultrasonic waterbath for 15 min and filtering over glass-wool. Conidial concentrations were determined with a hemocytometer.

Toxicity assays. MIC's of EBI's to growth of *P. italicum* isolates were determined in 6-cm-diameter Petri dishes with PDA amended with EBI's at various concentrations. The agar was inoculated with the isolates by streaking 10 μl of a conidial suspension with 10^7 conidia ml^{-1} evenly over the agar surface. Growth was assessed after 5 days of incubation at 24 °C. Scores used for evaluation of fungal growth, were: 5 = dense mycelial growth and heavy sporulation, 4 = dense mycelial growth but slight sporulation, 3 = 100-1000 colonies per Petri dish, 2 = idem 10-100, 1 = idem 1-10, and 0 = no colonies present.

Cross-resistance of fenarimol-resistant isolates to other EBI's was tested in radial growth tests on PDA amended with the toxicants. Inverted 5-mm agar discs with young mycelium were placed on the agar surface (in duplicate, 3 discs per 9-cm-diameter Petri dish). Radial growth was measured after 4 days of incubation at 24 °C.

Pathogenicity test. Spanish oranges without post-harvest fungicide treatment were obtained from Albert Heyn B.V. (Zaandam, the Netherlands). They were surface sterilized by dipping in 1% sodium hypochlorite for 1 min, treated in acetone for 1 min, rinsed in running tap water for 1 h and dried in paper towels. Oranges were injured with a needle (4 mm deep, 2 mm diameter, at three sites on the equator of the fruits,

inoculated per site with 10 μl of a conidial suspension (10^7 conidia ml^{-1}), and incubated in plastic bags for 7-10 days at 20 °C. Then, the diameter of the colony and the area of macerated tissue were measured. The colony diameter (sporulating tissue with a margin of white mycelium) was regarded as a measure for virulence. Spore production was determined as described for colonies from PDA.

Fungicide treatment of oranges. Curative fungicide treatment of oranges against blue mold was carried out by dipping fruits (in triplicate) at 24 h after inoculation in suspensions of Fungaflor or Rubigan at various concentrations for 1 min. The fruits were drained on paper towels and incubated in plastic bags. The effect of the treatment was assessed after 7 days of incubation at 20 °C as described for the pathogenicity test.

Phenotype analysis in mixed spore inocula. Preliminary results showed that the MIC of fenarimol for colony formation by conidia of the wild-type isolate W5 on PDA is 10 μg ml^{-1} (10^4 conidia per 9-cm-diameter Petri dish). This concentration also partly inhibits colony formation by conidia of EBI-resistant isolates. In order to calculate the number of resistant conidia in mixed spore inocula of the wild-type and resistant isolates, it is necessary to multiply the number of colonies, counted on PDA with 10 μg fenarimol ml^{-1} after 2 days of incubation at 24 °C, with a correction factor. This factor is defined as 100/A in which A is the percentage of fenarimol-resistant conidia of individual resistant isolates able to develop a colony on the agar medium.

Competition experiments. Conidia used in competition experiments were harvested from *in vitro* cultures of strains which were all re-isolated from artificially inoculated oranges at one time shortly before the beginning of the competition experiments. The initial mixed-inoculum pairs of wild-type W5 and each of different fenarimol-resistant isolates were prepared by combining conidial suspensions (10^7 conidia ml^{-1}) of both isolates in a 1 : 1 ratio. The actual proportion (50%) of each isolate in the initial mixed-inoculum was determined by plating conidial samples on PDA amended with 10 μg fenarimol ml^{-1}. Fruits were inoculated in duplicate with each of the inocula and incubated for 7-8 days at 20 °C. Then, conidial samples were collected from decay lesions and used for phenotype analysis (mixed pairs of isolates) or determination of A (individual lines) and used as inoculum for new series of oranges. This procedure was repeated four times. Percentages of resistant conidia in mixed inocula were calculated using the average value of A for each individual line (Table 6).

Results

Selection of EBI-resistant isolates. MIC's of fenarimol and imazalil to growth of *P. italicum* W5 in toxicity assays were between 3-10 and 0.3-1μg ml^{-1}, respectively. Therefore, mass selection of W5 conidia for resistance to these fungicides was carried out in PDA amended with 10 and 30 μg fenarimol ml^{-1} or 1 and 3 μg imazalil ml^{-1}. Colonies only developed on fenarimol-containing agar (10 μg ml^{-1}). The frequency of appearance was in the order of 1 to 2 per 10^6 conidia. From three experiments 32 isolates were made. Part of them were as sensitive to fenarimol as the wild-type W5, indicating that at high spore densities the selection media concerned did not fully discriminate

Table 1. Toxicity of fenarimol to growth of wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum*.

Isolate	Origin	Mutagenic treatment	Fenarimol concentration (μg ml^{-1})						
			0	1	3	10	30	100	300
W5	–	–	5[1]	5	3	0	0	0	0
A10-9	W5	none	5	5	5	4	3	2	2
B10-4	W5	none	5	5	5	4	3	2	2
C10-3	W5	none	5	5	4	3	2	0	0
C10-18	W5	none	5	5	4	3	2	0	0
D100-4	B10-4	none	5	5	5	4	3	2	2
E300-1	B10-4	MNNG[2]	5	5	5	4	3	2	1
E300-2	A10-9	MNNG[2]	5	5	5	4	2	2	2
E300-3	D100-4	UV[2]	5	5	5	5	5	4	4
E300-4	C10-18	UV[2]	5	5	5	3	2	2	2
E300-5	D100-4	UV[3]	5	5	5	5	4	3	3

[1] Figures indicate growth on PDA upon surface inoculation of 10^5 conidia per 5-cm-diameter Petri-dish; 5 = dense mycelial growth and heavy sporulation; 4 = dense mycelial growth but slight sporulation; 3 = 100-1000 colonies; 2 = 10-100 coloniies; 1 = 1-10 colonies; and 0 = no growth.

[2] Duration of treatment 30 min.

[3] Duration of treatment 5 min.

Tabel 1. Toxiciteit van fenarimol voor de groei van het wild-type W5 en van fenarimol-resistente isolaten van Penicillium italicum.

between sensitive and resistant strains. The remaining isolates with decreased sensitivity to fenarimol could be distinguished in two categories: isolates, coded as A or B, with MIC's above 300 μg ml^{-1} and isolates, codes as C, with MIC's between 30 and 100 μg ml^{-1} (Table 1). All isolates proved to be pathogenic on oranges; they were identified by the Centraalbureau voor Schimmelcultures (Baarn, the Netherlands) as *P. italicum,* although the C-coded isolates differed from the others in having pigmented hyphae. All isolates retained their relative insensitivity to fenarimol after re-isolation from oranges or after subculturing on fungicide-free PDA for more than one year. They were, therefore, regarded as fenarimol-resistant mutants of the wild-type isolate.

In further experiments it was tested whether mass selection within spore populations of isolates A10-9, B10-4 and C10-18 would result in a higher degree of resistance. To this end, colonies which developed on PDA with 100 and 300 υg fenarimol ml^{-1} (Table 1), were subcultured and tested for sensitivity to fenarimol. Sensitivity of seven of such isolates appeared to be almost similar to that of their respective parent strains. One of the isolates, D100-4 (Table 1), was used in further experiments.

Another attempt to select for strains with a relatively high degree of resistance was undertaken by mass selection of mutagen-treated conidia of isolates W5, A10-9, B10-4, C10-18 and D100-4. Conidia of these isolates were UV-irradiated or MNNG-treated and incorporated in PDA containing 300 μg fenarimol or 1 μg imazalil ml^{-1}. Colonies only developed on the fenarimol-amended medium inoculated with the fenarimol-

resistant isolates. Three out of five isolates (E300-3, E300-4 and E300-5), subcultured from such colonies, appeared to be more resistant to fenarimol than their respective parent isolates (Table 1). All isolates were pathogenic to oranges and were identified as *P. italicum* although it was noticed that the morphology of isolate E300-5 deviated from that of the wild-type. Resistance proved to be stable upon subculturing on oranges or PDA; therefore, these strains were also regarded as fenarimol-resistant mutants of *P. italicum*.

Cross-resistance. Toxicity of a number of chemically non-related EBI's to radial growth of *P. italicum* isolates was tested on PDA (Table 2). Etaconazole showed the highest toxicity to the wild-type isolate of all chemicals tested. Positively correlated cross-resistance in fenarimol-resistant isolates was observed to bitertanol, etaconazole, fenapanil, and imazalil; the highest and lowest resistance levels were observed with fenarimol and imazalil, respectively. Except for C10-18, all other isolates tested possessed negatively correlated cross-resistance to fenpropimorph.

Determination of in vitro growth parameters. In vitro growth characteristics of the wild-type W5 and fenarimol-resistant isolates were compared on PDA containing 5% orange juice (Table 3). Alle resistant isolates tested showed normal spore germination, but, with the exception of isolates C10-18 and E300-5 a slightly reduced germ tube growth. Radial growth rate and sporulation of C10-18 were also similar to that of the wild-type W5. All other resistant isolates possessed an increased radial growth rate, but a reduced spore production per mm^2 colony. B10-4 was the only isolate with a reduced spore production per colony as a whole (Table 3).

Virulence of fenarimol-resistant isolates. The size of fungal colonies on oranges in pathogenicity tests was regarded as a measure for virulence of the different isolates.

Table 2. Toxicity of various fungicides which inhibit ergosterol biosynthesis to radial growth of wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum* on PDA.

Isolate	EC_{50}($\mu g\ ml^{-1}$)					
	bitertanol	etaconazole	fenapanil	fenarimol	fenpropimorph	imazalil
W5	0.35 (1)[1]	0.037 (1)	0.24 (1)	0.30 (1)	0.074 (1)	0.07 (1)
A10-9	6.0 (17)	0.31 (8)	1.8 (8)	7.9 (26)	0.030 (0.4)	0.12 (1.7)
B10-4	4.8 (14)	0.31 (8)	1.5 (6)	5.8 (19)	0.022 (0.3)	0.10 (1.4)
C10-18	3.5 (10)	0.16 (4)	1.0 (4)	4.6 (15)	0.072 (1)	0.10 (1.4)
D100-4	5.2 (15)	0.29 (8)	1.6 (7)	5.7 (19)	0.042 (0.6)	0.14 (2.0)
E300-3	11.1 (32)	0.62 (17)	4.4 (18)	15.0 (50)	0.038 (0.5)	0.16 (2.3)
E300-5	10.0 (29)	0.46 (12)	2.7 (11)	10.0 (33)	0.038 (0.5)	0.13 (1.9)

[1] Between brackets: resistance level defined as ratio between EC_{50} of fungicide for resistant and wild-type isolate.

Tabel 2. Toxiciteit van verschillende fungiciden die de ergosterolbiosynthese remmen voor de radiale groei van het wild-type W5 en van fenarimol-resistente isolaten van Penicillium italicum op PDA.

Table 3. Germination, germ tube growth, mycelial growth, and sporulation of wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum* on PDA containing 5% orange juice.

Isolate	Germination (%)	Germ tube growth		Radial growth (mm)		Growth rate[1]		Spore production/colony[4]		Spore production mm^{-2}	
		(μm)	(%)	(22 h)	(88 h)	(mm)	(%)	(number $\times 10^{-8}$)	(%)	(number $\times 10^{-5}$)	(%)
W5	98.0	104	100	5.2	21.8	16.6	100	4.4 ± 0.21	100	11.9	100
A10-9	97.3	88[2]	85	5.0	24.5	19.5[3]	117	4.6 ± 0.25	104	9.8[3]	83
B10-9	97.8	88[2]	85	5.1	24.4	19.3[3]	116	3.3 ± 0.53[3]	75	7.1[3]	60
C10-18	98.2	93	89	5.0	21.7	16.7	100	4.5 ± 0.49	102	12.2	103
D100-4	98.1	92[2]	88	5.3	28.7	23.4[3]	141	5.1 ± 0.20[3]	115	7.9[3]	67
E300-3	99.3	92[2]	88	4.7	25.5	20.8[3]	125	4.7 ± 0.25	107	9.2[3]	78
E300-5	98.3	101[2]	97	4.6	26.1	21.5[3]	129	4.4 ± 0.57	100	8.2[3]	70

[1] Mycelial growth (mm) between 22 and 88 h of incubation.
[2] Significantly different from W5 at $P = 0.05$ (Student test).
[3] Significantly different from W5 at $P = 0.01$ (Student test).
[4] Determined after 88 h of incubation.

Tabel 3. Kieming, kiembuisgroei, myceliumgroei en sporulatie van het wild-type W5 en van fenarimol-resistente isolaten van Penicillium italicum op PDA met 5% sinaasappelsap.

Table 4. Virulence of wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum* on oranges.

Isolate	Diameter[1] (mm)		Visual assessment of sporulation[3]
	fungal colony	macerated tissue	
W5	36 ± 4	61 ± 5	+ + +
A10-9	39 ± 5	62 ± 5	+ +
B10-4	34 ± 4	58 ± 4	+ + +
C10-18	35 ± 3	60 ± 4	+ + +
D100-4	32 ± 12	57 ± 3	+ +
E300-3	38 ± 5	57 ± 7	+ + +
E300-5	13 ± 16[2]	58 ± 3	+

[1] Figures indicate average values and standard deviation of 18 colonies (6 fruits each with 3 inoculation sites).
[2] Significantly different from W5 at $P = 0.01$ (Student test).
[3] Normal sporulation: + + +; reduced sporulation: + +; slight sporulation: +.

Tabel 4. Virulentie van het wild-type W5 en van fenarimol-resistente isolaten van Penicillium italicum op sinaasappels.

Except for E300-5, all isolates did not differ significantly in colony size from W5. Visual assessment of sporulation in colonies suggested normal spore production by B10-4, C10-18 and E300-3 as compared with the wild-type (Table 4).

Stability of resistance and competitive ability of fenarimol-resistant isolates. Oranges were inoculated with conidial suspensions of individual isolates or mixed-pairs of isolates. Conidia produced on the decayed oranges were used as inoculum for new series or oranges. This procedure was repeated four times. The resistant strains could be re-isolated from the decayed oranges on PDA with 10 μg fenarimol ml^{-1} throughout the experiment. Results of experiments with isolates A10-9, C10-18 and E300-3 are presented in Table 5. The re-isolates retained their original of resistance (data not shown). These results indicate that resistance in all isolates is stable.

The resistant strains could also readily be re-isolated from decayed oranges inoculated with mixed-pairs of isolates (Table 5). However, upon quantitative phenotype analysis of the successive inocula a significant decrease in the proportion of resistant conidia was observed (Table 6). Although the decrease was relatively slow with isolate C10-18, apparently all mutants had a lower comparative fitness than the wild-type isolate under the test conditions used.

Spore production. One of the parameters involved in the gradual decrease of the proportion of resistant conidia may be an abnormally low spore production by resistant isolates on oranges. Therefore, in two preliminary tests spore production by some of the isolates used in the competition experiments, was counted (Table 7). Only isolate A10-9 produced a significantly lower number of conidia than the wild-type.

Table 5. Isolation of *Penicillium italicum* from decayed oranges 7 days after inoculation with the first and fifth inoculum of competition experiments. PDA amended with 10 μg fenarimol ml^{-1} was used as isolation medium[1].

Individual isolate	Diameter of colony (mm)[2]		Mixed-pair of isolates	Diameter of colony (mm)[2]	
	1st inoculum	5th inoculum		1st inoculum	5th inoculum
W5	0	0			
A10-9	22	26	W5 + A10-9	21	21
C10-18	15	21	W5 + C10-18	17	19
E300-3	30	26	W5 + E300-3	29	22

[1] Isolation on fenarimol-free PDA always resulted in colonies of 25-30 mm.
[2] Approximate average values of 3 replicates measured 5 days after isolation from tissue of non-sporulating borders of colonies.

Tabel 5. Isolatie van Penicillium italicum van rotte sinaasappels, 7 dagen na inoculatie met het eerste en vijfde inoculum in competitie-experimenten. PDA met 10 μg fenarimol ml^{-1} werd als isolatie-medium gebruikt.

Table 6. Competition between wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum* during successive inoculations of oranges.

Exp.	Pair of isolates	Percentage of resistant conidia in inoculum					A[1]
		1[2]	2	3	4	5	
I	W5 × A10-9	37.0	15.1	9.3	3.3	0.5	A10- 9: 18.0
	W5 × C10-18	49.9	42.2	38.6	16.5	12.5	C10-18: 7.7
	W5 × D10-4	60.2	32.0	26.8	2.7	<0.3	D10- 4: 15.2
II	W5 × C10-18	38.5	27.5	21.2	7.4	12.1	C10-18: 14.9
	W5 × E300-3	38.5	18.1	9.5	3.3	2.2	E300-3: 81.6

[1] A = average percentage of conidia in successive inocula of individual resistant isolates able to form a colony on PDA with 10 μg fenarimol ml^{-1}; conidia of W5 did not form colonies on this medium.
[2] The intended proportion of resistant conidia in the initial inoculum was 50%.

Tabel 6. Competitie tussen het wild-type W5 en fenarimol-resistente isolaten van Penicillium italicum gedurende opeenvolgende inoculaties op sinaasappels.

Disease control of Penicillium decay. In two different experiments disease controlling efficacy of fenarimol and imazalil was tested against decay caused by fenarimol-sensitive and resistant isolates. Results indicate that fungicide treatments effective against decay incited by the wild-type could be less effective against decay caused by resistant isolates (Table 8). In comparison with imazalil, control by fenarimol was always less effective.

Table 7. Spore production of wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum* on oranges.

Experiment	Isolate			
	W5	A10-9	C10-18	E300-3
I	10.4± 3.1[1] (5)[2]	7.6±3.5[3] (5)	10.4±3.7 (5)	9.7±4.6 (5)
II	22.9±10.3 (8)	nd[4]	nd[4]	17.4±5.7 (9)

[1] Average spore production ($\times 10^{-7}$) per colony.
[2] Between brackets: number of colonies assessed.
[3] Significantly different from W5 at P = 0.05 (Student test).
[4] nd = not determined.

Tabel 7. Sporeproduktie van het wild-type W5 en fenarimol-resistente isolaten van Penicillium italicum op sinaasappels.

Discussion

Laboratory resistance in *P. italicum* to EBI's can easily be detected by mass selection of conidia on fenarimol-amended agar. Isolates with varying degrees of resistance could be distinguished (Table 1). Isolates with a low and medium degree of resistance arose spontaneously. Isolates with a high degree of resistance were obtained after mutagenic treatment of conidia with UV and selection within isolate D100-4. In all cases resistance proved to be stable upon subculturing of the isolates in the absence of the fungicide. Therefore, resistance has probably a genetic basis and isolates have to be regarded as mutants of the wild-type strain W5. The presence of different degrees of resistance indicates that different mutations for resistance may be involved. The acquisition of isolates with a high degree of resistance after mutagenic treatment of D100-4 conidia suggests selection of a second mutation for resistance with a relatively low frequency, in addition to the one already present. The existence of different genes for resistance to EBI's and the additive effects of combining such genes has previously been demonstrated for resistance to imazalil in *Aspergillus nidulans* (Van Tuyl, 1977).

Fenarimol-resistant isolates proved to possess cross-resistance to imazalil (Table 2). However, efforts to select directly for imazalil-resistant isolates on imazalil-containing agar failed. Likewise, imazalil-resistance could not be observed in *P. digitatum* (Eckert, 1982). The explanation for this apparent inconsistency may be that the resistance level of the isolates for fenarimol was considerably higher than for imazalil (Table 2). This might imply that the imazalil concentrations used for selection may have been lethal or highly inhibitory to resistant mutants.

The normal virulence, the high comparative fitness on fungicide-free oranges, and the reduced control of decay incited by EBI-resistant laboratory mutants after dip treatment with EBI's suggest that such isolates may also develop in practice under severe selection pressure. However, practical use of EBI-fungicides has not yet led to loss of disease control. This may be explained by the fact that EBI-resistant isolates can hitherto be controlled by practically recommended dosage rates as a consequence of which such isolates remain unnoticed unless they are monitored in monitoring programmes (Fletcher and Wolfe, 1981). This hypothesis could also apply for

Table 8. Effect of a curative drench treatment of oranges with formulated fenarimol or imazalil against decay caused by wild-type W5 and fenarimol-resistant isolates of *Penicillium italicum*.

		Diameter of fungal colonies on fruits (mm)[1]							
		control	fenarimol (μg a.i. ml^{-1})			imazalil (μg a.i. ml^{-1})			
Exp.	Isolate		625	2500	10 000	25	100	250	500
I	W5	45 (75)	30 (60)	7 (24)	0 (0)	20 (47)	0 (0)	0 (0)	nd[4]
	A10-9	40 (74)	31 (57)	28[2] (57)[2]	9[2] (24)[2]	32[2] (60)[2]	6[2] (20)[2]	0 (0)	nd[4]
	B10-4	36 (74)	34 (64)[2]	27[2] (52)[2]	14[2] (27)[2]	24 (50)	0 (14)[2]	0 (0)	nd
	C10-18	42 (62)	35 (66)[2]	21[2] (56)[2]	11[2] (16)[2]	34[2] (62)[2]	10[2] (34)[2]	0 (0)	nd
	D100-4	39 (59)	29 (59)	18[2] (47)[2]	7[2] (18)[2]	31 (59)	6[2] (20)[2]	0 (0)	nd
II	W5	37 (63)	24 (49)	11 (29)	0 (16)	19 (43)	0 (13)[3]	0 (10)[3]	0 (9)[3]
	E300-3	36 (60)	33[2] (61)[2]	25[2] (48)[2]	14[2] (25)[2]	33[2] (61)[2]	15[2] (42)[2]	3 (17)[2]	0 (9)[3]
	E300-5	39 (61)	30[2] (56)[2]	18 (48)[2]	10[2] (24)[2]	25 (60)[2]	17[2] (41)[2]	6[2] (14)[2]	0 (9)[3]

[1] Average diameter of 6 colonies (2 fruits each with 3 inoculation sites); figures in parenthesis represent diameter of the macerated tissue.
[2] Significantly different from W5 at $P = 0.05$ (Student test).
[3] Brown coloured macerated tissue; size indicated already present at time of drench treatment.
[4] nd = not determined.

Tabel 8. De werking van een curatieve dompelbehandeling van sinaasappels met geformuleerd fenarimol of imazalil tegen rot veroorzaakt door het wild-type W5 en fenarimol-resistente isolaten van Penicillium italicum.

development of resistance to *P. italicum,* since the dosage of imazalil recommended in practice for control of *Penicillium* decay is 500 or 1000 μg ml^{-1} (Laville, 1974; Harding, 1976; McCornack et al., 1977; Dave and Petrie, 1981). A dip treatment with 500 μg imazalil ml^{-1} indeed controlled decay by all resistant isolates tested (Table 8). However, at increasingly lower concentrations loss of control was first noticed for the highly resistant isolates and thereafter for the isolates with a lower degree of resistance. The wild-type could be controlled at 100 μg ml^{-1}. The stepwise accumulation of resistance demonstrated in this way, might proceed further upon continuous selection pressure and give rise to resistance levels causing loss of disease control at the dosage rates of imazalil used in practice. Results obtained with fenarimol dip treatments were less pronounced than with imazalil (Table 8). Due to its low efficacy, fenarimol has also never been recommended for control of post-harvest diseases in citrus.

Development of resistance under practical conditions is also determined by the virulence of resistant mutants. Resistance in various fungal pathogens, e.g. in *C. cucumerinum* to triarimol (Fuchs and Viets-Verweij, 1975) was negatively correlated with virulence. Therefore, the likelihood of development of resistance to EBI's under practical conditions was assumed to be low (Fuchs and Drandarevski, 1976). However, resistance to these fungicides in *P. italicum* was not correlated with reduced virulence, even not in strains with a high degree of resistance (E300-3). The only difference of the resistant isolates with the wild type was their slightly increased radial growth rate and the decreased spore production in vitro (Table 3). Virulence of most resistant isolates on oranges was virtually normal, although with some strains a more or less reduced sporulation was observed (Table 4). The normal virulence of resistant isolates probably accounts for the fact that in competition experiments the mutants could still be isolated from oranges infected with the 5th successive inoculum of mixed-pairs of isolates (Table 5). However, phenotype analysis of the successive inocula evidently showed a gradual decrease in proportions of resistant conidia (Table 6). Apparently, comparative fitness of resistant isolates on fungicide-free fruit was lower than that of the wild-type W5. Isolate C10-18 with a low degree of resistance had a relatively high comparative fitness. No extensive attempts were made to explain these observations. One of the parameters involved migh be sporulation, since spore production in colonies of isolate A10-9 on oranges was less than by the wild type. No such pertinent data were obtained with isolate E300-3, while spore production of C10-18 was equal to that of W5 (Table 7). The rather high stability of resistant isolates in mixed inocula should be a matter of concern since comparative fitness on fruits with sublethal amounts of imazalil will be in favour of resistant mutants. During such conditions, selection of strains with normal fitness, e.g. with a higher sporulation capacity than that of one of the limited number of isolated tested, seems probable. The development in practice of EBI-resistant strains with an almost normal competitive ability on EBI-free citrus fruits may, therefore, not be excluded.

In view of the considerations mentioned above, development of resistance to imazalil in *P. italicum* in practice is a real risk. Therefore, the selection pressure of EBI's to which cross-resistance exists (Table 2), should be kept as low as possible. Recommendations made by Eckert (1982) may be useful in this respect. In addition, the presence of negatively correlated cross-resistance to fenpropimorph in all highly fenarimol-resistant isolates tested may be of practical importance (Table 2). In fact, fenpropimorph proved to be a highly effective fungicide against decay incited by both

the wild-type and all EBI-resistant isolates tested (unpublished results). Fenpropimorph may, therefore, be of value for disease controle strategies based on alternative use of different chemicals including other EBI's. In such disease control strategies the use of imazalil might also by of advantage because the levels of resistance in EBI-resistant isolates to this fungicide were very low as compared with the levels to other EBI's tested (Table 2).

The results described for *P. italicum,* which was used as a 'model' pathogen, may imply a warming for careful use of EBI's against other pathogens. General disease control strategies to delay or prevent fungicide resistance have recently been described by Dekker (1982).

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Samenvatting

Laboratoriumresistentie in Penicillium italicum tegen fungiciden die de ergosterolbiosynthese remmen

Laboratoriumisolaten van *Penicillium italicum* met uiteenlopende resistentieniveau's tegen fenarimol werden verkregen door massaselectie van conidiën op fenarimolbevattende PDA. Alle fenarimol-resistente isolaten vertoonden kruisresistentie tegen andere fungiciden die de ergosterolbiosynthese remmen (bitertanol, etaconazool, fenapanil, imazalil), maar niet tegen fenpropimorf. Alle isolaten met een relatief hoge graad van fenarimolresistentie waren zelfs gevoeliger voor dit laatste fungicide (negatief gecorreleerde kruisresistentie). De uiteenlopende graden van resistentie tegen ergosterolbiosynthese remmers (EBI's) suggereren dat verschillende mutaties een rol kunnen spelen. Isolaten met een hoge resistentiegraad werden geselecteerd in conidiënpopulaties van isolaten met een lage resistentiegraad. Dit duidt erop dat in dergelijke stammen verschillende mutaties gelijktijdig aanwezig zijn.

Er werden kleine verschillen in parameters voor *in vitro* groei tussen resistente en gevoelige isolaten geconstateerd. De virulentie van vrijwel alle stammen op sinaasappels was normaal; in competitie-experimenten met mengpopulaties van gevoelige en resistente isolaten konden laatstgenoemde isolaten nog na vijf opeenvolgende infectiecycli op fungicide-vrije sinaasappels worden geïsoleerd. Desalniettemin nam het percentage resistente conidiën in de opeenvolgende inocula geleidelijk af.

Penicillium-rot op sinaasappel, geïnoculeerd met EBI-resistente isolaten, kon nog worden bestreden door een curatieve dompelbehandeling met imazalil bij een dosering die in de praktijk wordt aanbevolen (500 μg ml^{-1}). Bij een halvering van deze dosering werd echter op sinaasappels, geïnoculeerd met de hoog-resistente isolaten nog rot waargenomen, hetgeen erop duidt dat de bestrijding bij de volle dosering slechts marginaal is.

Op grond van de beschreven resultaten kan worden verondersteld dat in de praktijk onder hoge selectiedruk van EBI's een geleidelijke accumulatie van verschillende mu-

taties, gepaard gaande met selectie van een normale fitheid, kan plaatsvinden, hetgeen uiteindelijk zou kunnen leiden tot onvoldoende bestrijding van *Penicillium*-rot. Bestrijdingsstrategieën met een lage selectiedruk van EBI's zijn daarom wenselijk.

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