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Stepwise development of laboratory resistance to DMI-fungicides in Penicillium italicum

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Abstract

Isolates of *Penicillium italicum* with differential levels of resistance to imazalil were obtained via step-wise mass selection of conidia of the fenarimol-resistant isolate E300-3 on imazalil-amended PDA. Three out of five selection steps were successful. The resistance level to imazalil of isolates acquired after the two last selection steps was on average 122 and 197. The differential level of resistance was also apparent in decay control on oranges by imazalil inoculated with the various isolates. The isolates showed a similar cross-resistance pattern to other fungicides which inhibit C-14 demethylation of sterols (DMIs), although the level of resistance to these fungicides was significantly higher. All isolates displayed negatively-correlated cross-resistance to tridemorph and dodine. Most isolates had a normal virulence on oranges. In competition experiments with mixed-inocula of the wild-type and a resistant isolate, the proportion of the wild-type increased in successive generations on untreated oranges and the proportion of the resistant isolate increased on imazalil-treated oranges. The lower competitive ability of the resistant isolate on untreated oranges may be due to a decrease in spore production as compared with the wild-type.

Since isolate E300-3 was obtained in two selection steps on fenarimol-amended PDA, the isolates obtained in the last selection steps on imazalil-amended PDA may have at least five different genes for resistance to DMIs. This is consistent with resistance to DMIs being under polygenic control, with the genes involved having an additive interaction, although this is not the only possible explanation of the results.

Additional keywords: fungicides, fenarimol, imazalil, sterol biosynthesis inhibitors, sterol C-14 demethylation.

Introduction

A fungus may acquire fungicide resistance either in one step, due to mutation of one gene, or in multisteps by the additive interaction of several mutant genes. In the latter case resistance is polygenically controlled and the effect of individual genes is generally small. A highly resistant strain can be obtained only by accumulating many mutant genes in the same nucleus, either by crossing different first step mutants or by stepwise selection (Georgopoulos, 1988). Resistance to fungicides which inhibit C-14 demethylation of sterols (DMIs) has been shown to be under polygenic control in several fungi. Polygenic systems for DMI-resistance have been documented well in *Aspergillus nidulans* and *Nectria haematococca* f.sp. *cucurbitae*. In both fungi eight genes were recognized

(Kalamarakis et al., 1986; Van Tuyl, 1977). In addition, many papers describe the selection of DMI-resistant mutants with low levels of resistance of which the supposed individual polygenes were not identified (cf De Waard and Fuchs, 1982).

Isolates of *A. nidulans* with a relatively high degree of DMI-resistance were made by crossing first step mutants. Recombinants possessing two different polygenes had significantly higher level of resistance, indicating additive effects (Van Tuyl, 1977). Additive interaction of polygenes for resistance to DMIs in *Erysiphe graminis* f.sp. *hordei* could also be deduced from frequency distributions of crosses from sensitive × resistant isolates, although the individual genes could not be identified (Hollomon et al., 1984).

The selection of isolates with a high degree of DMI-resistance made by stepwise selection of mutants with increasing levels of resistance has not been reported, except for one case which described a two-step selection of the fenarimol-resistant isolate E300-3 of *Penicillium italicum* (De Waard et al., 1982). This paper gives a continuation of this work and describes the isolation of mutants with a high degree of resistance to DMIs by stepwise selection in this fenarimol-resistant isolate using imazalil as the selecting agent.

Materials and methods

Fungal strains and culture methods. The *P. italicum* isolates used were the wild-type W5 and the fenarimol-resistant isolate E300-3. Isolate E300-3 was obtained by a two-step mass selection of conidia on potato dextrose agar (PDA) amended with fenarimol at 10 and 300 μg ml^{-1}, respectively (De Waard et al., 1982). The isolates were maintained on home-made PDA.

Chemicals. The fungicides used were generous gifts: bitertanol and tebuconazole from Bayer AG, Leverkusen, FRG; diclobutrazol from ICI Ltd, Bracknell, Berkshire, UK; dodine from Schering AAgrunol, Haren (Gr.), the Netherlands; etaconazole, penconazole and propiconazole from Ciba-Geigy AG, Basle, Switzerland; fenapanil from Rohm and Haas Company, Spring House, PA, USA; fenarimol from Lilly Research Centre, Erl Wood Manor, UK; imazalil sulphate (indicated in this paper as imazalil) from Janssen Pharmaceutica, Beerse, Belgium and tridemorph from BASF AG, Limburgerhof, FRG.

Toxicity assays. The determination of the minimal inhibitory concentration (MIC) of fungicides for growth on PDA, their toxicity to radial growth on PDA and their efficacy against blue mold development in curative dip treatments tests with oranges has been described previously (De Waard et al., 1982).

Stepwise selection for imazalil resistance. Mass selection of conidia for resistance to imazalil was carried out by inoculating PDA amended with imazalil at the minimal inhibitory concentration or 5× the minimal inhibitory concentration of a fungicide for a particular isolate with 10^7 conidia, spread evenly over the agar surface. Where indicated, conidial suspensions (1.5 ml with 10^8 conidia ml^{-1} in a 5-cm-diameter Petri dish) were irradiated with UV light (Hanovia UV lamp) at 254 nm at a distance of 2 cm for 4 min. Approximately 25% of the conidia survived this mutagenic treatment. The dishes were incubated at 24 °C and scored for growth of colonies for a period of up

to 3 weeks. Colonies were subcultured twice on imazalil-amended PDA at the same concentration as used in a particular selection step. Final isolates were maintained on PDA and also kept in storage on silica gel.

Phenotype analysis in mixed spore inocula. The competitive ability of one of the imazalil-resistant isolates, isolate H17, was determined by inoculating mixtures of conidia of isolate W5 and H17 on oranges (De Waard et al., 1982). The number of H17 conidia in the mixtures was determined by inoculating PDA without imazalil and PDA amended with imazalil at 0.1 μg ml^{-1} with 100 μl of a spore suspension containing 1000 conidia ml^{-1} (triplicate). This concentration of imazalil fully inhibits colony formation of the W5 isolate but does not reduce colony formation by isolate H17 conidia. The agar was always amended with dichloran (3 μg ml^{-1}) and oxytetracycline (25 μg ml^{-1}) in order to reduce colony size and to prevent bacterial growth, respectively. Oranges inoculated with the mixed spore inocula were incubated at 25 °C for one week. Then, conidia from resulting decay colonies were harvested and used for phenotype analysis and as inoculum for new series of oranges. This procedure was repeated four times. Where indicated, oranges were dip-treated with imazalil (500 μg ml^{-1}) one day after inoculation of the oranges.

Results

Selection of isolates on imazalil-amended PDA. Isolate E300-3 was obtained from wild-type W5 in previous studies by a two-step selection on PDA amended with fenarimol at 10 and 300 μg ml^{-1}, respectively (De Waard et al., 1982). The latter concentration is above the solubility level of fenarimol in PDA (about 50 μg ml^{-1}). Hence, continued selection on PDA amended with relatively higher concentrations of fenarimol would probably not be successful. The isolate showed cross resistance to all other DMIs tested but the level of cross resistance to imazalil was the lowest. Imazalil also had the highest fungitoxicity to isolate E300-3 of all DMIs tested. Therefore, continued selection for higher levels of resistance to DMIs in isolate E300-3 was carried out with this fungicide.

The first selection step with imazalil was carried out on PDA amended with imazalil at 1.0 and 2.5 μg ml^{-1} (Table 1). Representative resulting isolates such as F300-3 were not significantly less sensitive to imazalil as compared to E300-3 (Table 2). However, this isolate was used for a second selection cycle on PDA amended with imazalil at the same concentrations. This cycle yielded isolates with significantly increased levels of resistance to imazalil. A representative isolate is G7 (Table 2). Successive selection in G7 yielded isolate H17, which significantly differed in sensitivity to imazalil from G7 (Table 2). Further selection in H17 on PDA amended with imazalil at 10 and 25 μg ml^{-1} was only successful when conidia were irradiated with UV light before mass selection. In these cases, isolates with a significantly decreased sensitivity were obtained, such as I33. A successive selection step of UV irradiated I33 conidia yielded isolate J4. The increased level of resistance of this isolate was initially significantly different from that of the parent isolate (results not shown) but this could not be confirmed in following tests (Table 2). Further selection steps in isolate J4 on PDA amended with 50 μg imazalil ml^{-1} were not successful.

Most isolates were also tested for increased levels of fenarimol-resistance. Results proved to be similar as compared with those for imazalil (Table 2). Resistance levels

Table 1. Toxicity of imazalil to growth of *Penicillium italicum* wild-type isolate W5, fenarimol-resistant isolate E300-3 and of isolates selected in successive steps on imazalil-amended agar.

Isolate	Origin	Mutagenic treatment of conidia	Imazalil (μg ml^{-1})							
			0	0.5	1.0	2.5	5.0	10	25	50
W5			+[2]	−	−	−	−	−	−	−
E300-3[1]	D100-4	none	+	+	−[3]	−[3]	−	−	−	−
F300-3	E300-3	none	+	+	−[3]	−[3]	−	−	−	−
G7	F300-3	none	+	+	+	+	−[3]	−[3]	−	−
H17	G7	UV	+	+	+	+	+	−[3]	−[3]	−
I33	H17	UV	+	+	+	+	+	+	−[3]	−[3]
J4	I33	UV	+	+	+	+	+	+	+	−

[1] E300-3 is a DMI-resistant isolate obtained in two selection steps (in W5 and D100-4) on fenarimol-amended agar (De Waard et al., 1982).
[2] + and −: growth and no growth on PDA in MIC tests.
[3] Imazalil concentration used in selection step.

Table 2. Toxicity of imazalil and fenarimol to radial growth of *Penicillium italicum* wild-type isolate W5, fenarimol-resistant isolate E300-3 and of imazalil-resistant isolates selected in successive steps on imazalil-amended PDA.

Isolate	Imazalil			Fenarimol		
	EC_{50}[1,2]	n[3]	Q[4]	EC_{50}	n	Q
W5	0.062 ± 0.022[a]	11		1.24 ± 0.47[a]	11	
E300-3	0.34 ± 0.11[b]	8	5.5	46.3 ± 17.0[b]	6	37
F300-3	0.44 ± 0.21[b]	6	7.1	51.7 ± 9.2[b]	6	42
G7	1.19 ± 0.16[c]	4	19	199 ± 13.3[c]	3	160
H17	4.13 ± 1.8[d]	8	66	276 ± 53.5[c]	2	223
I33	7.54 ± 3.2[e]	7	122			
J4	12.2 ± 5.1[e]	6	197			

[1] EC_{50} for radial growth in μM.
[2] Figures followed by different letters are significantly different at $P = 0.05$.
[3] Number of replications.
[4] Q (resistance level): ratio between EC_{50} resistant isolate : EC_{50} isolate W5.

of isolates I33 and J4 for fenarimol were not determined in view of its limited solubility in PDA. An overview of all results (Table 2) shows that four steps in the stepwise selection procedure resulted in a significant increase of the resistance level. Final resistance levels of isolate I33 and J4 amounted to 122 and 197, respectively.

Inoculation of conidial suspensions of the various isolates on oranges yielded within one week of incubation typical blue mold colonies. This proves that the identity of the various isolates is indeed *P. italicum*. The identity of the isolates was confirmed by Dr R.A. Samson of the Centraalbureau voor Schimmelcultures (Baarn, the Nether-

lands). Isolate J4 differed from all other isolates with respect to its reduced radial growth rate on oranges (about 75% of that of W5) and its atypical colony growth on Czapek agar. However, growth on malt agar was normal (pers. comm. Dr R. A. Samson).

Cross resistance. Cross resistance of fenarimol-resistant isolate E300-3 and of the imazalil-resistant isolates G7, H17 and J4 to DMIs, morpholines and dodine was determined in radial growth tests. Resistance levels for all DMIs tested (imidazoles and triazoles) was higher than for imazalil (compare Q values in Table 2 and 3). Highest resistance levels were noted for the triazoles bitertanol, etaconazale, penconazole and propiconazole.

The imazalil-resistant isolates had a significantly increased sensitivity to the morpholine fungicide tridemorph. The degree of negatively-correlated cross-resistance varied from 0.15 in isolates E300-3 and G7 to 0.28 in isolate J4 (Table 3). The isolates also showed negatively correlated cross resistance to dodine.

In vivo experiments. Oranges inoculated with conidia of isolates W5, E300-3, H17 and I33 were dipped in imazalil solutions (0, 100 and 500 $\mu g\ ml^{-1}$) one day after infection. Results (Table 4) demonstrate that decay control is inversely correlated to the resistance level of the isolates to imazalil. The competitive ability of imazalil-resistant isolate H17 to wild-type W5 was tested by inoculating mixtures of conidia of both isolates on oranges. Conidia produced after one week on decayed oranges were harvested, analyzed for their composition (ratio W5 and H17) and used as inoculum for a new

Table 3. Toxicity of DMIs, morpholines and dodine to radial growth of *Penicillium italicum* wild-type isolate W5, fenarimol-resistant isolate E300-3 and of imazalil-resistant isolates selected in successive steps on imazalil-amended PDA.

Fungicide	Isolates								
	W	E300-3		G7		H17		J4	
	EC_{50}[1]	EC_{50}	Q^2	EC_{50}	Q	EC_{50}	Q	EC_{50}	Q
Imidazoles									
Fenapanil	1.03	15.5	15	129	125	157	153	245	238
Triazoles									
Bitertanol	0.80	22.8	29	253	316	496	619	602	752
Diclobutrazol	1.03	16.0	16	79	77	123	119	250	243
Etaconazole	0.098	1.1	11	7.7	79	57	578	99	1011
Penconazole	0.098	–	–	9.6	98	24	241	38	391
Propiconazole	0.091	2.2	24	10.2	112	38	418	84	918
Tebuconazole	0.29	6.0	21	–	–	34	116	55	191
Morpholines									
Tridemorph	0.195	0.03	0.15	0.030	0.15	0.053	0.27	0.054	0.28
Nonaromatics									
Dodine	135	25	0.18	16	0.12	34	0.25	–	–

[1] EC_{50} for radial growth in μM.
[2] Q (resistance level): ratio between EC_{50} resistant isolate : EC_{50} isolate W5.

Table 4. Efficacy of imazalil in curative control of blue mold decay caused by *Penicillium italicum* in dip treatments of oranges.

Isolate	Imazalil (μg ml^{-1})		
	0	100	500
W5	47.4 ± 9.5[1]	0	0
E300-3	42.6 ± 6.3	4.8 ± 2.1	0
H17	42.5 ± 3.8	29.4 ± 3.7	5.8 ± 6.7
I33	49.8 ± 6.5	37.2 ± 3.0	20.7 ± 6.5

[1] Average diameter (mm) of 12 decay colonies and standard deviation.

Table 5. Competition between wild-type W5 and imazalil-resistant isolate H17 of *Penicillium italicum* during successive growth cycles on oranges.

Isolate	Ratio[1]	Oranges[2]	Percentage of resistant conidia in growth cycle				
			1[3]	2	3	4	5
W5		−	0	0	0	1	3
W5		+	−[4]	−	−	−	−
H17		−	100	100	87	100	92
H17		+	100	100	90	100	100
W5 + H17	1 : 1	−	52	60	11	11	2
W5 + H17	99 : 1	+	0	28	109	99	90

[1] Ratio between W5 and H17 conidia in initial inoculum.
[2] Oranges treated (+) or not treated (−) with imazalil (500 μg ml^{-1}).
[3] Initial inoculum.
[4] No infection.

growth cycle on oranges. This procedure was repeated four times. One mixture (initial ratio of W5 : H17 conidia = 1 : 1) was maintained on untreated oranges and one mixture (initial ratio of W5 : H17 conidia = 99 : 1) on oranges dip-treated in a solution with 500 μg imazalil ml^{-1}. The proportion of H17 conidia rapidly declined on imazalil-free oranges, indicating a lower competitive ability of isolate H17 compared to isolate W5. In contrast, the proportion on imazalil-treated oranges rapidly increased, indicating that under these conditions the comparative fitness of isolate H17 was superior to that of the wild-type.

Discussion

This study shows that stepwise selection of resistance to DMI-fungicides is possible. In previous studies a highly fenarimol-resistant isolate was obtained in two selection steps (De Waard et al., 1982). In this study it has been shown that at least three additional selection steps lead to significant higher levels of resistance to imazalil in this fenarimol-resistant isolate, indicating that the resulting isolate I33 may have at least

5 different genes for resistance. The differential sensitivity of the various isolates to imazalil could be demonstrated both in vitro and in vivo experiments. The supposition that at least five genes for resistance are involved, could not be confirmed by means of genetic studies since *P. italicum* has no perfect stage and parasexual genetic analysis has not been described. Other explanations of our results may be that allelic mutations have an additive effect or encode different levels of resistance. If the latter hypothesis was the case, one would expect to be able to isolate mutants in the wild-type isolate W5 with a high level of resistance to DMIs in a single selection step. However, this was not observed (De Waard et al., 1982a).

Stepwise development of resistance to DMI-fungicides under field conditions has been reported for *Sphaerotheca fuliginea* (Schepers, 1985) and *E. graminis* f.sp. *hordei* (Heany et al., 1986). Therefore, the stepwise development of laboratory resistance as described in this paper may well be a model for the process which may proceed under field conditions. Attempts to obtain isolates with a higher degree of resistance than isolate I33 yielded isolate J4. The increase in resistance degree in isolate J4 was significantly higher than in isolate I33 immediately after its isolation (results not shown) but this property disappeared upon subculturing on fungicide-free PDA (Table 2). Therefore, resistance acquired in the last selection step may not have been stable. Further selection in J4 for higher resistance levels was not successful. It may be, that the resistance level of isolate J4 represents the highest one possible. This condition correlated with a change in growth characteristics, saprophytic fitness and virulence. It is likely that additional mutations in isolate J4 could not be detected because of the possibility that a further concomitant reduction in fitness parameters would be lethal. Recent research has shown that isolate J4 shows some unique properties with respect to growth on synthetic media. Results of these studies will be published elsewhere.

The imazalil-resistant isolates obtained during the stepwise selection procedure (Table 2) are cross-resistant to all other DMI-fungicides tested (Table 3). Therefore, the isolates can be regarded as DMI-resistant isolates. The degree of resistance in the highly resistant isolates is much higher than described earlier for isolates of other fungi with a (supposed) single gene for resistance to DMIs (De Waard and Fuchs, 1982; Köller and Wubben, 1986).

It is striking that the degree of cross-resistance in the DMI-resistant *P. italicum* isolates to almost all of these DMIs is higher than to imazalil. This is most notable for the triazoles bitertanol, etaconazole, penconazole and propiconazole. In part, these high Q-values may be an artifact due to the relatively low water solubility of some DMIs (bitertanol 5 μg ml^{-1}). However, the water solubility of etaconazole, penconazole and propiconazole is so high (respectively 80, 70 and 110 μg ml^{-1}) that this factor can not be the sole explanation. Köller and Wubben (1989) suggest that a structural requirement for low resistance levels is the presence of two phenyl rings separated from each other by at least three atoms. This is not the case with imazalil. Alternative explanations for the low resistance level to imazalil might be the presence of an additional target site for this fungicide or to its cationic character. No evidence for these hypotheses is available. Tebuconazole may have a second target site in sterolbiosynthesis of *Ustilago maydis* (Berg et al., 1987; 1988). If this was also the case in *P. italicum*, relatively low resistance levels to this fungicide would be expected. This is not obvious and hence the supposed additional target site probably does not play a decisive role in the sensitivy of the DMI-resistant isolates to tebuconazole.

All DMI-resistant isolates showed a significantly increased sensitivity to the morpholine tridemorph and to dodine. Negatively correlated cross-resistance to fenpropimorph and dodine has earlier been described for the fenarimol-resistant isolate E300-3 (De Waard et al., 1982; De Waard and Van Nistelrooy, 1982; 1983). All these chemicals have a cationic character. May be this factor is of importance for negatively-correlated cross-resistance to DMIs and these chemicals.

The virulence of all DMI-resistant isolates on oranges is the same indicating that stepwise resistance development is not coupled with an obvious reduction in fitness. However, in competition experiments with mixed inocula of wild-type isolate W5 and resistant isolate H17 on imazalil-free oranges, it appeared that isolate H17 has a lower competitive ability than wild-type W5. The reason for this phenomenon was not studied but preliminary observations suggest that this fact is probably due to a lower sporulation capacity of isolate H17 (unpublished results). However, this result should not lead to the conclusion that these isolates may not develop under practical conditions, since the competitive ability on imazalil-treated oranges is highest for isolate H17 and the latter condition will often occur upon storage of citrus fruit. However, imazalil-resistant isolates of *P. italicum* have not been found in practice. In contrast, such isolates of *P. digitatum* have recently been found (Eckert, 1987; 1990). The nature of resistance development to imazalil in this fungus is not known.

The mechanism of resistance to DMIs in the fenarimol-resistant isolate E300-3 is probably based on an energy-dependent efflux of the fungicide from mycelium (De Waard and Van Nistelrooy, 1984; 1988). The question whether the supposed genes acquired by additional selection on imazalil have a similar molecular mode of action or in act a completely different way, remains unanswered. This question is the topic of current research in our laboratory.

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