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GENETIC ASPECTS OF RESISTANCE TO IMAZALIL IN ASPERGILLUS NIDULANS

J.M. van Tuyl

Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands

Abstract

Mutant strains of Aspergillus nidulans have been isolated which display a low level of resistance to imazalil, a recently developed systemic fungicide. Agar growth tests showed that A. nidulans is about three times as sensitive to imazalil when growing on supplemented minimal medium (SM) as compared with complete medium. This effect was reduced by adding glutamic acid to the SM.

Imazalil resistance was found to be based on a multigenic system; 21 single gene mutations define 8 loci which were allocated to 6 different linkage groups. Mutations at different loci lead pleiotropically to one or more of the following properties: hypersensitivity or resistance to acriflavin, cycloheximide and neomycin, resistance to chloramphenicol and fenarimol, and to cold sensitivity. Of 120 cycloheximide-resistant strains isolated, 98 were also imazalil-resistant.

Recombination analysis of different imazalil-resistant strains with mutations at three loci resulted in additive effects, giving strains with a high level of resistance to imazalil.

The results indicate that imazalil may interfere either with protein synthesis like cycloheximide, chloramphenicol and neomycin or with synthesis or function of cell membranes. Interference with cell membrane synthesis might lead to altered sterol composition, resulting in selective permeability to different compounds.

Introduction

Imazalil (1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl)-1H-imidazole) is a recently developed fungicide (Laville, 1974). It acts against a number of phytopathogenic fungi especially within the group of the Ascomycetes and Deuteromycetes.

This paper deals with acquired resistance to imazalil in Aspergillus nidulans. The genetic analysis and the pleiotropic properties of the resistant mutants like hypersensitivity and resistance to various compounds are connected with the possible action of imazalil.

Materials and methods

Strains. All strains used are derived from the Glasgow stock (Clutterbuck, 1974). Mutants were isolated in two strains (biA1; acrA1) and (suA1adE20, yA2, adE20; acrA1; sB3; riboB2).

Media. The minimal and supplemented medium (MM, SM) have been described by Pontecorvo et al. (1953). As a complete medium (CM) the oxoid malt extract agar containing 3% malt extract, 0.5% mycological peptone and 1.5% agar no. 1 was used.

Chemicals. Imazalil phosphate was kindly supplied by Janssen Pharmaceutica, Beerse, Belgium. Fenarimol (α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methanol) was generously provided by Eli Lilly Company, Indianapolis, Ill., U.S.A. Acriflavin was obtained from Fluka AG, Buchs S.G., Switzerland; cycloheximide from Sigma, St. Louis, U.S.A.; chloramphenicol from Boehringer, Mannheim, Germany; neomycin sulphate and bacitracin from Calbiochem, Lucerne, Switzerland and oxytetracycline and streptomycin sulphate from Mycofarm, Delft, the Netherlands.

Growth tests. All growth tests were carried out on solid medium. Strains were stab-inoculated onto test media from master plates using a 13- or 26-point replicator. Growth was measured after 2 - 3 days at 37°C. Acriflavin, imazalil, cycloheximide and fenarimol were added to the complete medium, chloramphenicol and neomycin to the supplemented minimal medium (Gunatilleke et al., 1975).

Genetic techniques. The general genetic methods of Pontecorvo et al. (1953) were used. Mutants were allocated to linkage groups by mitotic haploidization (McCully and Forbes, 1965). A heterozygous diploid was synthesized from the mutant strain and a master strain (suA1adE20, yA2, adE20; phenA2; pyroA4; lysB5; nicB8; benA10, fwA1, carB2) (Van Tuyl, 1975a). The diploid was inoculated on complete medium plates containing 1 µg/ml benomyl (Hastie, 1970). After 3 - 4 days the haploid segregants were isolated and tested for the distribution of genetic markers.

Selection of mutants. Imazalil-resistant mutants which emerged spontaneously, or after UV irradiation or N-methyl-N'-nitro-N-nitrosoguanidine (NG) treatment (Martinelli and Clutterbuck, 1971) were selected on complete medium con-

taining 2 µg/ml imazalil. The percentage of conidia surviving the mutagenic treatment varied between 10 and 30.

Results

Growth tests on supplemented minimal medium. Growth of the wild type A. nidulans was completely inhibited on SM at a concentration of 0.4 µg/ml imazalil and on CM at a concentration of 1.2 µg/ml. By testing all nutrients present in CM, it appeared that at the same pH L- or D-glutamic acid or glutaric acid added to the SM reduced the toxicity of imazalil to the same extent as CM did. Fig. 1 shows this effect for the wild-type and an imazalil-resistant strain (IMA-4) growing on SM with and without glutaric acid (400 µg/ml). This effect is in agreement with observations of Van den Bossche (1974) in studies with miconazole, a compound related to imazalil. Interference with the uptake and/or utilization of glutamine could be involved in the mode of action of miconazole.

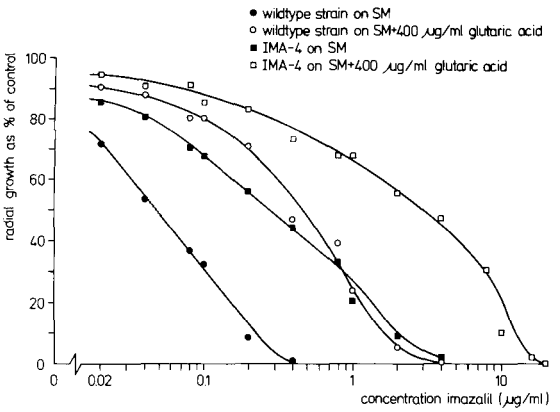

Fig. 1. The influence of glutaric acid on the toxicity of imazalil to the wild-type strain and an imazalil-resistant strain (IMA-4) of A. nidulans.

Isolation and characterization of the imazalil-resistant mutants. The frequency with which imazalil-resistant mutants occurred spontaneously was about one per ten million conidia and after mutagenic treatment about one per 100,000 surviving conidia. The level of resistance of the strains measured as the minimal inhibitory concentration (MIC) on complete medium varied from 2.5 - 12 µg/ml imazalil.

A number of pleiotropic effects are detected in the imazalil-resistant

strains. Different mutations gave rise to resistance or hypersensitivity to acriflavin, cycloheximide and neomycin, to resistance to chloramphenicol and fenarimol, and to cold sensitivity. No effect was found with three other inhibitors of protein synthesis, viz. bacitracin, oxytetracycline and streptomycin. In Table 1 the properties of the 21 imazalil-resistant mutants have been summarized. Fig. 2 shows the dosage-response curves of the wild-type and five imazalil-resistant strains to imazalil, cycloheximide, neomycin, acriflavin and fenarimol.

Genetic analysis of the imazalil-resistant mutants. In the multigenic system of imazalil resistance eight loci were located to six different linkage groups (Table 1). Eleven mutations were found to be alleles at the imaA locus which is located to linkage group VII. ImaA was mapped 12 units from wetA6 and 42 units from benC28 (Van Tuyl, 1975b). Four strains were allelic for a mutation imaB also leading to cycloheximide hypersensitivity and chloramphenicol resistance (IMA-9 in Fig. 2). It appeared that imaB was also allelic with camD (Gunatilleke et al., 1975), a chloramphenicol resistance which showed the same pleiotropic effects as imaB. ImaG18, a mutation also leading to cold sensitivity (no growth at 20°C, Waldron and Roberts, 1974a, b) and to cycloheximide resistance, was located to linkage group III at the same locus

Table 1. Properties of the imazalil-resistant mutants in A. nidulans.

Resistant mutant with mutant number	Locus involved	Linkage group	Pleiotropic effects[1]		
			resistance	hypersensitivity	additional remarks
IMA-1,2,3,4,5,6, 7,8,16,20,21	imaA	VII	neo, acr, fen		IMA-4 Fig. 2
IMA-9,11,12,17	imaB	V	cam, fen	acr, act, neo	IMA-9 Fig. 2
IMA-10	imaC	II	cam, act, fen		
IMA-13	imaD	VIII	fen	act, neo	Fig. 2
IMA-14	imaE	II	act, cam, fen, neo		Fig. 2
IMA-15	imaF	I	fen	act, neo	
IMA-18	imaG	III	act, cam		cold-sensitive
IMA-19	imaH	III	fen, neo		

[1] acr = acriflavin; act = cycloheximide; cam = chloramphenicol; fen = fenarimol; neo = neomycin.

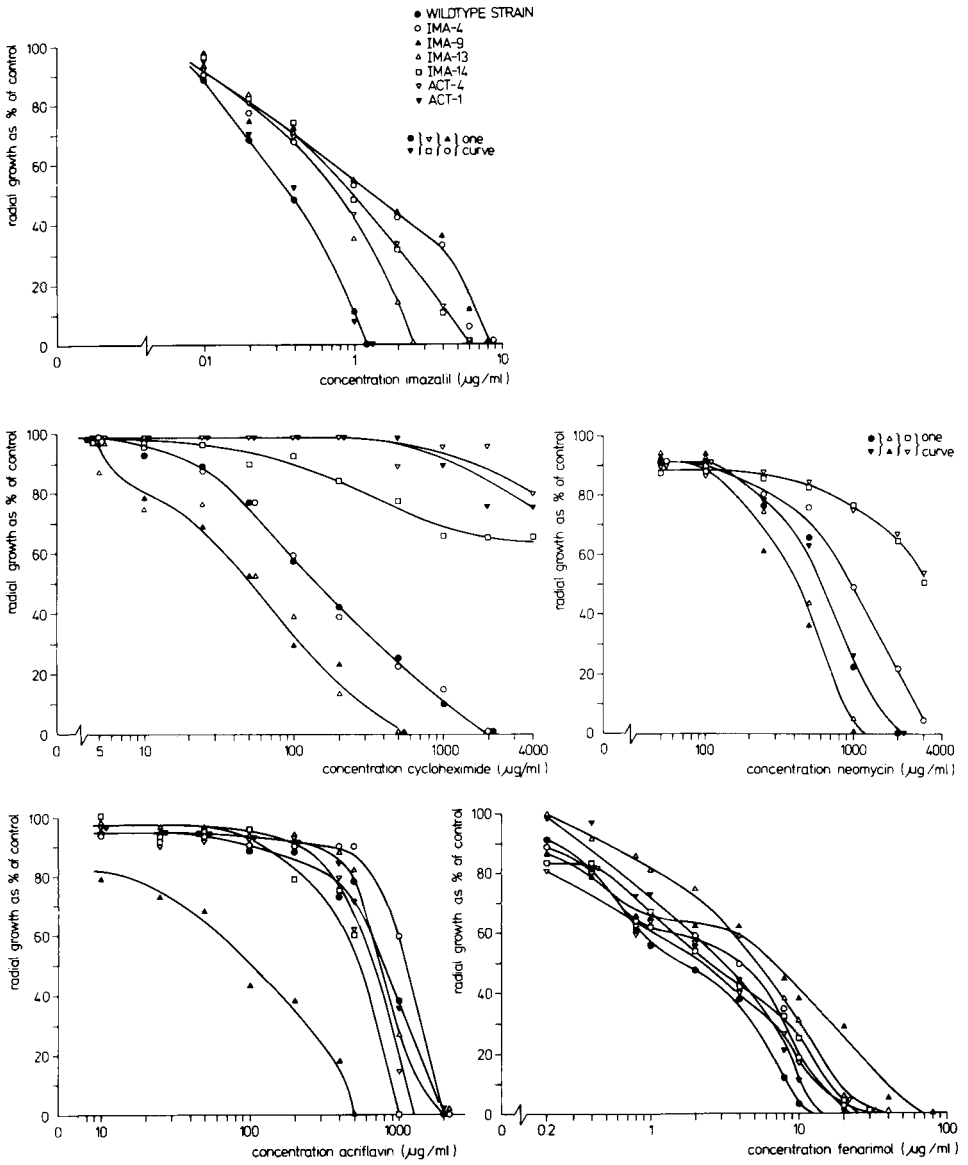

Fig. 2. Dosage-response curves of four imazalil-resistant, two cycloheximide-resistant and the wild-type strain of A. nidulans with respect to imazalil, cycloheximide, neomycin, acriflavin and fenarimol, showing some pleiotropic effects of these strains.

or closely linked to actA (Warr and Roper, 1965). ActA confers also cycloheximide resistance but this mutation showed no cross-resistance to imazalil.

Isolation and cross-resistance of cycloheximide-resistant strains. In order to investigate whether cross-resistance between cycloheximide and imazalil is general or exceptional, cycloheximide-resistant strains were selected. Of 120 spontaneous and NG-induced resistant strains 98 were cross-resistant to imazalil. In Fig. 2 ACT-1 and ACT-4 are two of those strains one (ACT-4) being cross-resistant to imazalil, the other (ACT-1) not.

Additive effects of different genes for resistance. All the single gene mutants conferring imazalil resistance show a relatively low level of resistance with a maximum MIC of 12 µg/ml compared with 1.2 µg/ml for the wild-type strain. When two genes were present in one strain, the level of resistance was increased to an MIC of 50 µg/ml. Three genes recombined in one strain resulted in an MIC up to 200 µg/ml imazalil. This positive interaction in recombinants is also known in the multigenic system for resistance to cycloheximide in Saccharomyces cerevisiae (Wilkie and Lee, 1965) and in the dodine resistance in Nectria haematococca (Kappas and Georgopoulos, 1970). Sisler and Siegel (1967) suppose that positive interaction of genes might conceivably involve a lowered permeability combined with increased resistance at the site of action.

Discussion

Pleiotropic effects of nuclear gene mutations, as shown here in hypersensitivity and cross-resistance to various inhibitors, can reveal the role of a mutation. Georgopoulos and Sisler (1970) reported negatively correlated cross-resistance between antimycin-A and oxathiins in certain mutants of Ustilago maydis. They showed that by a mutational elimination of a shift of the electron transport before the antimycin-A block, the mutant lacks an alternate pathway of electron transport and the antibiotic becomes very toxic to growth. Negative cross-resistance in A. nidulans (Van Tuyl et al., 1974; Van Tuyl, 1975b) between benomyl and thiabendazole was based on allelic mutations and appeared to be useful in studying the mechanism of action of methyl benzimidazole-2-yl carbamate (MBC) (Davidse, 1975).

Hypersensitivity and cross-resistance to cycloheximide in A. nidulans have also been reported by Waldron and Roberts (1974a) and Gunatilleke et al. (1975) in chloramphenicol resistance and cold sensitivity, respectively. Waldron and

Roberts have identified four cold-sensitive mutants which produce abnormal ribosome sedimentation profiles. Strain IMA-18 of this study, being cold-sensitive and cycloheximide-resistant, might have similar properties. Cycloheximide inhibits cytoplasmic protein synthesis in A. nidulans (Turner, 1973; Waldron and Roberts, 1974b) and chloramphenicol and neomycin are also known as inhibitors of protein synthesis. However, there is no evidence that protein synthesis is the basis of the growth inhibition of imazalil.

In Saccharomyces cerevisiae the pleiotropic effects of a single nuclear mutation, viz. increased resistance to eight diverse inhibitors of mitochondrial functions and an inhibitor of cytoplasmic protein synthesis, namely cycloheximide, could be explained by a reduced plasma membrane permeability (Rank et al., 1975). In studies with miconazole, a compound related to imazalil, it was assumed that miconazole induced permeability changes of the cell membrane (Van den Bossche et al., 1975). These data indicate that the action of imazalil might be due to its effects on membrane permeability of the cell membrane.

It is remarkable that without exception there is cross-resistance between imazalil and fenarimol. For triarimol, a compound related to fenarimol, it is believed that the sterol biosynthetic pathway is a primary site of action (Ragsdale and Sisler, 1973). Further work is necessary to establish whether the relatively low resistance to fenarimol is related with this primary action.

So far, eight loci responsible for imazalil resistance have been identified. In such a multigenic system imazalil may act at different sites of which membrane permeability, possibly in relation with sterols and protein synthesis, can be mentioned in view of the pleiotropic effects of different mutants.

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