GENETICS OF FUNGAL RESISTANCE TO SYSTEMIC FUNGICIDES

J. M. VAN TUYL

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. IR. J. P. H. VAN DER WANT,
HOOGLERAAR IN DE VIROLOGIE,
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 25 MAART 1977
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN

H. VEENMAN & ZONEN B.V. – WAGENINGEN – 1977

STELLINGEN

I

Het verdient aanbeveling om bij de ontwikkeling van nieuwe systemische fungiciden te onderzoeken of schimmels het vermogen bezitten om resistentie te verkrijgen tegen deze middelen.

II

Bij de bestudering van de resistentie ontwikkeling van een schimmel tegen een fungicide en van het werkingsmechanisme van het fungicide, dienen genetisch gekarakteriseerde stammen te worden gebruikt.

III

Resistentie van *Aspergillus nidulans* mutanten tegen imazalil berust waarschijnlijk op membraanmutaties.

IV

De termen dominant, semi-dominant en recessief ter aanduiding van de expressie van een kenmerk in een heterozygote diploid van *Aspergillus nidulans* worden veelal ten onrechte gebruikt.

J. R. WARR en J. A. ROPER, 1965. J. Gen. Microbiol. **40**: 273–281
A. C. HASTIE en S. G. GEORGOPOULOS, 1971. J. Gen. Microbiol. **67**: 371–373
S. SRIVASTAVA en U. SINHA, 1975. Genet. Res. Camb. **25**: 29–38

V

Het falen van Chidambaram en Bruehl om benomylresistentie in *Cercosporella herpotrichoides* te induceren is te wijten aan een te hoge dosis van het door hen gebruikte mutagens.

P. CHIDAMBARAM en C. W. BRUEHL, 1973. Pl. Dis. Reptr. **57**: 935–936

VI

De carboxinresistentie bij *Ustilago hordei* welke volgens Ben-Yephet, Henis en Dinoor polygeen bepaald wordt, berust waarschijnlijk op één locus en een aantal modificerende genen.

Y. BEN-YEPHET, Y. HENIS en A. DINOOR, 1975. Phytopathology **65**: 563–567

VII

Om ondubbelzinnig vast te stellen of een resistentiemutatie tevens leidt tot verminderde pathogeniteit en "fitness" van een pathogene schimmel, dient de mutant bij voorkeur zonder mutagene behandeling uit het wild type verkregen te zijn.

VIII

De methoden om bij plantpathogene schimmels resistentie tegen fungiciden te bepalen zouden gestandaardiseerd moeten worden, zoals dat voor resistentie tegen insekticiden gebeurd is.

FAO Plant Prot. Bull., 1974, **22**: 101–137

IX

Het gebruik van selectieve pesticiden, waarvan een mutagene werking op micro-organismen is aangetoond, schept een milieu waarin onvoorziene effecten op voor de mens nuttige en schadelijke micro-organismen te verwachten zijn.

M. BIGNAMI, G. MORPUGO, R. PAGLIANI, A. CARERE, G. CONTI en G. DI GIUSEPPE, 1974. Mutation Res. **26**: 159–170
J. P. SEILER, 1975. Mutation Res. **32**: 151–168
S. G. GEORGOPOULOS, A. KAPPAS en A. C. HASTIE, 1976. Phytopathology **66**: 217–220

X

Bij het veredelen van planten op ziekteresistentie krijgt het schimmelgenetische aspect te weinig aandacht.

XI

Het onderzoek naar geïntegreerde gewasbescherming is tot op heden voornamelijk gericht op de bestrijding van *plagen*. Teneinde het gestelde doel te bereiken zal de geïntegreerde *ziekten*bestrijding in de toekomst de volle aandacht moeten krijgen.

J. M. VAN TUYL
Wageningen, 25 maart 1977

VOORWOORD

Bij de voltooiing van dit proefschrift wil ik graag allen die aan het tot stand komen ervan hebben bijgedragen, daarvoor hartelijk danken.

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Ten slotte wil ik al diegenen, die hier niet genoemd zijn, hartelijk danken voor de steun en belangstelling die ik heb ontvangen.

CONTENTS

1. INTRODUCTION

1.1. General

In the control of fungal plant diseases fungicides play an important role since the end of the 19th century. Until recently the basic principle of protection with fungicides was prevention: the surface of the healthy plant was covered with a fungicidal compound which prevented the fungal organisms to enter the plant tissue. In addition to such important conventional fungicides as copper compounds, dithiocarbamates, organic tin compounds, captan, dinocap and dodine there are many other inorganic as well as organic compounds.

Since 1968 various new organic fungicides have been introduced in practice, which in contrast to the conventional fungicides, are taken up by the plant and transported in the 'system' of the plant; therefore they are called 'systemic' fungicides. They may eradicate pathogens from plant tissue even after infection has already taken place. The ability of controlling wilt diseases, caused by pathogens living inside the plant vessels and the protection of plant tissue which has not been hit directly by the fungicidal spray, are among other advantages of systemic fungicides. After the introduction of the systemic fungicides in practice, frequently reports appeared about development of resistance of plant pathogens to these fungicides. In the past development of resistance to conventional, non-systemic fungicides, which are mostly non-specific in their action, occurred only in rare cases, and moreover resistance was usually of a low level. With the selective systemic fungicides, which act mostly at a specific site in the metabolism of the cell, a single genetic change can result in a high level of resistance.

1.2. Review of the literature

1.2.1. *Mechanism of action of systemic fungicides*

Effects of systemic fungicides on fungal pathogens should, preferably, be explainable in terms of their action. Interference with various metabolic processes such as energy production and biosynthetic processes, and disruption of cell structure has been described (Kaars Sijpesteijn, 1972). Some fundamental facts about the main groups of systemic fungicides, viz. benzimidazoles, oxathiins, pyrimidines, organophosphorus compounds, piperazines and some antibiotics will be summarized below.

The benzimidazole and thiophanate fungicides are active towards a wide spectrum of fungi (Bollen and Fuchs, 1970; Edgington et al., 1971; Bollen, 1972). Probably a common mechanism of action is responsible for their fungitoxicity. Methyl benzimidazol-2-yl carbamate (MBC) is the breakdown

product of benomyl, methyl 1-(butylcarbamoyl) benzimidazol-2-yl carbamate, (CLEMONS and SISLER, 1969) and of thiophanate methyl, 1,2-bis(3-methoxycarbonyl)-2-(thioureido)-benzene (SELLING et al., 1970), and is assumed to be the toxic principle of the benzimidazole and thiophanate fungicides. Interference with mitosis (DAVIDSE, 1973, 1974, 1975; HAMMERSCHLAG and SISLER, 1973) has proved to be the primary site of action of these fungicides. Additional toxic effects, however, have been demonstrated for benomyl and are due to the formation of butyl isocyanate, which compound is toxic to *Ustilago maydis* and *Saccharomyces cerevisiae* (HAMMERSCHLAG and SISLER, 1972). In addition to these effects for thiabendazole, 2-(4'-thiazolyl) benzimidazole, inhibition of respiration is found (ALLEN and GOTTLIEB, 1970).

Oxathiins are especially toxic to Basidiomycetes (EDGINGTON and BARRON, 1967; SNEL et al., 1970) and inhibit energy production (MATHRE, 1968, 1970; RAGSDALE and SISLER, 1970). Further investigations by MATHRE (1971) and WHITE (1971) have demonstrated that carboxin, 5,6-dihydro-2-methyl-1,4 oxathiin-3-carboxanilide, inhibits mitochondrial respiration at or close to the site of succinate oxidation.

For the pyrimidine derivative triarimol, α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidinemethanol, sterol biosynthesis is assumed to be the primary site of action (RAGSDALE and SISLER, 1973; RAGSDALE, 1975). Recently, it has been shown that various fungicides chemically unrelated to triarimol viz. triforine, N,N'-bis (1-formamido-2,2,2-trichloroethyl) piperazine (SHERALD et al., 1973; SHERALD and SISLER, 1975); Denmert, S-n-butyl S'-p-tert-butylbenzyl N-3-pyridyldithiocarbonimidate (KATO et al., 1975) and triadimefon, 1-(4-chlorophenoxy-3,3-dimethyl-1(1,2,4-triazol-1-yl)-2-butanone (BUCHENAUER, 1975), also interfere with ergosterol biosynthesis.

The organophosphorus compound pyrazophos, O,O-diethyl O-(5-methyl-6-ethoxycarbonylpyrazolo [1,5-α] pyrimidin-2-yl) phosphorothioate, which is highly effective against powdery mildews (MARIOUW SMIT, 1969), is metabolically converted to PP, 2-hydroxy-5-methyl-6-ethoxycarbonyl-pyrazolo (1,5-α) pyrimidine. PP is regarded as the actual fungitoxic principle and believed to affect both oxygen uptake and biosynthetic processes (DE WAARD, 1974).

Chloroneb, 1,4-dichloro-2,5-dimethoxybenzene, an aromatic hydrocarbon fungicide, inhibits DNA synthesis in *Rhizoctonia solani* (HOCK and SISLER, 1969) and in *U. maydis* (TILLMAN and SISLER, 1973). Probably all aromatic hydrocarbon fungicides interfere with hereditary processes, as is shown by the induction of sectoring in diploid strains of *Aspergillus nidulans* (GEORGOPOULOS et al., 1976).

The toxicity of the polyene antibiotic pimaricin, which is produced by the actinomycete *Streptomyces natalensis*, is probably due to binding with certain sterols in the cell membrane (DEKKER, 1969b, 1971a).

Cycloheximide, β-(2[3,5-dimethyl-2-oxocyclohexyl]-2-hydroxyethyl) glutarimide, and other glutarimide antibiotics have been demonstrated to inhibit protein synthesis in *Saccharomyces pastorianus* (SIEGEL and SISLER, 1963) and

many other fungi (SISLER and SIEGEL, 1967; SISLER et al., 1967). Inhibition of incorporation of amino acids into protein has been shown to be the primary action of the glutarimide antibiotics (SIEGEL and SISLER, 1964).

1.2.2. *Development of resistance to systemic fungicides*

Development of resistance to systemic fungicides has been recently dealt with in a large number of review articles: DEKKER (1976), FEHRMANN (1976a, b), GEORGOPOULOS (1977) and HOFFMANN and KIEBACHER (1976). Some reviews of earlier data are those by GEORGOPOULOS and ZARACOVITIS (1967), GEORGOPOULOS (1969), DEKKER (1969a, 1971b, 1972, 1974), WOLFE (1971) and WOLFE and DINOOR (1973).

Often a close biochemical relationship has been found between the mechanism of action of the toxicant and the mechanism of resistance of the fungus. This is illustrated by the fact that a fungicide only rarely fails to reach the active site of action. Then, resistance is due to detoxification of the compound or to decreased permeability of the cell membrane. However, many examples are known in which resistance can be explained by a modification of the active site of the toxicant. Owing to the high specificity of the systemic fungicides, fungi can become resistant by only one genetic change (mutation) of one step in the metabolism of the fungus. The effect of such a mutation can be explained by a mechanism of resistance which reflects the mode of action of the fungicide (1.2.1.).

Resistance to benzimidazole derivatives has been frequently reported to occur in practice (SCHROEDER and PROVVIDENTI, 1969; NETZER and DISHON, 1970; BOLLEN, 1971; BOLLEN and SCHOLTEN, 1971; JARVIS and HARGREAVES, 1973; GEORGOPOULOS and DOVAS, 1973; WUEST et al., 1973; WICKS, 1974; BOLLEN and VAN ZAAYEN, 1975; TALBOYS and DAVIS, 1976), as well as in the laboratory (BARTELS-SCHOOLEY and MACNEILL, 1971; HASTIE and GEORGOPOULOS, 1971; BEN-YEPHET et al., 1974; BORCK and BRAYMER, 1974; BRASIER and GIBBS, 1975; VAN TUYL, 1975b; MEYER, 1976). The genetic basis of benzimidazole resistance has been studied in only a few fungi viz. *A. nidulans* (HASTIE and GEORGOPOULOS, 1971; VAN TUYL, 1975b), *Ceratocystis ulmi* (BRASIER and GIBBS, 1973), *Neurospora crrassa* (BORCK and BRAYMER, 1974) and *Ustilago hordei* (BEN-YEPHET et al., 1974, 1975a). These studies seem to justify the assumption that the high level of benzimidazole resistance, known from practice, is due to nuclear mutations at only one locus.

Reports on oxathiin resistance so far are mainly known from laboratory experiments with *U. maydis* (GEORGOPOULOS and SISLER, 1970; GEORGOPOULOS et al., 1972, 1975), *U. hordei* (BEN-YEPHET et al., 1974, 1975a, b) and *A. nidulans* (GUNATILLEKE et al., 1975a; VAN TUYL, 1975a). These genetic studies showed that resistance to oxathiins is determined multigenically; in fact in *U. maydis* and *U. hordei* two and in *A. nidulans* three loci have been identified.

Resistance to aromatic hydrocarbons can readily be obtained in the laboratory (GEORGOPOULOS, 1962, 1963a, b; THRELFALL, 1968; TILLMAN and SISLER, 1971), but no reports are known of emergence of resistance in the field.

Although resistance to triarimol and triforine (SHERALD et al., 1973; FUCHS and VIETS-VERWEY, 1975; SHERALD and SISLER, 1975) has been obtained in the laboratory, it seems rather unlikely that resistance to fungicides which inhibit ergosterol biosynthesis will develop in practice (FUCHS and DRANDAREVSKI, 1976). This conclusion is based on observations with resistant mutants. It appeared that mutants have several deficiencies with regard to spore formation or spore viability, which may greatly reduce their fitness. Similar results have already been known for a long time in medical practice with regard to polyene antibiotics, which also interfere with sterol synthesis (HAMILTON-MILLER, 1974). Reports about polyene resistance (AHMED and WOODS, 1967; MOLZAHN and WOODS, 1972; GRINDLE, 1973, 1974; KIM et al., 1974; HSUCHER and FEINGOLD, 1975) are concerned either with the genetics of the resistant mutants or with the identification of sterols.

Although development of field resistance to agricultural antibiotics (MISATO and KO, 1974) is reported in several cases viz. for streptomycin (WAKIMOTO and MUKOO, 1963), kasugamycin (ITO et al., 1974) and polyoxins (NISHIMURA et al., 1973), it is mainly restricted to in vitro experiments (NAKAMURA and SAKURAI, 1968; UESUGI et al., 1969). Several genetic studies about cycloheximide and polyene resistance in *S. cerevisiae* (WILKIE and LEE, 1965; COOPER et al., 1967; WOODS and AHMED, 1968) and *N. crassa* (HSU, 1963; GRINDLE, 1973; VOMVOYANNI, 1974) showed that this resistance is based on a multigenic system.

1.2.3. *Genetic effects of systemic fungicides*

With respect to the practical application of systemic fungicides three aspects should be considered. In the first place for the approved utilization in plant disease control the compounds should be toxicologically safe; secondly there should be no fungal resistance to fungicides; only recently a third aspect, namely the genetic or carcinogenic activity has been distinguished. Genetic activity can manifest itself by the induction of point mutations or chromosome damage like non-disjunction and crossing-over (FREESE, 1971; ROPER, 1971).

A very sensitive method for detecting those chemicals that cause point mutations is provided by a coupled system of mammalian liver homogenate and *Salmonella typhimurium* tester strains (AMES et al., 1973), which system takes into account the activation by chemical modification which may occur within the human liver. Benzimidazoles have proved to induce mutations in *Fusarium oxysporum* (DASSENOY and MEYER, 1973) and *S. typhimurium* (SEILER, 1972, 1973, 1975). In other studies with *S. typhimurium*, CARERE et al. (1976) have obtained inconclusive results with captan, N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide, and benomyl, while nine other pesticides turned out to be non-mutagenic. Gene mutation has also been studied through induction of 8-azaguanine-resistant mutants in *A. nidulans* by AULICINO et al. (1976). Here, out of ten pesticides only captan appeared to be mutagenic.

Induction of non-disjunction and crossing-over has been studied in diploid strains of *S. cerevisiae* and *A. nidulans*. SIEBERT et al. (1970) examined the in-

duction of mitotic gene conversion in *S. cerevisiae* by 14 fungicides, two of which turned out to be strongly active, viz. folpet, N-(trichloromethylthio) phthalamide and methiram, a dithiocarbamate fungicide. With diploids of *A. nidulans*, non-disjunction (haploidization) and crossing-over induced by chemicals have been reported (HASTIE, 1970; BIGNAMI et al., 1974; KAPPAS and GEORGOPOULOS, 1974, 1975; KAPPAS et al., 1974; AZEVEDO and PACHECO SANTANA, 1975; GEORGOPOULOS et al., 1976). Using this method, GEORGOPOULOS et al. (1976) showed that the benzimidazoles, the aromatic hydrocarbons and griseofulvin induced sectoring; they took this as a criterion for toxicity of these fungicides which interfere with hereditary processes. Recently, KÄFER et al. (1976) have presented well-marked diploid strains of *A. nidulans*, which permit identification of a wide range of genetic effects.

1.3. AIM AND OUTLINE OF THE PRESENT STUDY

The aim of this study was to contribute to the knowledge of the genetic background of development of fungicide resistance. This seemed of interest not only for scientific reasons, but also because of the strategy of handling fungicide resistance in practice and in the development of new systemic compounds.

For these studies different types of systemic fungicides and various fungi have been used. Information was gathered about the potential of fungi to acquire resistance by studying the mutation frequency and the degree of resistance to the different compounds in the various fungi (chapter 3).

Pathogenicity is for a fungal plant pathogen the most characteristic property with which it can compete (or not) in a population. Pathogenicity tests were carried out to check whether the resistant strains, obtained after mutagenic treatment of a pathogenic wild-type fungus, were still pathogenic. Moreover, in cases where pathogenicity was retained resistance in vitro was compared with resistance in vivo (chapter 4).

Characteristics of the resistant strains such as dosage response relationships in fungicide resistance, patterns of cross-resistance and the influence of exogenous factors on toxicity are indispensable data both for genetic studies and for biochemical studies on the mode of action of a fungicide (chapter 5).

In studying the genetics of fungicide resistance it is important to work with a genetically well-defined fungus, which is easy to handle. *Aspergillus nidulans*, a non-pathogen has been chosen. Crossing experiments can show whether a single gene is responsible for a certain character. With this fungus it is possible to locate genes to-, and to localize them on anyone of the eight linkage groups (chromosomes). Further dominance or recessiveness of genes can be determined in a heterozygous diploid. Often more than one gene is involved in the resistant mutants investigated. In that case more than one mechanism of resistance might play a role. Here, it should be investigated whether the combined genes cause an additive effect, so that the level of resistance is increased (chapter 6).

Many pathogenic fungi belong to the group of Fungi Imperfecti. With these fungi no genetic analysis can be done by crossing. But since the parasexual cycle has been discovered as an alternative genetic system, genetic information can still be gathered. This sort of genetic analysis was carried out here with a fungus for which a parasexual cycle is known viz., *Aspergillus niger*, and another fungus used in many phytopathological studies *Cladosporium cucumerinum* for which it is not known (chapter 7).

2. MATERIALS AND METHODS

2.1. PLANTS

Cucumber seeds (*Cucumis sativus* L.) cv 'Lange Gele Tros' were sown in pots with sterilized sand at 28°C. After three days the pots were placed in a climate room, at 17–18°C with a 12 hours light regime (Philips TL 40 W/33 RS). After seven to ten days when the cotyledons had developed fully but no leaves had been formed yet, the seedlings were placed in beakers with 50 ml Hoagland solution and used for the experiments.

Seeds of maize (*Zea mays* L.) cvs. 'Caldera' and 'Golden Bantam' were germinated on wet filter papers at 30°C for 72 hours. After inoculation, the seedlings were planted in pots with sterilized sand supplemented with a mineral salt solution and incubated in a climate cabinet (Weiss; volume 0.3 m^3) at 28°C, 90% RH and continuous illumination (Philips HPL 400 W).

Rye (*Secale cereale* L.) seedlings cv 'Dominant' were grown in glass tubes (diameter 3.5 cm, 20 cm long) with sterilized sand containing Hoagland solution at 17°C. When the plants (3–4 per tube) were 8–10 cm long, they were used for the experiments and incubated in a Weiss climate cabinet at 5°C and 90% RH, with 12 hours light/day.

Carnation plants (*Dianthus caryophyllus* L.) cv 'William Sim', one well-rooted cutting per pot, were grown in steamed soil in a greenhouse at 18–20°C.

2.2. FUNGI

Strains of the following fungi were kindly provided by Mr. G. J. Bollen and Mr. E. P. van der Hoeven, Laboratory of Phytopathology, Wageningen, The Netherlands. *Aspergillus niger* v. Tieghem (from hyacinth bulbs): *Aureobasidium bolleyi* (Sprague) v. Arx (from roots of rye); *Cercosporella herpotrichoides* Fron (from stubble of rye); *Fusarium nivale* (Fr) Ces. (from stubble of rye); *Penicillium expansum* Link ex. Thorn (from greenhouse soil) and *Phialophora cinerescens* (Wollenw.) Van Beyma (from roots of carnation). The strain of *Cladosporium cucumerinum* Ellis & Arth. is the one in common use at this laboratory.

A strain of *Rhodotorula rubra* (Demme) was obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands.

Two compatible strains of *Ustilago maydis* (DC) Corda, viz. 213 with an adenine (*ad1*) and 220 with a methionine (*me15*) and a pantothenic acid (*pan1*) requirement, were kindly provided by Dr. S. G. Georgopoulos, Nuclear Research Center 'Demokritos', Athens, Greece.

The *Aspergillus nidulans* Eidam (Wint.) strains, listed in Table 1, were generously supplied by Dr. H. N. Arst, University of Cambridge, Department of

Genetics, Cambridge, England (strains No. 21–26); Dr. B. W. Bainbridge, Queen Elisabeth College, University of London, London, England (strain No. 20); Mr. C. J. Bos, Agricultural University, Department of Genetics, Wageningen, The Netherlands (strains No. 1–3); Dr. A. J. Clutterbuck, University of Glasgow, Institute of Genetics, Glasgow, Scotland (strains No. 4–7, 13–17) (CLUTTERBUCK, 1969); Dr. A. C. Hastie, University of Dundee, Department of Biological Sciences, Dundee, Scotland (strains No. 18–19) and Dr. W. N.

TABLE 1. List of strains of *Aspergillus nidulans*.

Number	Genotype*	Depositor**
1	*suA1adE20, yA2, adE20; acrA1; phenA2; pyroA4; lysB5; nicB8*	B1
2	*suA1adE20, yA2, adE20; acrA1; sB3; riboB2*	B2
3	*biA1; acrA1*	B3
4	*ornB7, fwA1*	G 81
5	*yA2; cnxB11, fpaD43*	G 814
6	*pabaA1; galC7, facB101, riboB2, palB7, chaA1*	G 823
7	*pabaA1; wA2; tsD15, ornB7, galC7, facB101, riboB2*	G 87
8	*biA1; benA10, fwA1*	T
9	*biA1; wA2; ornB7*	T
10	*biA1; wA2; carA1*	T
11	*suA1adE20, yA2, adE20; phenA2; pyroA4; lysB5; nicB8; benA10, fwA1, carB2*	T T
12	*biA1; benB29; phenA2; lysB5*	T
13	*suA1adE20, adE20, biA1; wA2; choA1; chaA1*	G 78
14	*phenB6, pantoB100, methE6*	G 710
15	*yA2; malA1, wetA6, nicB8, palD8*	G 712
16	*yA2; adD3; sA1*	G 315
17	*proA1, biA1; wA3, acrB2*	G 221
18	*proA1, pabaA1, yA2; wA3*	H
19	*proA1, pabaA1, yA2; wA3; benA1*	H 3A
20	*proA1, pabaA1, yA2, biA1; actA1*	BWB 229
21	*yA2; camB105; pyroA4; cnxC3*	A
22	*yA2, camC108; pyroA4; cnxC3*	A
23	*yA2; pyroA4; camD27; cnxC3*	A
24	*yA2, adE20; phenA2, cbxA17*	A
25	*pabaA1; cbxB28*	A
26	*pabaA1; cbxC34*	A
27	*biA1; acrA1, wA3, abA1, cnxE16, adD3*	FGSC 254
28	*nicA2, hxA1, riboD5*	FGSC 258
29	*pabaA1, yA2; oliC3*	FGSC 450
30	*acrA1; lysB5, pA2, riboD5*	FGSC 504
31	*riboA1, yA2, adE20; methG1, suC1adE20, palC4, pabaB22, pyroA4*	FGSC 517

* Symbols separated by a semi-colon mean that genes are on different chromosomes.
** Providers (cf. 2.2.) and their stock number.

A = H. N. Arst
B = C. J. Bos
BWB = B. W. Bainbridge
G = A. J. Clutterbuck
H = A. C. Hastie
FGSC = Fungal Genetics Stock Center
T = The author

TABLE 2. List of mutations of *Aspergillus nidulans*.

Mutation	Linkage group	Phenotype*
abA1	II	α-amino butyric acid (= isoleucine) req.
acrA1	II	acriflavin res.
acrB2	II	acriflavin res.
actA1	III	cycloheximide res.
adD3	II	adenine req.
adE20	I	adenine req.
benA1 = benA10	VIII	benomyl res.
benB29	II	benomyl res.
biA1	I	biotin req.
camB105	II	chloramphenicol res.
camC108	I	chloramphenicol res.
camD27	V	chloramphenicol res.
carA1 = cbxB28	VII	carboxin res.
carB2 = cbxC34	VIII	carboxin res.
carC9 = cbxA17	III	carboxin res.
chaA1	VIII	chartreuse conidia
choA1	VII	choline req.
cnxB11 = cnxC3	VIII	nitrate and hypoxanthine non-ut
cnxE16	II	nitrate and hypoxanthine non-ut
facB101	VIII	acetate non-ut, and fluoroacetate res.
fpaD43	VIII	p-fluorophenylalanine res.
fwA1	VIII	fawn conidia
galC7	VIII	galactose non-ut
hxA1	V	hypoxanthine non-ut
lysB5	V	lysine req.
malA1	VII	maltose non-ut
methE6	VII	methionine req.
methG1	IV	methionine req.
nicA2	V	nicotinic acid req.
nicB8	VII	nicotinic acid req.
oliC3	VII	oligomycin res.
ornB7	VIII	ornithine req.
pA2	V	pale conidia
pabaA1	I	p-aminobenzoic acid req.
pabaB22	IV	p-aminobenzoic acid req.
palB7	VIII	alkaline phosphatase deficient
palC4	IV	alkaline phosphatase deficient
palD8	VII	alkaline phosphatase deficient
pantoB100	VII	pantothenic acid req.
phenA2	III	phenylalanine req.
phenB6	VII	phenylalanine req.
proA1	I	proline req.
pyroA4	IV	pyrodoxine req.
riboA1	I	riboflavin req.
riboB2	VIII	riboflavin req.
riboD5	V	riboflavin req.
sA1	III	sulphate non-ut
sB3	VI	sulphate non-ut
suA1adE20	I	suppressor of *adE20*
suC1adE20	IV	suppressor of *adE20*
tsD15	VIII	temperature sensitive
wA2 = wA3	II	white conidia
wetA6	VII	wet-white conidia
yA2	I	yellow conidia

* req. = requiring. res. = resistant. ut. = utilizing. for further explanation s. 2.5.4..

Ogata, Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, California, U.S.A. (strains No. 27–31) (BARRATT et al., 1975). The nomenclature of the locus symbols is according to the proposals made by CLUTTERBUCK (1973, 1974). This means that since then newly described mutations are to be abbreviated by three letters followed by a capital locus-specific letter and the mutant number. Table 2 lists the mutations used with the designation of the linkage group and description of the phenotype (cf. 2.5.4.). Symbols separated by a semi-colon mean that the genes are on different chromosomes.

2.3. MEDIA

The Hoagland nutrient solution used for the hydroponic culture of cucumber seedlings contained per litre: $Ca(NO_3)_2$ 590 mg; KNO_3 257 mg; $MgSO_4.7H_2O$ 246 mg; KH_2PO_4 68 mg; iron sodium salt of sequestrene 30 mg; H_3BO_3 0.61 mg; $MnCl_2.4H_2O$ 0.39 mg; $CuSO_4.5H_2O$ 0.055 mg; $ZnSO_4.7H_2O$ 0.055 mg; $Na_2MoO_4.2H_2O$ 0.028 mg; KI 0.028 mg; KBr 0.028 mg.

The mineral salt solution used to drench sand grown maize seedlings contained per litre: $NaNO_3$ 200 mg; Na_2HPO_4 20 mg; $MgSO_4.7H_2O$ 20 mg; $Fe_3(PO_4)_2$ 5 mg.

In all experiments 'Oxoid' malt extract agar (Oxoid CM 59) was used as a solid complete medium (CM). The formula per litre is: malt extract (Oxoid L 39) 30 g; mycological peptone (Oxoid L 40) 5 g; agar No. 1 (Oxoid L11) 15 g; pH 5.4. All auxotrophic mutants (Table 2) grew very well on this medium, except the *A. nidulans pabaA1* mutant needed more p-aminobenzoic acid for good sporulation. To obtain a more solid CM for plating and spore harvesting 5 grams extra of agar were added per litre of this medium.

The minimal medium (MM) used in genetic experiments with *A. nidulans* and *A. niger* was that described by PONTECORVO et al. (1953b) and contained: $NaNO_3$ 6.0 g; $MgSO_4.7H_2O$ 0.52 g; KCl 0.52 g; KH_2PO_4 1.52 g; glucose 10 g; traces $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$; distilled water to 1 l: pH adjusted to 6.5 with KOH. For solid MM 20 g 'Oxoid' agar No. 3 per litre was added. To test particular metabolic mutants other carbon, nitrogen and sulphur sources were used. In the MM glucose was replaced by acetate, galactose or maltose; nitrate by ammonium or hypoxanthine and sulphate by sulphite or thiosulphate, respectively (cf. Table 4). In some fungitoxicity tests glucose was replaced by acetate, citrate, fumarate, malate or succinate as the carbon source. With substitution, the total molar quantity of carbon was remained unchanged.

Czapek Dox (Oxoid CM 97) was used as MM in experiments with *C. cucumerinum*, containing per litre $NaNO_3$ 2.0 g; magnesiumglycerophosphate 0.59 g; KCl 0.5 g; K_2SO_4 0.35 g; $FeSO_4.7H_2O$ 0.01 g; sucrose 20.0 g; agar No. 3 (Oxoid L 3) 12.0 g; pH 6.8.

The MM described by HOLLIDAY (1961a) and employed for *U. maydis* contained per litre: NH_4NO_3 3.0 g; KH_2PO_4 2.0 g; Na_2SO_4 0.5 g: KCl 1.0 g;

$MgSO_4.7H_2O$ 0.25 g; $CaCl_2.6H_2O$ 0.2 g; glucose 10 g; trace element solution 4 ml and pH 5.8. The trace element solution contained per litre: H_3BO_3 30 mg; $MnCl_2.4H_2O$ 70 mg; $ZnCl_2$ 200 mg; $CuSO_4.5H_2O$ 200 mg; $FeCl_3.6H_2O$ 50 mg; $Na_2MoO_4.2H_2O$ 20 mg.

To obtain supplemental medium (SM) for growth of auxotrophs the minimal media were supplemented per litre with the respective growth factors; amino acids 200 mg (alanine, arginine, aspartic acid, cysteïne, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan and valine), purines (adenine, guanine) and pyrimidines (cytosine, thyminę, uracil) 50 mg; biotin 0.2 mg; choline 2 mg; folic acid 1 mg; inositol 20 mg; nicotinic acid 1 mg; p-aminobenzoic acid 1 mg; Ca-pantothenate 1 mg; pyrodoxine 0.5 mg; riboflavin 1 mg; thiamine 1 mg.

2.4. Chemicals

Chemicals were obtained from several companies the names of which have been abbreviated in the list as given below: B : Boehringer, Mannheim GmbH, Germany; C : Calbiochem A.G., San Diego, Calif., U.S.A.; F : Fluka A.G., Buchs S.G., Switzerland; G : G.T. Gurr Ltd., London, England; H : Hoffmann – LaRoche, Basel, Switzerland; M : Merck, Darmstadt, Germany; GB : Gist-Brocades N.V., Mycofarm, Delft, The Netherlands; O : Institute for Organic Chemistry TNO, Utrecht, The Netherlands; SM : Schuchardt, München, Germany; S : Sigma Chemical Co., St. Louis, Mo., U.S.A.; P : Pfizer & Co., New York, N.Y., U.S.A..

All nutrients mentioned under Media (2.3.) were of an analytical grade (H, M, S): acriflavin (2,8-diamino-10-methyl-acridinium chloride) (F); antimycin-A (C); bacitracin (C); chloramphenicol (B); cycloheximide (β-[2(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide (S); diazoblue (di-orthoanisidine, tetrazotized) (G); DL-p-fluorophenylalanine (F); Giemsa solution (M); griseofulvin (S); neomycin sulphate (B grade) (C); N-methyl-N'-nitro-N-nitrosoguanidine (F); oligomycin (P); 3-phenylindole (O); quintozene (90-96% pentachloronitrobenzene) (SM); vendarcin (GB); violet salt B (Na-α-naphthyl phosphate) (S); streptomycin sulphate (GB).

The chemical name and formulation of the following fungicides are listed in Table 3. Benomyl (technical), Benlate and chloroneb were kindly provided by E. I. Dupont de Nemours & Co., Wilmington, Del., U.S.A.. Carboxin and oxycarboxin were manufactured by Uniroyal, US Rubber Chemical Division, Naugatuck, Conn., U.S.A.. Fenarimol was generously provided by Eli Lilly Company, Indianapolis, Ill., U.S.A.; imazalil by Janssen Pharmaceutica, Beerse, Belgium; pimaricin by Gist-Brocades N.V., Mycofarm, Delft, The Netherlands and thiabendazole by Ligtermoet Chemie B.V., Rotterdam, The Netherlands.

TABLE 3. Name and formulation of the fungicides used.

Common name	Trade name or code	Formulation	Chemical name*
benomyl		technical	methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate
	Benlate	WP 50%**	
carboxin	Vitavax	WP 75%	5,6-dihydro-2-methyl-1,4 oxathiin-3-carboxanilide
chloroneb	Demosan	WP 65%	1,4-dichloro-2,5 dimethoxybenzene
fenarimol	EL-222	technical	α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol
imazalil phosphate		technical	1-β-(alkyloxy)-2,4 chlorophenetyl-imidazole phosphate
oxycarboxin	Plantvax	WP 75%	5,6-dihydro-2-methyl-1,4 oxathiin-3-carboxanilide-4,4-dioxide
pimaricin	516-E-201	85%	
thiabendazole		90%	2-(4'-thiazolyl) benzimidazole

* Structural formulas s. Fig. 3.
** WP = wettable powder.

2.5. CULTURE METHODS

2.5.1. *Incubation and storage of cultures*

A. nidulans and *A. niger* were incubated at 35–37°C; *U. maydis* at 30°C; *C. cucumerinum*, *P. expansum* and *R. rubra* at 23°C and the other fungi mentioned under 2.2. at 20°C.

Stock cultures were maintained on CM agar slants and stored at 4°C except for *U. maydis*, which was maintained at 10°C and regularly subcultured. Stock cultures of all kinds of fungi and bacteria (PERKINS, 1962; HOLLIDAY, 1974; SHERMAN and LAURENCE, 1974; YOUNG and WILSON, 1974) are nowadays successfully preserved on silica gel. This method was therefore applied in this study. To this end, a loopful of a freshly sporulating culture was suspended in a drop of diluted milk and transferred to small tubes (10 cm long and 1 cm in diameter) with anhydrous sterile silica gel granules (Merck) ground to 1–5 mm size. The tubes were stored at a dry place at 4°C. To recover a culture a few granules with the dried spores were put on a CM agar plate. Stocks of *U. maydis* sporidia which on a slant survive for only a few months have been preserved up to ten years by this method (HOLLIDAY, 1974). A further advantage of this preservation method is that stocks can be recovered many times from the same tube.

2.5.2. *Spore plating and counting*

Spores were collected from well sporulating cultures and shaken in a mixer to break up spore chains. When necessary Tween-80 was added, without exceeding the toxic level of 10 μg/ml. Spore suspensions were counted in a Neubauer haemocytometer. To determine percentage of survival after mutagenic

treatment, treated as well as untreated spore suspensions were diluted and plated at two dilutions on CM agar. Colony counts provided the survival percentages (relative to the control).

2.5.3. *Method for mutant and phenotype classification*

In the determination of the phenotypes of isolated mutants (auxotrophic or resistant), in crossing analysis, diploid analysis and in fungitoxicity tests a number of colonies had to be tested on various media. For this reason a number of replicators each consisting of a metal plate at the size of a Petri-dish (diameter 9 cm) carrying 3, 5, 6, 7, 9, 10, 13, 21, 26, 37 and 50 needles were used. The required colonies were inoculated on to a CM 'master plate' in an array corresponding to the position of the needles on the multipin replicator. When after inoculation the colonies on the master plate were sporulating well, they were transferred with the replicator to various test media. Fig. 1 gives a picture of a replica, with all the different replicators, of *A. nidulans* strain No. 3 after two days of incubation. One needle of the replicator was found to be able to transfer as many as 5000 conidia of *A. nidulans* from the master plate to a replica. This method appeared to be applicable for all fungi used in this study.

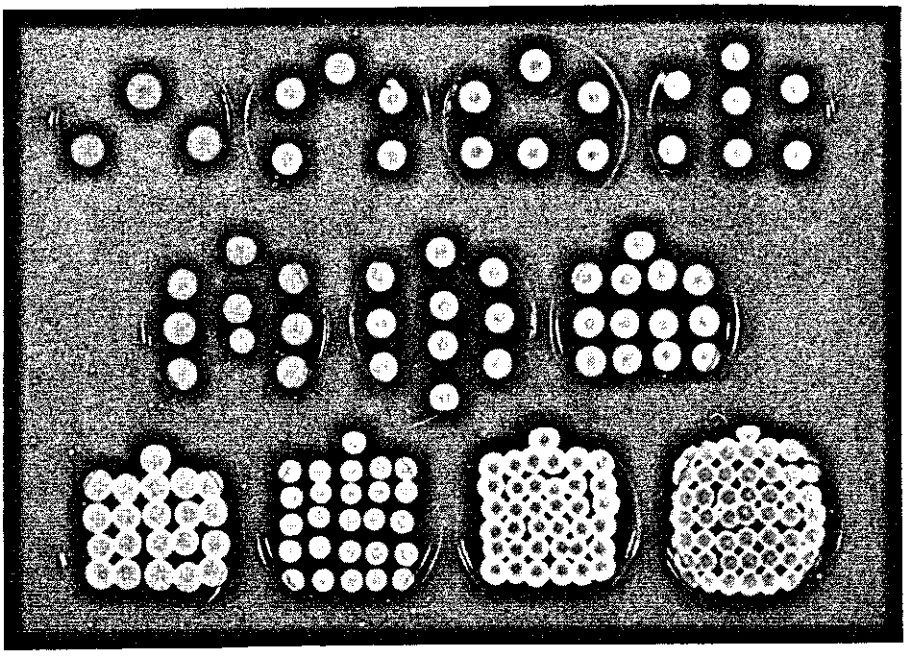


FIG. 1. The replica method; using different multipin replicators a variable number of colonies was tested by replication from a master plate, as shown for *Aspergillus nidulans* strain No. 3 two days after incubation.

2.5.4. *Phenotype testing of the Aspergillus nidulans mutants*

Table 2 lists the mutations of *A. nidulans* used in this study. The phenotypes are indicated as 'requiring', 'non-utilizing', 'deficient', 'resistant' and 'sensitive', terms which will be explained below.

For growth of requiring mutants the MM was supplemented with respective growth factors (2.3., Media); to test a requirement the relevant growth factor was omitted. In the case of the non-utilizing mutants a particular carbon, nitrogen or sulphur source cannot be used for growth of the mutant. Thus, to test the mutant for the deficiency the non-utilized source was used; however, for growth another source as listed in Table 4 was necessary.

To test the alkaline phosphatase-deficient mutants (DORN, 1965) which have a reduced phosphatase activity in an alkaline milieu, these mutants were grown on SM without KH_2PO_4 for one day. Then, the mycelium was stained by pouring per plate a solution of 4 ml 0.1 M Tris-buffer pH 8.3 containing 2 mg Na-α-naphthyl phosphate and 20 mg violet salt B; within 10 min in the mycelium of strains without the *pal*-mutation a redish-brown precipitate was formed, indicating a phosphatase activity, which was absent in the case of the mutant.

The temperature sensitive mutant (*tsD15*) did not grow at 37 °C, but at 25 °C growth was similar to that of other mutants.

The suppressor mutations *suA1adE20* and *suC1adE20* suppress the effect of the adenine requirement *adE20*. The suppressor marker, only expressed when *adE20* is present, has often been used for the selection of haploids from a heterozygous diploid. A diploid homozygous for *adE20* and heterozygous for the suppressor will not grow on adenine-free medium, but segregant haploids carrying the suppressor can do so.

The resistant mutants were tested by adding the toxicant to CM or SM agar in the following concentrations: acriflavin 50 μg/ml; benomyl 2 μg/ml; chloramphenicol (in SM) 5 mg/ml (GUNATILLEKE et al., 1975b); carboxin 50 μg/ml; cycloheximide 1 mg/ml; p-fluorophenylalanine 125 μg/ml; oligomycin 2 μg/ml; neomycin (in SM) 0.5 and 2 mg/ml.

TABLE 4. Non-utilizing mutants in *Aspergillus nidulans*.

Locus symbol of mutations	Non-utilized source	Utilized source
cnx	nitrate, hypoxanthine	ammonium
hx	hypoxanthine	nitrate, ammonium
fac	acetate	glucose
gal	galactose	glucose
mal	maltose	glucose
s	sulphate	thiosulphate, sulphite

2.6. Mutation induction and characterization of mutants

2.6.1. *Mutation induction*

Mutants were obtained spontaneously as well as after mutagenic treatment by UV irradiation and N-methyl-N'-nitro-N-nitrosoguanidine (NG) treatment. Spore suspensions (10^6–10^8 spores/ml) of freshly grown cultures were used (s. 2.5.2.).The UV irradiation was carried out with a Hanovia chromatolite (2537 nm, 30 W) portable UV lamp. Eight small dishes (3 cm diameter; 1.5 cm high), containing 1.5 ml spore suspension and placed in direct contact with the lamp were irradiated simultaneously. After 10–60 min of irradiation the survival percentage varied from 5–50 depending on spore concentration and fungal species. NG is a very powerful mutagen and was applied in two ways. Spore suspensions were harvested in buffer and NG was added as a ten times concentrated solution in buffer to a final concentration of 100–500 μg/ml. The first procedure was carried out at pH 6.9 (0.05 M Sørensen-phosphate buffer); after 10–60 min incubation and regular shaking, the suspension was centrifuged, the pellets were resuspended and washed in sterile water and centrifuged again. The resulting spore suspension was used for the experiments. The second procedure described by Délic et al. (1970) and Martinelli and Clutterbuck (1971) was carried out at pH 9.0 (0.05 M Tris-maleic acid buffer) which causes inactivation of NG as it acts during the treatment. The spore suspension containing 100–500 μg/ml NG was incubated in a shaker for six hours after which the suspension could be used without centrifugation.

2.6.2. *Isolation of resistant mutants*

To obtain resistant mutants a spore suspension, either treated or not, of the sensitive fungus was plated on CM containing the toxicant in a concentration which inhibited the wild-type strain completely. When the toxicant is not completely inhibitory at any concentration the so-called 'sandwich' method was used. With the sandwich method the spore suspension was spread between two layers of CM containing the toxicant. After incubation for at most twice the period necessary to obtain a sporulating colony on CM, the number of resistant colonies was counted and isolated. Mutation frequencies were calculated from the number of resistant mutants and the number of spores that had survived the mutagenic treatment and expressed as number per ten million surviving spores.

2.6.3. *Isolation of auxotrophic mutants*

For the selection of auxotrophs in *A. niger* and *C. cucumerinum* different methods were used. The simplest procedure is the total analysis, according to which treated spores plated on CM at dilutions giving about 50 colonies per dish, were tested directly on MM supplemented with the nutrients required by the original strain. From inoculated points showing little or no growth after 1–2 days spores were isolated on CM. The identification of a new auxotroph is described in 2.6.4..

Some enrichment techniques were applied which were based on the principle that survival of auxotrophic mutants should be favoured above that of prototrophs. With the filtration technique treated spores were incubated in a liquid MM; when prototrophic spores had germinated the suspension was filtered through glasswool and the filtrate was incubated again. After repeating this procedure three or four times the suspension was plated out.

The starvation technique described by PONTECORVO et al. (1953b) makes use of the fact that a vitamin-requiring mutant when incubated on MM dies much faster than a mutant with an amino acid requirement. According to this method, treated spores of a vitamin-requiring strain were plated on solid MM and after five or more days of incubation a layer of CM was poured over the MM. By that time, 99% or more of the spores of the original strain have been killed and auxotrophic strains can grow out. This method can give a percentage of auxotrophics of more than 70, that means an enrichment of 70 compared with a maximum of 1% using the total analysis. A disadvantage of this method is that most mutants isolated are of the same type.

The last technique applied, makes use of toxicants which exclusively kill growing spores as described by DITCHBURN and MACDONALD (1971) using nystatin and BAL et al. (1974, 1975) using N-glycosyl-polyfungin. Since both compounds are polyene antibiotics like pimaricin, in this study the latter antibiotic was used. Incubation of treated spores took place in liquid medium containing pimaricin; at different times the spores were centrifuged and plated on CM.

2.6.4. *Identification of new auxotrophic mutants*

To identify an unknown auxotroph a method (HOLLIDAY, 1956) was used with which 36 growth factors can easily be tested at the same time. Of twelve stock solutions divided in two groups of six each containing six different nutrients as given in Table 5, two solutions one from each group contained one growth factor in common.

To establish the requirement of a mutant, a spore suspension was plated on solid SM (supplemented only with the required nutrients); on the surface of the agar plate 12 filter paper discs were placed, each of which contained one

TABLE 5. Determination of a new auxotrophic mutant, for explanation s. text.

	1	2	3	4	5	6
7	adenine	biotin	phenylalanine	alanine	arginine	leucine
8	hypoxanthine	folic acid	serine	cysteïne	ornithine	glycine
9	cytosine	pantothenic acid	tryptophan	threonine	aspartic acid	isoleucine
10	guanine	pyrodoxine	tyrosine	thiosulphate	proline	histidine
11	thymine	thiamine	p-aminobenzoic acid	methionine	glutamic acid	lysine
12	uracil	riboflavin	nicotinic acid	choline	inositol	valine

of the twelve stock solutions. When after incubation growth appeared around two discs the data of Table 5 disclosed which one was the required growth factor, this being the compound present in both of the discs. Many new mutants were recognized simply by using twelve solid SM plates each containing the six nutrients corresponding to one of the twelve solutions. Colonies were transferred to these plates with a multipin replicator.

2.6.5. *Fungitoxicity tests on agar media*

Sensitivity and resistance of fungal strains to various toxicants were usually determined by measuring radial growth on agar in the following way. Chemicals were added as a suspension or solution in acetone, methanol or water to molten agar medium. The final acetone or methanol concentration in the medium, identical in control and treatment, never exceeded 1%. Carboxin, oxycarboxin and thiabendazole were dissolved in methanol; benomyl, chloroneb, fenarimol and oligomycin in acetone and the other chemicals in water. Vendarcin (50 μg/ml) was added to the medium to suppress bacterial growth. Inoculation took place using the replica method (2.5.3.) according to which a maximum of nine strains were tested at the same time on series of concentrations of the toxicants, always at least in duplicate. Radial growth was measured after two to ten days of incubation depending on the fungal species and the number of strains to be tested at the same time. Radial growth on different concentrations of the fungicide was expressed as a percentage of the control without fungicide. The data were plotted in graphs; the concentration on a logarithmic scale on the ordinate and the growth as percentage of the control on a linear scale on the abscissa. Relative toxicity was expressed as percentage of growth in presence of the toxicant or more specifically as ED_{50}, that is the concentration causing 50% reduction in radial growth and as MIC, that is the minimal inhibitory concentration. ED_{50} and MIC were determined using the dosage response curves.

2.6.6. *Fungitoxicity tests in liquid medium with Ustilago maydis*

For the assessment of the fungitoxicity of chemicals to fungi with a yeast-like growth, tests in liquid medium were carried out as described by GEORGOPOULOS and SISLER (1970) for *U. maydis*. With this method the effect of a toxicant on growth was determined by measuring optical density (OD) changes with a Klett-Summerson colorimeter. The OD-value is linearly related with the number of sporidia and the dry weight. These experiments were carried out in test tubes, containing 7.5 ml supplemented liquid medium. Glucose and $MgSO_4.7H_2O$ were added to the test tubes only after autoclaving to prevent discoloration of the medium. A series of concentrations of the fungicide was made in the tubes. The strains of *U. maydis* to be tested were subcultured in liquid medium in a New Brunswick shaker (175 rev/min) at 30°C and the sporidia filtered before inoculation. The tubes were inoculated with the appropriate strain to a fixed initial OD. The cultures were shaken in a waterbath at 30°C and the OD-changes were recorded at intervals. The amount of growth

was estimated from the OD-increase as percentage of control, during an incubation period of 36–48 hours.

2.7. Methods of genetic analysis in *Aspergillus nidulans* and *Aspergillus niger*

The general genetic methods for *A. nidulans* and *A. niger* have been described by Pontecorvo et al. (1953b) and Lhoas (1967), respectively. Genetic analysis of *A. nidulans* can be carried out by means of the sexual cycle (2.7.4.) and the parasexual cycle. The parasexual cycle, consisting of heterokaryon formation, fusion to a heterozygous diploid (2.7.1.) and haploidization (2.7.2.), has also proved to be applicable to many imperfect fungi, like *A. niger*.

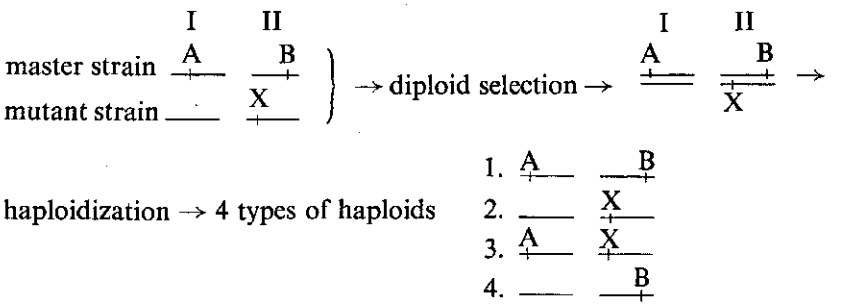
2.7.1. *Heterokaryon formation and diploid selection*

Heterokaryons of *A. nidulans* and *A. niger* can readily be formed when forced by balanced auxotrophies in the parents. To this end a mixture of conidia of two parent strains with different auxotrophic and conidial-color markers were inoculated on solid CM and after one day small pieces of the mixed mycelium were transferred to solid MM (three pieces per dish). In general, after 2–5 days heterokaryons were formed, however, this may be mimicked by cross-feeding. Sometimes it happens that the nuclei of the parents in a heterokaryon fuse to form a diploid nucleus. Since the conidia of *A. nidulans* and *A. niger* are uninucleate, heterokaryons will produce only (haploid) conidia like one of the two parent strains, being also auxotrophic. Therefore, diploids can be isolated from a heterokaryon by plating a conidial suspension of a heterokaryon between two layers of MM. Diploids are estimated to occur at a rate of one in 10^7 conidia in *A. nidulans* and of one in 10^6 in *A. niger*. A diploid is recognized by growth on MM, conidial color (two parent strains each with one different conidial-color marker will give a wild-type colored diploid) and the size of the conidia (2.9.).

2.7.2. *Diploid analysis*

In *A. nidulans* a new mutation is located to a linkage group by means of the parasexual cycle. For making the appropriate diploids, master strains have been developed (McCully and Forbes, 1965), which have markers on each of the eight linkage groups. Diploids can be completely haploidized by inoculating diploid conidia on CM containing either p-fluorophenylalanine (Lhoas, 1961, 1967), arsenate (Van Arkel, 1963), Benlate (Hastie, 1970), griseofulvin (Kappas and Georgopoulos, 1974), chloroneb (Azevedo and Pacheco Santana, 1975) or other agents. Throughout this study benomyl was used as the haploidizing agent, since it gave the best results compared with the other agents mentioned. Three to four days after inoculating a diploid on CM plates containing 0.5 or 1 μg/ml benomyl with either no or one benomyl-resistance marker present in one of the parent strains of the diploid, haploid

segregants could be isolated. The master strains used were strains No. 1, 2 and 11 of Table 1. Since crossing-over and haploidization do not normally occur at the same time, linkage groups segregate as units and a new marker will be found almost totally linked to one of the master-strain markers or rather to his wild-type allele. Thérefore, only small numbers of haploids were needed to locate a mutant. An example of this principle is given in the diagram below, where it is shown with two linkage groups (chromosomes) I and II, at which a master strain carries two markers A and B; a mutant strain with an unknown marker X:



Conclusion: X is located to chromosome II, because it is never associated with the B marker.

Linkage between any two of the master-strain markers themselves indicate a translocation in the tested strain (KÄFER, 1962, 1965). After the location of a new mutation to a linkage group, detailed meiotic mapping by cleistothecium analysis (2.7.4.)is needed to exactly localize the mutation from linkages with known markers. From *A. niger* no master strains are available, but here a new marker can also be found linked with a known marker; however, crossing-over and haploidization do occur more frequently here simultaneously than in *A. nidulans* (LHOAS, 1967).

2.7.3. *Dominance tests*

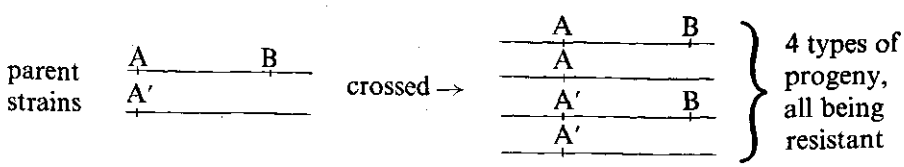
Using the heterozygous diploids, synthesized to carry out the diploid analysis (2.7.2.),the expression of mutations to resistance in diploids was determined. Dosage response curves of the diploids were made (2.6.5.) and compared with those of the haploid resistant strains. Using these it was established whether the mutant was dominant, recessive or intermediate.

It should be noted that complementation tests could not be successfully carried out, because mutations to resistance generally show a more or less intermediate character in a heterozygous diploid.

2.7.4. *Cleistothetium analysis*

A. nidulans is a homothallic fungus with a perfect state, *Emericella nidulans*

(Eidam.) Wint. Cleistothecia can be selfed or hybrid. To recognize hybrid cleistothecia two parent strains were usually chosen with different auxotrophic and spore-color markers. Besides crosses made on MM with different auxotrophs on a thick agar layer and with a certain anaerobiosis favoured the formation of hybrid cleistothecia. Crosses were often made by mixing conidia of the two parent strains on a thick CM agar layer in a plate sealed with cellulose adhesive tape. Cleistothecia were then mature in 6–8 days. When crosses were made with pieces of mixed mycelium in the center of a dish with a thick MM agar layer, cleistothecia needed 10–14 days to mature, with a higher chance to get hybrid cleistothecia. Always single cleistothecium analysis was carried out. Cleistothecia were carefully cleaned from adhering conidia and Hülle-cells by rolling them on 3% water agar and then crushed against the wall of a tube, which contained 2 ml sterile water. The dark-red ascospores do not cluster if the cleistothecium is mature. A streak of every rolled cleistothecium was made (eight on one CM plate) to determine whether the cleistothecium was hybrid or selfed. Then, platings were made at dilutions giving about 50 colonies per dish. The progeny of a cross was classified using a 21 or 26 needle replicator (2.5.3.). For meiotic analysis at least 200 colonies from every cross were tested. To test allelism between two different resistant strains usually 100 colonies of a cross of these mutants were examined. When all the 100 tested colonies appeared to be resistant, there was assumed to be allelism. This principle is shown in the diagram below, where two allelic mutations to resistance called A and A′ and another mutation B on the same linkage group recombine.



2.8. Methods of genetic analysis in *Ustilago maydis*

The genetic techniques generally used with *U. maydis* have been described by Holliday (1961a). The haploid phase of *U. maydis* consists of uninucleate sporidia which divide by yeast-like budding to form compact colonies on artificial media. *U. maydis* is heterothallic, so inoculation of haploid strains of opposite mating type into the host, viz. young seedlings of *Zea mays*, results in the formation of an infective dikaryon within the host tissue.

To make crosses, sporidial suspensions were obtained from cultures in liquid medium (2.6.6.). An overnight culture generally contained 5.10^7 sporidia/ml.

Suspensions of the two compatible strains were mixed before inoculation. About 1 mm of the tip of the coleoptile of each maize seedling was removed and the mixed sporidial suspension was inoculated, using a Calibra hypodermic syringe of 2 ml capacity, just above the first node until a droplet of suspension emerged from the cut end of the coleoptile. For every cross at least 20 seedlings were inoculated and incubated in a Weiss climate cabinet (2.1.). Galls generally appeared on leaves and stems 5 days after inoculation. Brandspores were formed between 10 and 14 days after inoculation but usually they were produced by only a few galls. Galls containing brandspores were ground in a small mortar to which gradually 5–10 ml 1.5% copper sulphate solution was added. The suspension was filtered through cotton wool and to kill contaminants and vegetative cells, the suspension was left in the copper sulphate solution at room temperature for 18 hours and was then centrifuged. The brandspores were suspended in sterile water and plated on solid CM. Since every brandspore (diploid) produces four basidiospores (haploid) which do not develop simultaneously, the colonies originated from the plated brandspores were harvested as soon as they became just visible after about 36 hours. The suspension so obtained was well mixed to get single sporidia which were plated at a concentration to give 100–200 colonies per dish. After 3 days of incubation the colonies were inoculated on to master plates using the 37 needle replicator and another 3 days later these plates were replicated to the appropriate test media (2.5.3.).

2.9. Cytological observations

Spore size has frequently been used to distinguish diploid from haploid strains (Pontecorvo et al., 1953b; Lhoas, 1967). Spore measurements were carried out with a haemocytometer using a measuring-ocular. *A. nidulans* and *A. niger* which have round conidia, could be measured in chains of three conidia. The spores of *C. cucumerinum* on the other hand, having an oblong shape, showed a considerable variation in size. According to Clutterbuck and Roper (1966) who showed that haploid and diploid strains of *A. nidulans* differed in nuclear size, measurements of nuclear size were made using the staining method described by Robinov and Caten (1969). Therefore conidia were grown on sheets of dialysis tubing, placed on the surface of solid CM. After incubation for 1–2 days the sheets were removed and fixed in a mixture of acetic acid and ethanol 1:3 for 10 min and washed successively in 75, 50 and 25% aqueous ethanol for one min each, then left in 1 N HCl for 20 min at room temperature followed by 1 N HCl at 60°C for 10 min. After washing with distilled water the material was stained for at least one hour with Giemsa (Giemsa solution diluted in 0.1 M Sørensen-phosphate buffer pH 7.0 1:25) and then washed in buffer and observed.

2.10. Determination of DNA and RNA content of conida of *Cladosporium cucumerinum*

A double DNA content of nuclei is a good proof of having isolated a diploid strain. The method used has been described by Davidse (1973). Spore suspensions of both types of strains were made, concentrated to give a total of about 4.10^9 conidia and washed twice with distilled water. The conidia were incubated in 5 ml ice-cold 0.2 N perchloric acid (PCA) for 10 min (to remove low molecular weight compounds) and centrifuged. This process was repeated twice. The pellet was resuspended in 4 ml 0.3 N KOH and kept overnight at 37°C to hydrolyze RNA. To precipitate DNA 0.6 ml 3.75 N PCA was added to the suspension cooled in an ice-bath. After centrifugation the pellet was washed twice with 4 ml ice-cold 0.2 N PCA. The supernatant and the combined washings were considered to be the RNA fraction. The RNA content was determined by the orcinol method (Munro and Fleck, 1966) with yeast RNA as a standard.

The pellet remaining after extracting RNA was treated with 1 ml 1.5 N PCA for 20 min at 70°C to extract DNA. After centrifugation the pellet was extracted again. The supernatants were combined and made up to 3 ml with 1.5 N PCA. This fraction was considered to be the DNA fraction; the DNA content was determined with the diphenylamine method of Burton (1956) as modified by Giles and Myers (1965) with calf thymus DNA being the standard.

The number of nuclei in the conidial suspensions was determined after staining the conidia with Giemsa as described under 2.9..

2.11. Pathogenicity and in vivo fungitoxicity tests

2.11.1. *Application of the fungicide*

The effect of fungicides against plant pathogens was tested by root application. After inoculation of plants, fungicides were either applied to the liquid nutrient medium or by soildrenching.

Fungitoxicity tests to *P. expansum* on apple were carried out by dipping the apples in a fungicide solution for 1 min before inoculation.

2.11.2. *Re-isolation of fungal test strains from diseased host plants*

At the end of the pathogenicity and fungitoxicity tests, re-isolation of the inoculated strain was usually attempted. Pieces of hypocotyl, stem or leaf were surface-disinfected with 0.3–1 % hypochlorite solution for several minutes washed in sterile water and transferred to solid CM containing 50 μg/ml vendarcin. After incubation and isolation colonies were tested according to the phenotype of the inoculated strain (degree of resistance, auxotrophy).

2.11.3. *Cladosporium cucumerinum*

Growth of cucumber seedlings, cv 'Lange Gele Tros', is described in 2.1.. In pathogenicity tests at least six seedlings were inoculated with a conidial suspension of the appropriate strain of *C. cucumerinum* by means of a De Vilbiss sprayer. After incubation in a moist environment at 18°C for 5 to 7 days the hypocotyl showed disease symptoms. Disease index of every plant was estimated after seven days as described by VAN ANDEL (1958). The index was graded in a scale from 0–6, in which 0 means healthy and 6 heavily diseased; averaged for the six test plants and expressed as a percentage of that of control, which was inoculated with the wild-type strain. After determination of the disease index the fungal strains were re-isolated from the hypocotyls.

2.11.4. *Cercosporella herpotrichoides and Fusarium nivale*

C. herpotrichoides and *F. nivale*, both pathogens of rye, were tested in the same way on young rye plants, cv 'Dominant' (2.1.). Tubes with 3 or 4 seedlings were inoculated with three 5 mm agar discs of freshly grown mycelium. Every tested strain was treated in duplicate. After incubation for 5 weeks at 5°C and 90% RH final observations were made.

2.11.5. *Penicillium expansum*

The apple cultivar 'Cox Orange Pippin' was used as host for *P. expansum*. After harvesting the apples were stored at 4°C. Before inoculation apples were washed in sterile water and surface-sterilized with 70% ethanol. The apples were point-inoculated with a needle, which was first dipped in a sporulating colony of the strain to be tested, about 3 mm deep through the skin of the apple. To avoid errors due to physiological differences between apples strains to be compared were inoculated into the same apple. Incubation took place in a moist environment at room temperature (22°C). After 2 to 3 days the first growth of the fungus appeared; the final observation was made after eight days, when the apples showed a sporulating rot with a diameter of 25 mm.

2.11.6. *Phialophora cinerescens*

Carnation plants, cv 'William Sim', were used as a susceptible host of *P. cinerescens* (2.1.). Inoculation was carried out by immersing the carnation plants with their roots in a spore suspension (about 10^6 sp/ml) for some hours. After inoculation the plants were placed separately in pots and grown in the greenhouse at 20°C. After 7 weeks control plants were completely killed by the disease and the experiment was finished with re-isolaton of the various inoculated strains.

3. MUTATION FREQUENCY AND DEGREE OF RESISTANCE TO SYSTEMIC FUNGICIDES IN RELATION TO TYPE OF FUNGICIDE AND FUNGAL SPECIES

3.1. INTRODUCTION

Resistance of a fungal strain is here defined as a stable, inheritable property characterized by a reduced sensitivity to a toxicant as compared with the strain from which it has been derived. Resistance in a fungal population may arise through mutation either spontaneously or by mutagenic treatment. Whether a fungicide-resistant population will arise in the field depends on various factors. One of these is the potential of a fungus to acquire resistance to a certain fungicide. This potential can be studied in laboratory experiments using mutagenic agents.

Nutritional reversion and mutation to resistance provide the best selective, mutational systems. Reverse mutation in an auxotrophic mutant is easy to measure, since mutants of this type can efficiently be detected on MM. The average rate of spontaneous mutation per locus in *Neurospora crassa* has been determined with this method as to be about 10^{-8} (GILES, 1951). MORPUGO (1962) estimated forward mutation in *Aspergillus nidulans* with respect to resistance to 8-azaguanine and p-fluorophenylalanine. Another mutational system in *A. nidulans* is based on change in conidial pigmentation in the presence of 2-thioxanthine, which allows estimation of forward mutation to at least eight loci (ALDERSON and SCAZZOCCHIO, 1967).

In genetically well-defined fungi, the number of loci which may mutate to give resistance to a certain toxicant can be estimated indirectly. This method has been described by BALASSA (1969) for the quantitative survey of sporulation mutants of *Bacillus subtilis* and was adopted by MARTINELLI and CLUTTERBUCK (1971) for conidiation mutants of *A. nidulans*. The number of loci can be calculated by comparing the frequency of resistant mutants with the frequency of auxotrophic mutants obtained in the same induction experiment. The total number of auxotrophic loci in *A. nidulans* is known to be about 70. Using this method SRIVASTAVA and SINHA (1975) estimated a number of approximately 28 loci for p-fluorophenylalanine resistance in this fungus. The method, however, presupposes equal mutability of loci and equal probability of recovery of all kinds of mutants; often however, this assumption is incorrect.

With respect to differences in frequency of development of resistance in fungi, DEKKER (1974) mentioned various causative factors e.g. type of fungicide, type of fungus and type of disease. In this chapter a comparison is made between the frequency of development of resistant mutants on one hand and the degree of their resistance in relation to type of fungus and fungicide under comparable experimental conditions on the other hand.

3.2. MUTAGENIC TREATMENT AND SURVIVAL PERCENTAGE IN RELATION TO MUTATION FREQUENCY

For a better understanding of the data on mutation frequency to different fungicides and in different fungi, it is necessary to consider first the relation of mutation frequency to mutagenic treatment and survival percentage. The relation between mutation frequency and survival percentage has been studied

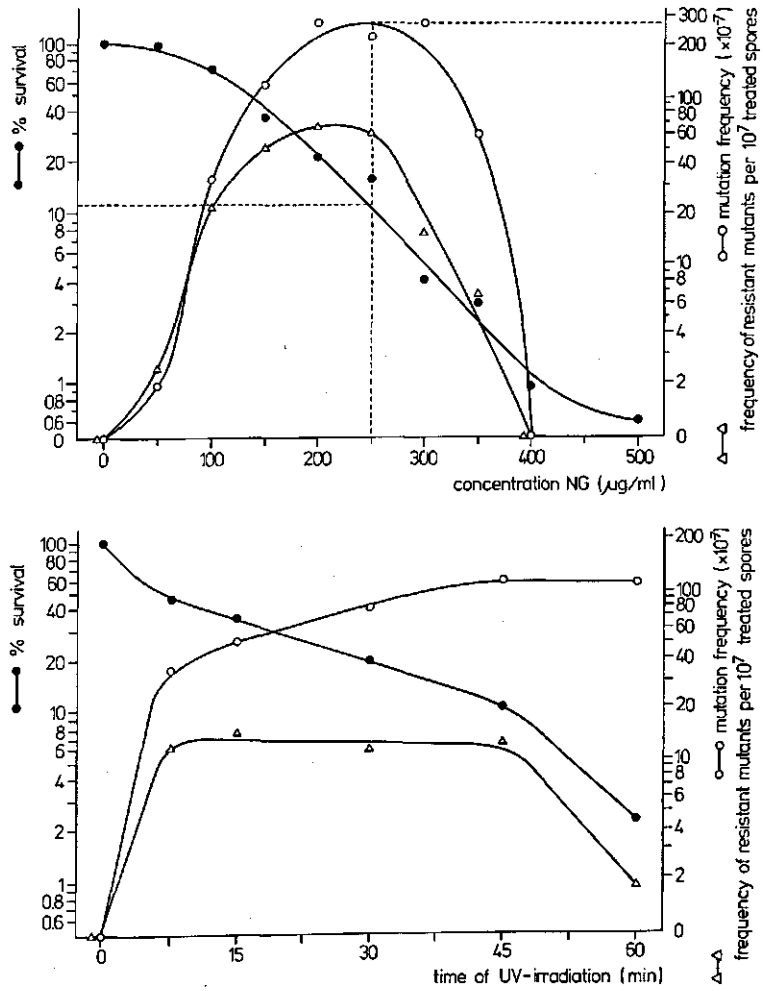


FIG. 2. Effect of mutagenic treatment (NG, UV) on survival percentage and mutation frequency (frequency of mutants per 10^7 surviving spores) to benomyl resistance in *Aspergillus niger*.

Ordinate: survival percentage (●—●); mutation frequency (o—o) and frequency of resistant mutants per 10^7 treated spores (△—△).

Abscissa: NG concentration and time of UV irradiation.

by CHADWICK and LEENHOUTS (1976) in several organisms e.g. *A. nidulans*, *Escherichia coli* and *N. crassa*.

In experiments dealing with the induction of benomyl resistance in *A. niger* the effect of mutagenic treatment (UV irradiation and NG treatment) and survival percentage in relation to mutation frequency was investigated (Fig. 2). The mutation frequency is expressed as the number of resistant mutants per ten million spores which survived the mutagenic treatment. In addition, the ratio between the number of resistant mutants and the total number of treated spores is given. Spore suspensions were treated with NG in a buffer of pH 9 or with UV (Fig. 2). Both treatments were carried out in one experiment using the same selective agent (5 μg/ml benomyl) and the same spore suspension (2.10^7 spores/ml). Fig. 2 shows that an increase of the mutagenic dose resulted in a decrease of survival percentage and an increase of mutation frequency for NG to a maximum at 250 μg/ml. In order to obtain mutants in otherwise undisturbed genetic background, as will be shown of importance with respect to pathogenicity of mutant strains in chapters 4 and 7, it is necessary to choose a mutagenic dose resulting in a mutation frequency below 10 (for instance, as in Fig. 2, a concentration of NG of 75 μg/ml or 5 min UV irradiation). In the mutation experiments (3.3.) survival percentages varied from 3–60% (Table 6). These percentages correspond with mutation frequencies of 70–(via 250 at 10%)– 75 and 100 – 10 per ten million spores using NG treatment and UV irradiation, in Fig. 2, respectively. Variations of this kind must be taken into account in considering the data of the following section.

3.3. INDUCED RESISTANCE TO SYSTEMIC FUNGICIDES IN DIFFERENT FUNGI

Resistance to systemic fungicides was studied with seven compounds, viz. benomyl and thiabendazole, which are both benzimidazole derivatives; carboxin, an oxathiin; chloroneb, an aromatic hydrocarbon; imazalil, an imidazole derivative and two antibiotics, cycloheximide and pimaricin, a glutarimide and a polyene antibiotic, respectively. The structural formulas of these compounds are listed in Fig. 3, the chemical names and formulations are given in Table 3. In Table 6 these compounds are arranged together with the fungi in which resistance was induced. Dependent on the sensitivity of the wild-type strain of the fungus (indicated in the third column as the ED_{50} of the wild-type strain), the selection concentration was chosen (fourth column). After the mutagenic treatment (UV of NG, as indicated in the fifth column) the survival percentage (sixth column) was determined by plating a series of dilutions of the treated and untreated spore suspensions. From the number of colonies counted on the selection media (Fig. 4) the mutation frequency (expressed as a number per ten million surviving spores) was calculated. The highest level of resistance found in the strains selected in these experiments is listed in the last column of Table 6 to give an idea about the potential of fungi to develop a certain level of resistance by a single gene mutation (as will be shown in

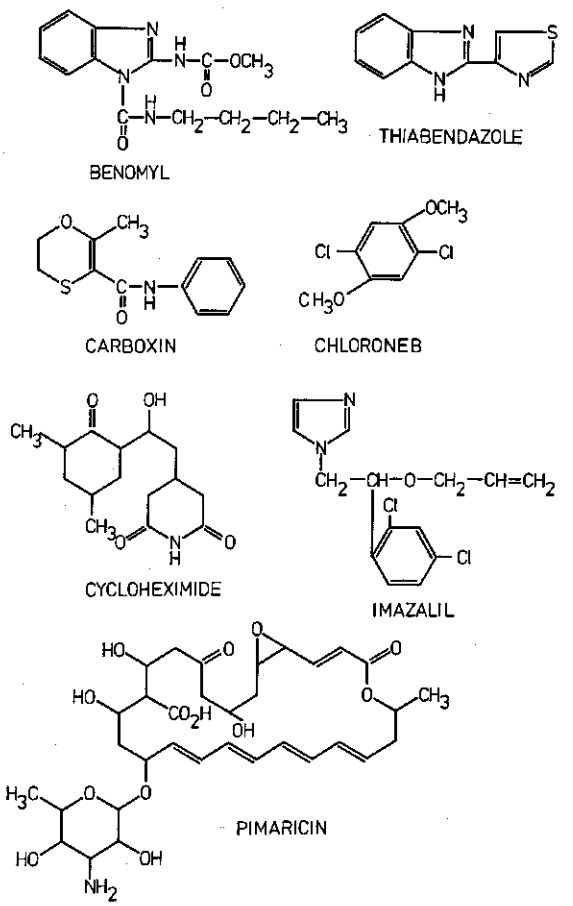
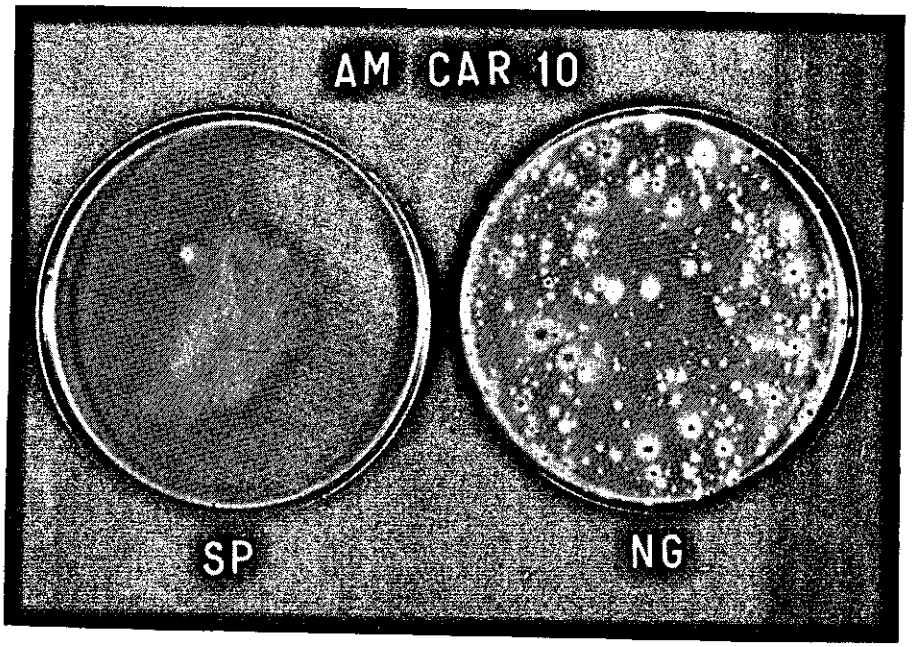


FIG. 3. Structural formulas of the systemic fungicides used in the mutation experiments. Chemical names are listed in Table 3.

chapters 6, 7). In addition to Table 6 it should be noted that resistance occurred in all cases which were examined on basis of sensitivity tests of the wild-type fungi. The selection concentration, however, had to be just above the MIC (minimal inhibitory concentration) of the wild-type fungus and the mutagenic treatment had to be effective with a survival of e.g. at least 10^7 spores per experiment.

3.3.1. *Benomyl and thiabendazole*

Resistance to benomyl was induced in a number of fungi, among which seven plant-pathogenic fungi (Table 6). Until lately no reports on resistance in the latter fungi in the field have appeared in the literature; recently, how-



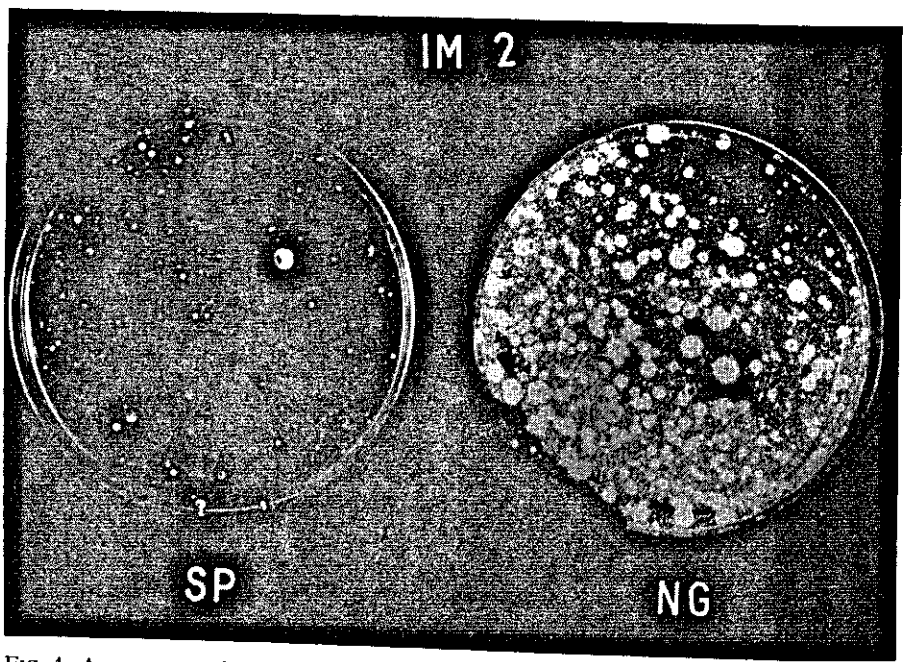


FIG. 4. Appearance of mutants of *Aspergillus nidulans* resistant to carboxin (10 μg/ml in SM with acetate as carbon source; CAR) and to imazalil (2 μg/ml; IM), arisen spontaneously (SP) or after NG treatment (NG), respectively.

TABLE 6. Induced resistance to systemic fungicides in different fungi.

Compound	Fungus	ED_{50} wild type (μg/ml)	Selection concentration (μg/ml)	Mutagens*	Survival %	Mutation frequency ($\times 10^{-7}$)	Number of strains	Highest degree of resistance (ED_{50} in μg/ml)
Benomyl	*Aspergillus nidulans*	0.7	2	UV	7	5	400	30
	*Aspergillus niger***	0.6	5	UV	25	25	200	>500
	Aureobasidium bolleyi	0.04	1	UV	5	2	4	40
	Cercosporella herpotrichoides	0.1	1	UV	8	100	4	>500
	Cladosporium cucumerinum	0.15	1	UV	35	1.2	5	70
	Fusarium nivale	0.1	1	UV	10	5	9	100
	Penicillium expansum	0.15	1	UV	40	10	200	>500
	Phialophora cinerescens	0.1	1	UV	8	30	25	40
	Rhodotorula rubra	2.0	10	UV	10	50	40	>500
	Ustilago maydis	1.5	10	UV	10	5	10	>500
Thia-bendazole	*A. nidulans*	8	25	UV	7	9	100	125
	A. niger	5	25	UV	25	25	38	250
	P. expansum	0.5	5	NG	20	15	305	25
	R. rubra	15	100	UV	40	66	180	150
	U. maydis	20	100	UV	25	50	40	200
Carboxin	*A. nidulans****	1.5	10	NG	17	260	158	200
	C. cucumerinum	2	50	UV	40	170	10	50
	R. rubra	1	10	UV	18	15	25	50
	U. maydis	0.5	10	UV	30	1	8	15
Chloroneb	*A. nidulans*	13	100	UV	50	225	25	>1000
	A. niger	10	100	UV	25	280	6	>1000
	P. expansum	10	100	UV	40	250	6	>1000
	U. maydis	8	25	UV	50	2000	10	>1000
Cyclo-heximide	*A. nidulans*	130	2000	NG	30	23	150	>4000
	A. niger	40	250	NG	3	40	13	400
	C. cucumerinum	5	25	NG	6	10	6	10
	R. rubra	1	10	UV	18	5	45	100
Imazalil	*A. nidulans*	0.4	2	NG	30	82	200	4
	A. niger	2	10	NG	10	2	6	5
	C. cucumerinum	0.5	5	NG	5	26	25	4
	P. expansum	0.3	1	NG	15	700	25	1.2
	P. cinerescens	0.5	1	UV	8	250	30	5
Pimaricin	*A. nidulans*	1.4	5	NG	10	25	25	6
	A. niger	2.5	10	NG	10	3	4	8
	C. cucumerinum	1.0	2	UV	60	1.1	10	2.5
	U. maydis	4.0	10	UV	25	2	4	12

* UV = ultraviolet irradiation.
NG = N-methyl-N'-nitro-N-nitrosoguanidine treatment.
** This is a different experiment from that in Fig. 2.
*** On acetate medium (s.5.5.1.).

ever, also in nature *Cercosporella herpotrichoides* developed resistance to benzimidazoles (RASHID and SCHLÖSSER, 1975). Wild-type strains of the ten investigated fungi are known to be very sensitive to benomyl. The resistance varied from low to high levels (5.2.1., Fig. 16). One of the non-pathogenic fungi is *Aureobasidium bolleyi*, known from the mycoflora of cereal roots (PLATENKAMP and BOLLEN, 1973), which has been used as a benomyl indicator for a long time. The mutation frequency per ten million spores found for the different fungi varied from 1 to 100. The value 100 found with *C. herpotrichoides* may be overrated, however, because the spores of this fungus are multicellular and therefore the number of surviving spores in the control may be underestimated. BARTELS-SCHOOLEY and MACNEILL (1971) reported benzimidazole fungicide resistance in *Fusarium oxysporum* to arise spontaneously with a mutation frequency of 0.1 (one resistant mutant in 8.6×10^7 spores) and after UV irradiation a mutation frequency of 22 (one resistant mutant in 4.6×10^5 spores). Upon UV irradiation of *A. nidulans* HASTIE and GEORGOPOULOS (1971) obtained 9 resistant mutants in a sample of 8 million surviving conidia; that means a mutation frequency of 11. The results shown here are in agreement with these data. The results of MEYER (1976) with physiological races of *Colletotrichum lindemuthianum* are especially worth mentioning. These strains differed among each other with regard to mutation rates to MBC (methyl benzimidazol-2-yl carbamate), varying from 1.10^{-6} to $< 2.10^{-8}$ and 2.10^{-5} to $< 2.10^{-8}$ depending on whether they arose spontaneously or after UV treatment. Of two strains no resistant mutants could be recovered on a medium containing 1 µg/ml MBC, which has been explained by the higher sensitivity of these strains compared with the other strains to the benzimidazole fungicides.

Complete cross-resistance of benomyl-resistant strains to thiabendazole was found in all species, except in *A. nidulans* and *A. niger*, where about 1% of the benomyl-resistant mutants were as sensitive to thiabendazole as the wild-type strain (5.2.1., Fig. 16, strain No. 3). In section 5.2.1. this will be discussed in more detail. Attempts to induce resistance to thiabendazole in five fungi gave results comparable to those obtained with benomyl. Here 2–20% of the thiabendazole-resistant strains did not show cross-resistance to benomyl, but on the other hand were more sensitive to benomyl than the wild-type strain (5.2.1.). Generally, fungi are not as sensitive to thiabendazole as to benomyl, but on the other hand the degree of resistance to thiabendazole did not attain the same level as that to benomyl.

3.3.2. *Carboxin*

Resistance to carboxin was induced in all fungi tested (Table 6). Carboxin has proved to be ten times more fungitoxic in a medium with acetate than in a medium with glucose as carbon source (RAGSDALE and SISLER, 1970; VAN TUYL, 1975a). Therefore, in order to obtain a complete inhibition of the wild type, *A. nidulans* was grown on a carboxin-containing medium with acetate as carbon source (5.5.1.). With NG as mutagenic agent this resulted in a muta-

tion frequency of 260 (Table 6). Using CM the sandwich method (2.6.2.) was applied with a carboxin concentration of 100 μg/ml. With UV as mutagenic agent this resulted in a mutation frequency of 2 (VAN TUYL, 1975a). With the latter method, however, only highly resistant strains were recovered. The stronger mutagenic action of NG as compared with UV, together with the lower selection concentration, explains the difference in mutation frequency between these two experiments. *U. maydis* showed the lowest mutation rate to carboxin resistance, which could be explained by the high selection concentration chosen in spite of the high sensitivity of this fungus to carboxin, as compared with the other fungi used.

3.3.3. *Chloroneb*

Chloroneb resistance has already been reported for *U. maydis* (TILLMAN and SISLER, 1971, 1973). High-level resistance to chloroneb was easily induced in all fungi tested (Table 6). Mutagenic treatment resulted in a high mutation frequency, but relatively many mutations appeared already spontaneously (Table 7).

3.3.4. *Cycloheximide*

Resistance to cycloheximide was obtained in all fungi used, the wild types of which differ considerably in sensitivity to this compound (Table 6). Although no important differences were found in mutation frequency, the degree of resistance varied appreciably. In *C. cucumerinum* only a low level of resistance was obtained; however, by repeating mutation experiments with resistant strains that were isolated subsequently, it was possible to increase the resistance to a rather high level.

3.3.5. *Imazalil*

The degree of resistance to imazalil appeared to be of a low level in all fungi studied (Table 6). On the other hand the frequency with which mutation to imazalil resistance occurred was high, except in the case of *A. niger*. In comparison with the fungicides mentioned before, this compound, however, might cause less problems in practice, as far as development of resistance is concerned.

3.3.6. *Pimaricin*

In all four fungi tested mutation frequency and level of resistance were low (Table 6 and unpublished results). These results confirmed the view of HAMILTON-MILLER (1974) in a review about polyene antibiotics, stating that 'from a clinical point of view the problem of polyene resistance is non-existent'.

3.4. THE MUTATION FREQUENCY OF RESISTANCE TO DIFFERENT FUNGICIDES IN *ASPERGILLUS NIDULANS*

In order to compare the rates of mutation to resistance to different fungicides in one fungus, experiments were carried out with *A. nidulans*, in which a number of toxicants and different mutagenic treatments were examined, at a given survival percentage (different from experiments presented in Table 6). The frequency of spontaneous as well as UV- and NG-induced mutations were determined using six different fungicides. The results of two experiments are shown in Table 7. The highest frequency was found with chloroneb, followed by imazalil, whereas the lowest was obtained with carboxin and benomyl.

Using the frequency of auxotrophic mutants as a reference, SRIVASTAVA and SINHA (1975) estimated the number of loci involved in p-fluorophenylalanine resistance in *A. nidulans*. Without any knowledge of the frequency with which a certain mutation occurs, it is difficult to translate frequencies found into numbers of loci, that have mutated. The number of loci found in *A. nidulans*, to be discussed in more detail in chapter 6, however, can be compared with these frequencies. Analysis of the resistance to the different fungicides resulted in the identification of the following number of loci: benomyl 3, carboxin 3, chloroneb 1, cycloheximide 5, imazalil 8 and pimaricin 2 (s. 6.3.). In addition to these results it must be mentioned that the alleles investigated are

TABLE 7. Mutation frequency of resistance to different fungicides in *Aspergillus nidulans*.

Experiment*	1			2		
Spore concentration/ml	10^8			7.10^7		
Mutagenic treatment**	SP	UV	NG	SP	UV	NG
Survival %	100	50	50	100	80	50
Mutation frequency ($\times 10^{-7}$)*** and number of mutants on selection media:						
CM benomyl (2 μg/ml)	0.02 (1)	7 (53)	22 (147)	0 (0)	5 (12)	37 (45)
AM carboxin (10 μg/ml)	0.25 (8)	12 (351)	80 (600)	0.2 (2)	5 (20)	45 (212)
CM chloroneb (100 μg/ml)	2.3 (22)	225 (225)	500 (494)	20 (86)	430 (311)	5000 (160)
CM cycloheximide (2 mg/ml)	2.5 (47)	20 (60)	35 (527)	5.7 (12)	9 (24)	55 (174)
CM imazalil (2 μg/ml)	4.0 (76)	160 (110)	250 (200)	1.0 (4)	35 (14)	475 (271)
CM pimaricin (5 μg/ml)	3.1 (97)	10 (160)	15 (18)	5.7 (49)	29 (114)	150 (296)

* Experiment 2 is a replicate of experiment 1.

** SP = spontaneous mutation, UV = ultraviolet light, NG = N-methyl-N'-nitro-N-nitrosoguanidine.

*** Mutation frequencies are expressed as number per 10^7 surviving spores; in parenthesis the absolute number of mutants out of the experiments; CM = complete medium, AM = acetate medium; thiabendazole is left out, because of the similarity with benomyl (Table 6).

often not equally distributed over the number of loci. The high mutation frequency of the chloroneb resistance for which only one locus could be established is not according to expectation. A high mutability of the locus to chloroneb resistance is probably responsible. Moreover in cases that more loci were found (especially for benomyl and imazalil resistance, 5.2.2., Table 13) it is doubtful whether an equal probability of recovery of all kinds of mutants may be supposed.

3.5. Discussion

In order to obtain an impression about the potential of fungal pathogens to acquire resistance to a given fungicide, the mutation frequency with which resistance develops was studied in connection with the degree of resistance, using seven fungicides and various fungi. The spontaneous mutation frequency was increased by using mutagenic agents. It was shown that the mutation frequency was a function of the survival percentage as a result of mutagenic treatment (Fig. 2). This implies that in different experiments considerable differences in mutation rate to resistance may be found.

The determination of mutation frequencies of induced resistance in the fungi studied, resulted in extreme values for the different fungicides ranging from 10^{-7} to 2.10^{-4} in the following sequence: pimaricin, benomyl, thiabendazole, cycloheximide, carboxin, imazalil and chloroneb (Table 6). With respect to differences in frequency of emergence of resistance in fungi Dekker (1974) considered various factors e.g. type of fungicide, type of fungus and type of disease. The results presented here indicate that the mutation frequency differed considerably among the fungal species. With respect to the type of fungicide, however, not only differences in mutation frequency, but also considerable differences in the level of resistance were found. Expressing the level of resistance of the most resistant strain of each fungus examined as a ratio between the ED_{50} values of that strain and the wild-type strain(Table 6), the following values with variations for different fungi were obtained: benomyl 50–5000; thiabendazole 10–50; carboxin 25–130; chloroneb 100: cycloheximide 2–100; imazalil 2.5–10 and pimaricin 2.5–4. It is evident that the potential of a fungal species to acquire resistance to a toxicant is dependent on level of resistance obtained after one mutation, rather than on mutation frequency. In view of this, it can be concluded that pimaricin and imazalil may be suitable for agricultural use, while on the other hand because of the possibility of development of a high level of resistance to them, benomyl, thiabendazole, carboxin and chloroneb are less desirable in plant disease control.

Using different mutagenic treatments in the same experiment (Table 7), a comparison was made between the frequencies of occurrence of resistance to different fungicides in a genetically well-defined fungus *A. nidulans*. Frequency of spontaneous as well as UV- and NG-induced mutations were determined using six fungicides. With spontaneous mutations the mutation frequency of

resistance to benomyl was very low (only one mutant in two experiments). Similar observations were made by BEN-YEPHET et al. (1974) with *Ustilago hordei*; they did not find any resistant mutant in 1.57×10^8 plated sporidia, although benomyl-resistant mutants could readily be obtained by mutagenic agents. In studies with *A. nidulans* (MARTINELLI and CLUTTERBUCK, 1971; SRIVASTAVA and SINHA, 1975) an estimation was made of the number of loci involved in a mutation experiment. Using the genetic analysis of the resistant mutants as described in chapter 6, it was established that chloroneb resistance, though showing the highest mutation frequency, was defined only by one locus. This can be explained by the fact that the mutability of various loci may be very different. Moreover, this estimation is rather loosely based on the supposition of an equal probability of recovery of all kinds of resistant mutants, as appears from an unequal distribution of the mutants over the loci in the analysis of benomyl and imazalil resistance.

4. PATHOGENICITY OF FUNGI RESISTANT TO SYSTEMIC FUNGICIDES

4.1. INTRODUCTION

When a pathogen has the potential to acquire fungicide resistance (chapter 3), this does not necessarily imply that resistance problems will arise in practice. Whether resistant mutants will have the capacity to build up a resistant population in the field depends on various factors. Of great importance in this respect are the pathogenicity and fitness of the resistant mutants.

In recent years many reports have appeared about fungicide-resistant strains arisen under field conditions (DEKKER, 1972; WOLFE and DINOOR, 1973; IIDA, 1975). On the other hand, pathogenicity of laboratory-induced resistant strains is often found reduced. In tests with ten mutants resistant to chlorinated nitrobenzenes of *Hypomyces solani f. cucurbitae*, GEORGOPOULOS (1963a) demonstrated that strains with mutations to resistance at the same locus may give both highly and weakly pathogenic strains. KAPPAS and GEORGOPOULOS (1971) examined six dodine-resistant mutants of *Nectria haematococca*, four of which were found to be less pathogenic than the wild type. In this case genetic analysis revealed that in a mutant strain a mutation to reduced pathogenicity inherited independently from the mutation to resistance. One example is known in which resistance and non-pathogenicity were found to be linked. LEBEN et al. (1955) isolated a highly antimycin-A-resistant strain of *Venturia inaequalis*, arisen by a single gene mutation, in which case resistance and non-pathogenicity did not recombine.

Disease is a consequence of a host-parasite interaction, involving a number of factors in both host and parasite (DAY, 1974; BURNETT, 1975). Several attempts have been made to find a correlation between pathogenicity and characteristics of mutants in vitro. The nutrition-inhibition hypothesis of pathogenicity as put forward by GARBER (1956), appeared to be inadequate as stated in pathogenicity tests of nutritionally deficient mutants of *Penicillium italicum* and *P. digitatum* (BERAHA et al., 1964), *Penicillium expansum* (BERAHA and GARBER, 1965; MACNEILL and BARRON, 1966), *Fusarium oxysporum* (TUVESON and GARBER, 1959; SANCHEZ et al., 1975); *Pseudonomas solanacearum* (COPLIN et al., 1974) and *Cladosporium cucumerinum* (chapter 7), although in some of these cases nutritional deficiency accounted for non-pathogenicity of a mutant which could be related to relatively low concentrations of the required nutrient at the site of inoculation. MANN (1962) induced mutations in *F. oxysporum f. sp. lycopersisi* unable to produce extracellular pectic enzymes and she concluded that this might play a role in pathogenicity. By comparing an induced non-pathogenic strain and a revertant of *Erwinia carotovora* BERAHA and GARBER (1971) showed that non-pathogenicity was associated with a greatly reduced production of several extracellular enzymes.

In order to establish possible relations between acquired resistance and changes in pathogenicity, greenhouse experiments were carried out with resistant strains of the following plant-pathogenic fungi: *C. cucumerinum*, *Cercosporella herpotrichoides*, *Fusarium nivale*, *P. expansum* and *Phialophora cinerescens*.

4.2. *CLADOSPORIUM CUCUMERINUM*

Five benomyl-resistant mutants of *C. cucumerinum* were tested for benomyl sensitivity and pathogenicity on cucumber seedlings. The data of the in vitro experiments are presented in Table 8 and Fig. 5, and those of the in vivo tests in Table 8 and Fig. 6. Use was made of the disease index as given by VAN ANDEL (1958). The results showed that the pathogenicity of the benomyl-resistant strains was similar to that of the wild type. It further appeared that a close correlation existed between the degrees of sensitivity of *C. cucumerinum* to benomyl in the in vitro and in vivo experiments.

In the same way six UV-induced carboxin-resistant strains were tested. The

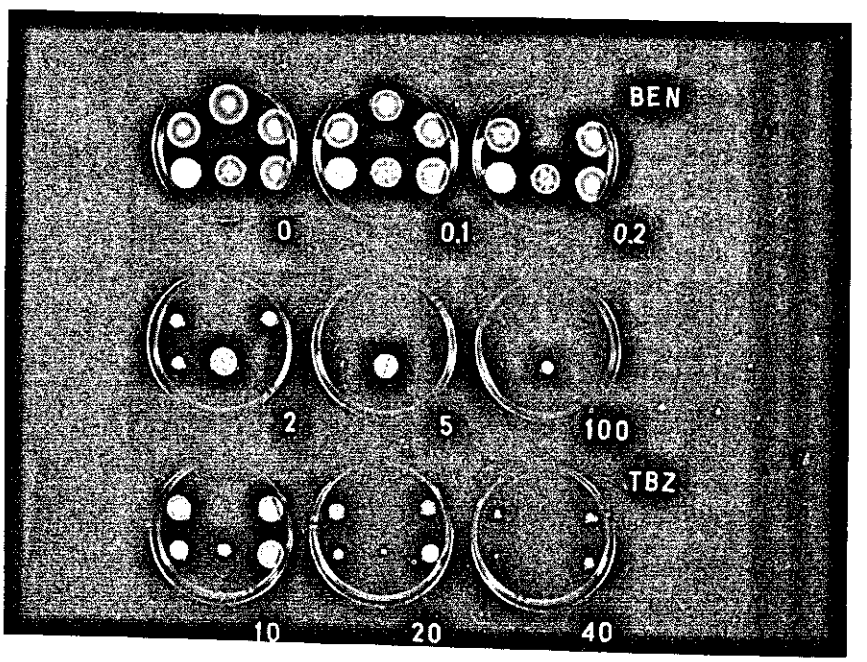


FIG. 5. The wild type (at the top of each plate) and five benomyl-resistant strains of *Cladosporium cucumerinum* grown on CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml). The mutant strains carry the following mutations: *ben1*, *ben3*, *ben2*, and *ben5* (from left to right and top to bottom, respectively; origin and characteristics of these strains are given in Table 8, 24).

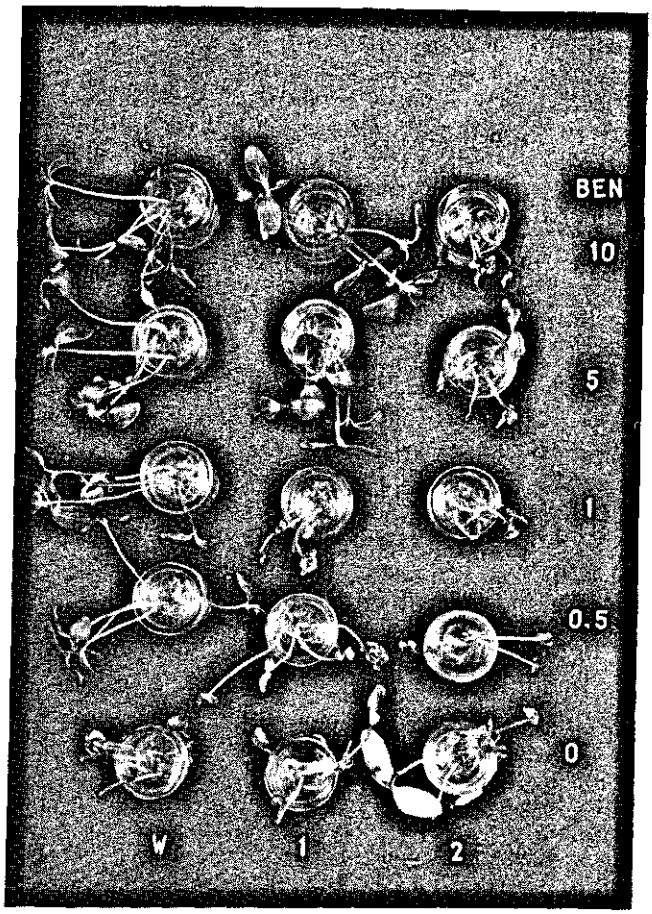


FIG. 6. Pathogenicity test of the wild type and two benomyl-resistant strains of *Cladosporium cucumerinum* on cucumber. Cucumber seedling were inoculated with (from left to right) the wild type (W) and two resistant strains carrying *ben2* (1) and *ben4* (2); benomyl concentrations ranged from 0 to 10 μg/ml.

data of in vitro tests are presented in Fig. 7. All strains were as pathogenic as the wild-type strain. A pimaricin-resistant mutant, however, derived from strain (*ben2*; *pal2*; *met1*) (7.4., Table 24) was considerably less pathogenic than the parental strain, the percentage pathogenicity being 15 versus 70 for the parental strain.

A decreased pathogenicity was also found for nine imazalil-resistant mutants which were obtained by NG-treatment of strain (*ben2*; *pal2*; *whi1*; *met5*). One strain had lost its pathogenicity, while the others showed 20–67% pathogenicity in comparison with the parental strain. It should be considered that NG is a more efficient mutagenic agent than UV irradiation (cf. 3.2.). More specific mutations also mean more additional mutations in the genetic background, which can cause a decrease in pathogenicity.

TABLE 8. In vivo and in vitro toxicity to benomyl of the wild type and five benomyl-resistant mutants of *Cladosporium cucumerinum*.

Strains*	Percentage disease in in vivo test at different concentrations of benomyl (μg/ml)					Inhibition of growth in vitro for benomyl (μg/ml)	
	0	0.5	1	5	10	ED_{50}	MIC
W	100	0	0	0	0	0.15	0.2
BEN-1	100	100	100	25	0	2	5
BEN-2	100	100	100	25	0	2	5
BEN-3	100	100	100	75	0	2	5
BEN-4	100	100	100	100	80	100	>500
BEN-5	100	100	75	20	0	1.5	2.5

* W = wild-type strain; BEN = benomyl-resistant strain, carrying the respective *ben* mutations (Table 24).

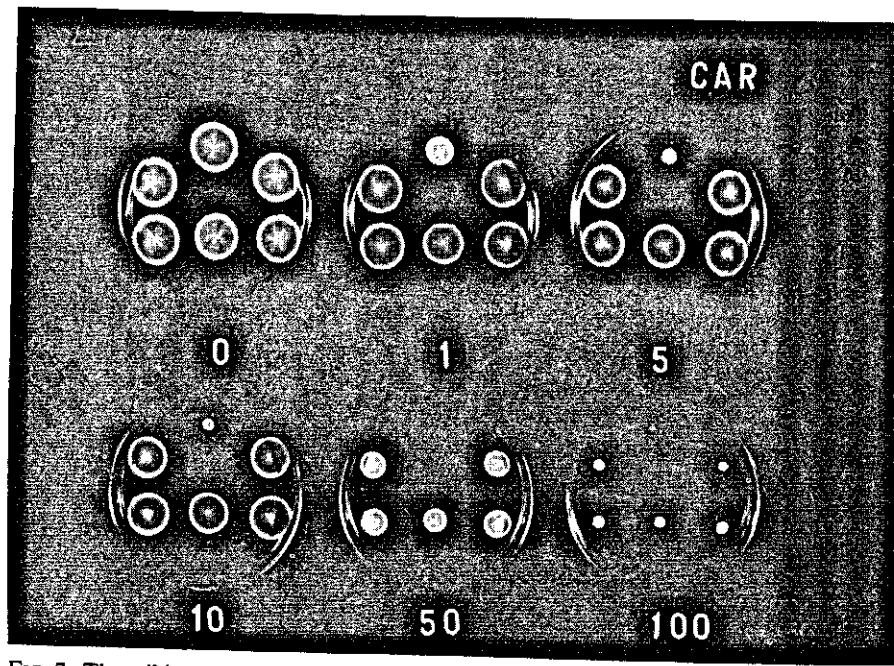


FIG. 7. The wild type (at the top of each plate) and five carboxin-resistant strains of *Cladosporium cucumerinum* grown on CM containing various concentrations of carboxin (CAR) (in μg/ml).

4.3. *CERCOSPORELLA HERPOTRICHOIDES* and *FUSARIUM NIVALE*

Pathogenicity tests on rye plants were carried out with benomyl-resistant strains of *C. herpotrichoides* and *F. nivale*. Also the effect of benomyl on disease development was studied in tests in which the fungicide was administered in

solutions to the roots of rye seedlings. After five weeks of incubation disease symptoms on the rye plants were evaluated and fungal growth was recorded. In the case of *F. nivale* (Fig. 8) the rye plants turned yellow and showed discontinued growth, with an abundant growth of the pathogen. *C. herpotrichoides* showed less abundant growth on the seedlings and it was uncertain whether infection had taken place. Table 9 presents the effect of benomyl on disease development caused by the tested strains, in which, for the sake of comparison, also the ED_{50} values for growth inhibition of these strains by benomyl in vitro are given (Fig. 9). Two resistant strains of *F. nivale* had lost their pathogenicity, while others were less pathogenic. This method of testing pathogenicity seemed inadequate for *C. herpotrichoides*. The low incubation temperature (5°C), which is favourable for infection of *F. nivale* (HOLMES and CHANNON, 1975)

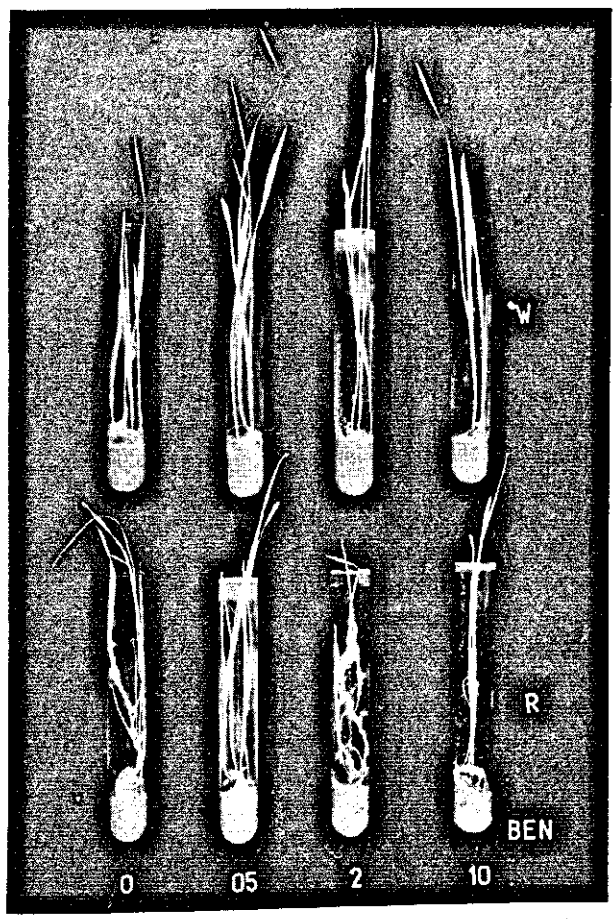


FIG. 8. Pathogenicity test of the wild type (W) and a benomyl-resistant (R) strain of *Fusarium nivale* on rye. Rye plants were inoculated with these strains. Benomyl concentrations ranged from 0 to 10 μg/ml.

TABLE 9. In vivo and in vitro toxicity to benomyl of the wild type and benomyl-resistant mutants of *Cercosporella herpotrichoides* and *Fusarium nivale*.

Strains*	Disease index of in vivo test at different concentrations of benomyl (μg/ml)**					Inhibition of growth in vitro for benomyl (μg/ml)
	0	0.5	2	10	50	ED_{50}
F. nivale						
W	+++	−	−	−	−	0.1
BEN-1	+++	++	++	+	−	1.5
BEN-2	++	+	+	−	−	1.5
BEN-3	+++	+++	+++	+	+	7
BEN-4	++	+++	+++	++	+	50
BEN-5	++	++	++	+	−	1
BEN-6	++	++	+	+	−	1
BEN-7	++	++	++	−	−	5
BEN-8	−	−	−	−	−	1
BEN-9	−	−	−	−	−	1
C. herpotrichoides						
W	+++	++	+	−	−	0.1
BEN-1	+	+	+	−	−	1.5
BEN-2	++	+	+	+	+	60
BEN-3	++	+	+	+	+	>500

* W = wild-type strain; BEN = benomyl-resistant strain.

** The symptoms are recorded relative to W: +++ = symptoms as caused by W; ++ and + = lower degrees of disease; − = no disease symptoms.

might have been less suitable for *C. herpotrichoides*. The procedure described by EVANS and RAWLINSON (1975) might give better results with the latter pathogen. Re-isolations from the rye plants showed no change in benomyl resistance of any of the tested strains.

4.4. *PENICILLIUM EXPANSUM*

In genetic studies with *P. expansum* MACNEILL and BARRON (1966) and BERAHA and GARBER (1965) tested thousands of mutants of this fungus for pathogenicity, only a few of which had lost pathogenicity. Also in my experiments with six benomyl- and thiabendazole-, six chloroneb- and three imazalil-resistant strains, no considerable losses in pathogenicity were found. The chloroneb-resistant strains showed less sporulation in vivo (Fig. 10) as well as in vitro (Fig. 11).

Four strains with a different benomyl sensitivity were compared for pathogenicity. Apples were dipped in suspensions containing 0, 100, 1000 and 5000

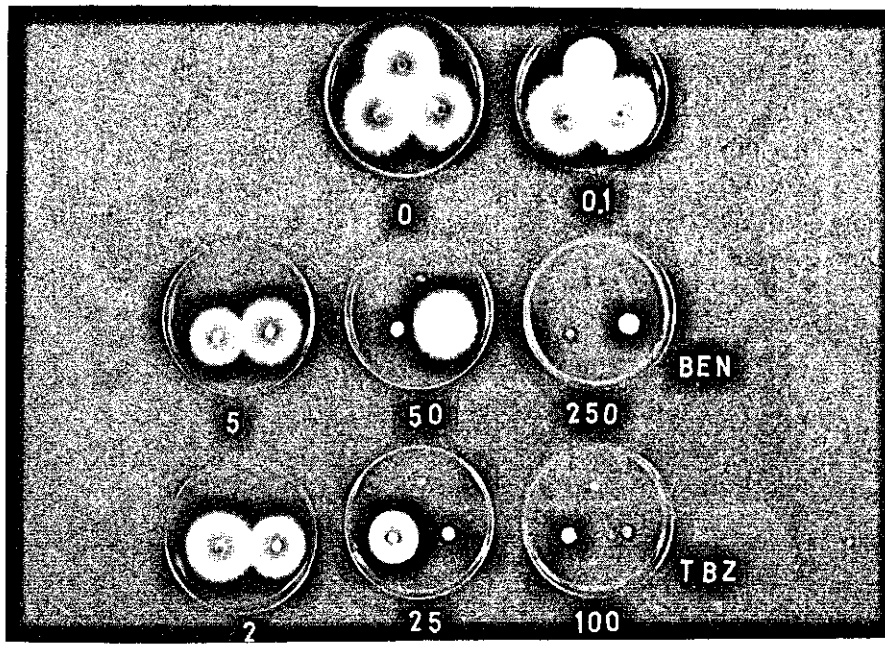


FIG. 9. The wild type (at the top of each plate) and two benomyl-resistant strains of *Fusarium nivale* grown on CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml).

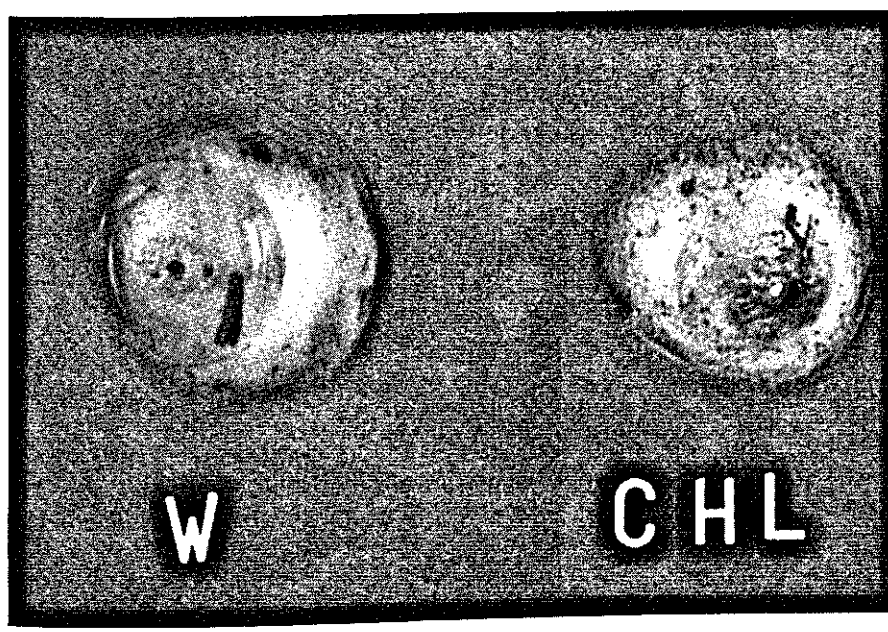


FIG. 10. Pathogenicity test of the wild type (W) and a chloroneb-resistant strain (CHL) of *Penicillium expansum* on apple, 8 days after inoculation.

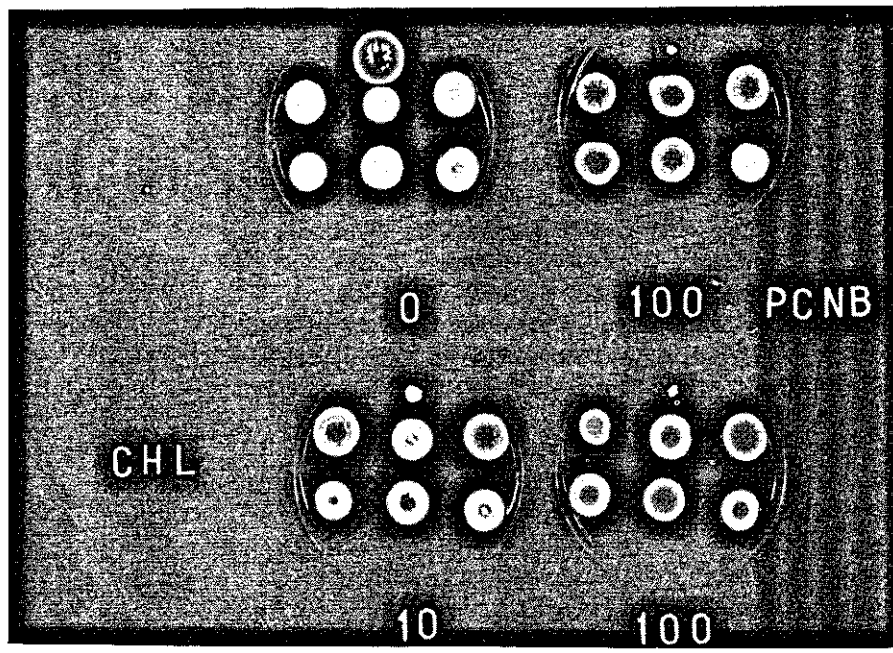


FIG. 11. The wild type (at the top of each plate) and six chloroneb-resistant strains of *Penicillium expansum* grown on CM containing various concentrations of pentachloronitrobenzene (PCNB) or chloroneb (CHL) (in μg/ml). Note the poor sporulation of the resistant strains on the control plate (cf. 5.6.1.).

μg/ml of benomyl and then inoculated with a highly sensitive, a sensitive (wild type), a moderately resistant and a highly resistant strain, respectively. Also with the mutants of this pathogen there was a fair correlation between the effect of benomyl in vivo (Fig. 12) with that obtained in vitro (Fig. 13).

4.5. *PHIALOPHORA CINERESCENS*

Six benomyl-resistant strains of *P. cinerescens* were examined in vitro (Fig. 14) and for pathogenicity on carnation (Fig. 15). One strain appeared to have lost its pathogenicity. After re-isolation the other strains proved to be still resistant. The effect of benomyl against development of the disease on plants, inoculated with two of the resistant strains was studied. Potted plants were inoculated with water, the wild type and two benomyl-resistant strains. Pots were soil-drenched with a benomyl suspension giving a final concentration of 0, 0.5, 1.5, 5, 15 and 50 μg/ml benomyl in the soil, respectively. Fig. 15 shows this experiment five weeks after inoculation. After 48 days the control plants and the ones treated with 15 and 50 μg/ml benomyl and inoculated with the wild type were healthy, while all the other plants were completely killed by the disease. These results indicate that the effect of benomyl on this fungus in

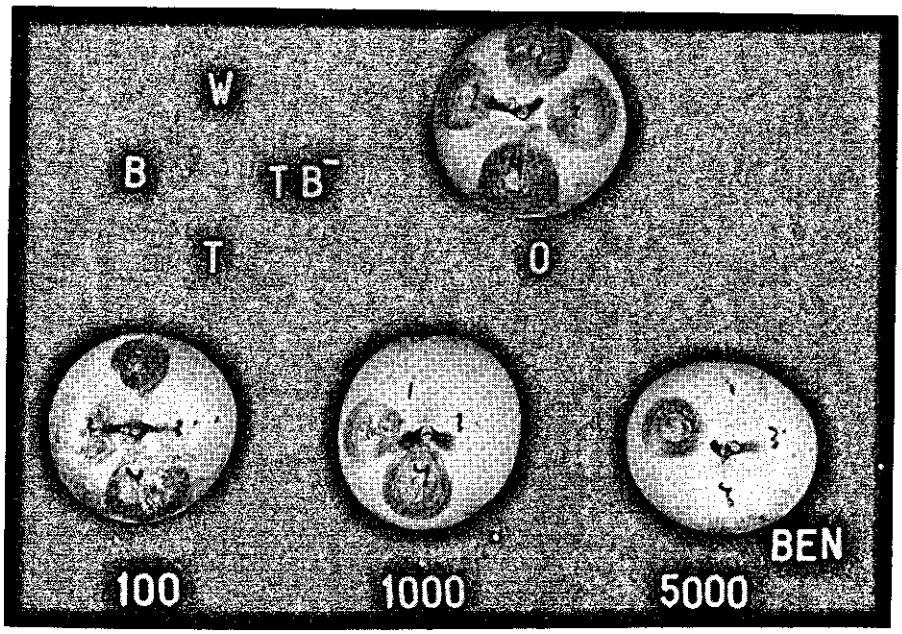


FIG. 12. Pathogenicity test of four strains of *Penicillium expansum* on apple, 7 days after inoculation. Each apple was inoculated once with each of the four strains. The apples were dipped in suspensions of benomyl, with concentrations varying from 0 to 5000 μg/ml. W represents the wild type, B a benomyl-resistant strain, T a thiabendazole-resistant one and TB⁻ a thiabendazole-resistant strain with negative cross-resistance to benomyl (cf. Fig. 13).

vitro is much stronger than in vivo. This phenomenon may be due to inactivation of the fungicide by adsorption to the soil or to insufficient uptake by the plants.

4.6. DISCUSSION

In studies with fungicide-resistant mutants of five pathogenic fungi, viz. *Cladosporium cucumerinum*, *Cercosporella herpotrichoides*, *Fusarium nivale*, *Penicillium expansum* and *Phialophora cinerescens*, some strains were as pathogenic as their wild type, others were less pathogenic and a few had lost their pathogenicity. Similar results have been reported for chlorinated nitrobenzene-resistant mutants of *Hypomyces solani f. cucurbitae* (GEORGOPOULOS, 1963) and *Botrytis allii* (PRIEST and WOOD, 1961), for dodine-resistant mutants of *Nectria haematococca* (KAPPAS and GEORGOPOULOS, 1971), for 6-azauracil-resistant mutants of *C. cucumerinum* (DEKKER, 1974) and for polyoxin-resistant mutants of *Alternaria kikuchiana* (SAKURAI and SHIMADA, 1974). In these cases loss of pathogenicity in induced-resistant strains is likely a consequence

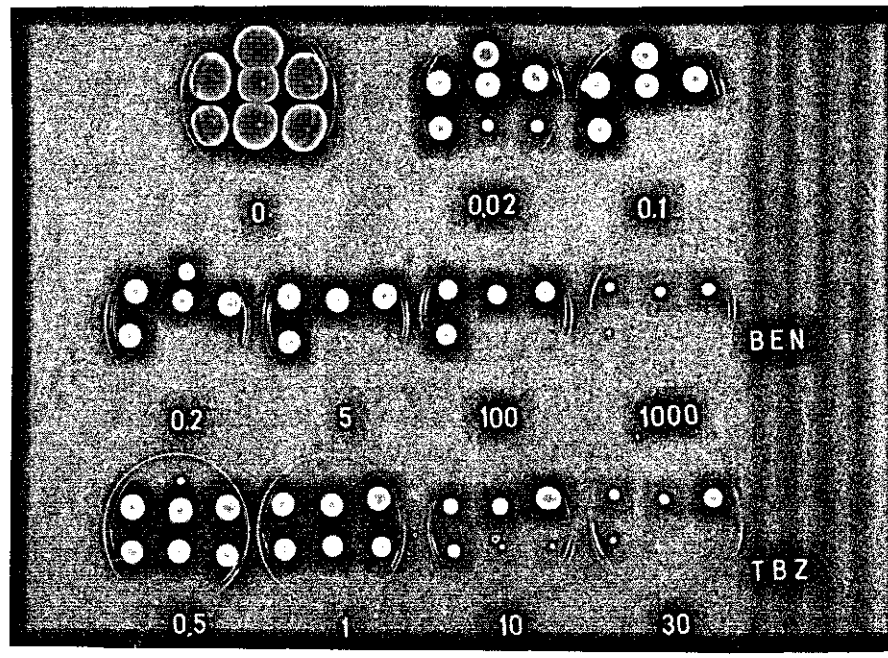


FIG. 13. Negative cross-resistance between benomyl and thiabendazole in *Penicillium expansum*. The wild type (at the top of each plate), three strains isolated from a benomyl- (middle) and three strains isolated from a thiabendazole-containing medium (bottom) grown on CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml) (s. Fig. 12 for a pathogenicity test of four of these strains).

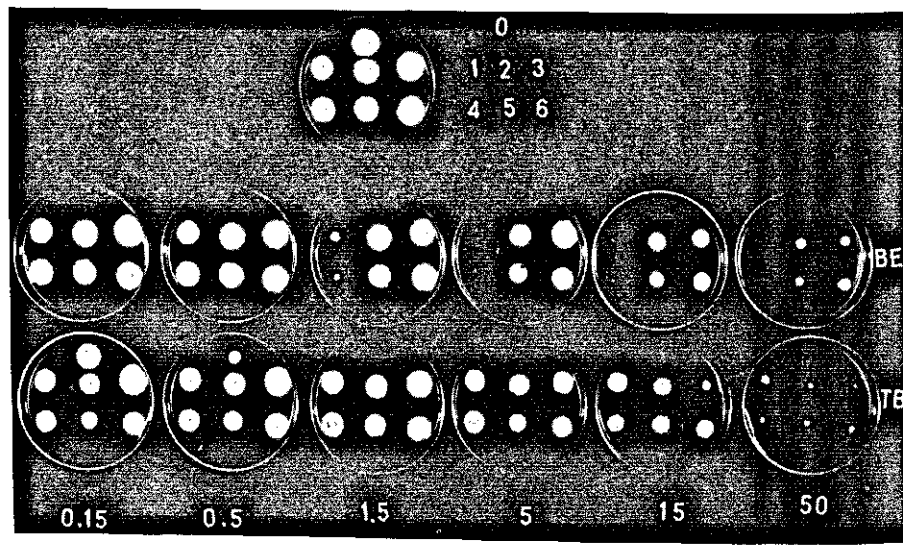


FIG. 14. The wild type (on the top of each plate, O) and six benomylresistant (1–6) strains of *Phialophora cinerescens* grown on CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml).

of the mutagenic treatment, which caused additional mutations affecting the pathogenicity of the pathogen.

The results obtained with benomyl-, carboxin-, imazalil- and chloroneb-resistant strains in my experiments did not show an inverse relationship between degree of resistance and level of pathogenicity. The possibility that increased resistance might be correlated to decreased pathogenicity and fitness may, however, exist for other fungicides. Indications for the occurrence of this phenomenon have been obtained for triarimol- and triforine-resistant mutants of *C. cucumerinum* (FUCHS and VIETS-VERWEY, 1975; FUCHS et al., 1977) and for fenarimol-resistant mutants of *Aspergillus nidulans* (DE WAARD and GIESKES, 1976). In order to prove this, however, more research has to be done on field behaviour of mutants resistant to these and other inhibitors of ergosterol biosynthesis.

Even if a resistant mutant appears to be as pathogenic as the wild type in greenhouse experiments it is doubtful whether this mutant can compete with the wild type in pathogenicity and fitness under field conditions. WOLFE (1971) suggests that a mutant gene conferring fungicide resistance may have selective

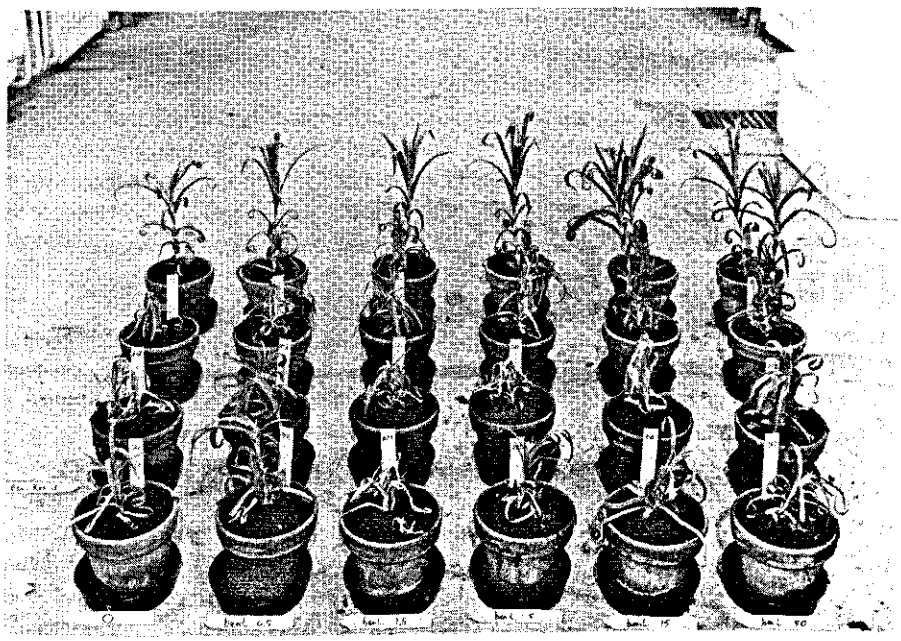


FIG. 15. Pathogenicity test of the wild type and two benomyl-resistant strains of *Phialophora cinerescens* on carnation plants. Potted plants were inoculated with (from top to bottom) water, the wild type and two benomyl-resistant strains (cf. Fig. 14, strains No. 1, 2). Pots were soil-drenched with a benomyl suspension giving (from left to right) a final concentration of 0, 0.5, 1.5, 5, 15 and 50 μg/ml benomyl in the soil, respectively. Five weeks after inoculation the plants treated with 15 and 50μg/ml benomyl were heavily diseased when inoculated with the two benomyl-resistant strains but healthy, when inoculated with the wild type.

advantage in a natural population, but that the whole population does not easily shift towards this direction since in a population so many interacting genes are maintained in a complex balance to its environment.

The competitive capacity of a resistant mutant has been studied by inoculating the host plant with a mixture of sensitive and resistant spores. This was repeated for several generations and afterwards the ratio sensitive/resistant spores was determined. In such experiments a decreased survival of fungicide-resistant strains has been reported for benomyl-resistant strains of *Cercospora beticola* on sugar beet (RUPPEL, 1975) and of *Sphaerotheca fuliginea* on cucumber (DEKKER, 1976), and for kasugamycin-resistant strains of *Pyricularia oryzae* on rice (MISATO and KO, 1975). This does, however, not exclude the possibility that mutants may emerge with high competitive ability, even in the absence of the fungicide. GEORGOPOULOS (1976) reports that the population of benomyl-resistant *Cercospora beticola* on sugar beet in Greece did not show signs of decline three years after application of benomyl had been stopped. In the case of *Verticillium malthousei* on mushroom a benomyl-resistant strain with an even higher pathogenicity than the wild type has been found by BOLLEN and VAN ZAAYEN (1975).

From the pathogenicity experiments described here and from the literature it can be concluded that in the case of benzimidazole fungicide resistance, resistant strains may emerge and survive in practice. Whether this will be true for other systemic fungicides as well depends on the behaviour of the resistant strains under field conditions. Therefore, much more information has to be gathered about pathogenicity and fitness of resistant mutants under natural conditions.

5. CHARACTERIZATION OF THE RESISTANT MUTANTS

5.1. INTRODUCTION

In studies dealing with the mechanism of action of a toxicant, mutants resistant to this compound have been used almost generally (GEORGOPOULOS, 1963b; DEKKER, 1968; GEORGOPOULOS and SISLER, 1970; GEORGOPOULOS et al., 1972; TILLMAN and SISLER, 1971, 1973; THRELFALL, 1972; BORCK, 1973; DAVIDSE, 1974, 1975, 1976; BEN-YEPHET et al., 1975b; HOPPE et al., 1976). Resistance can be caused by detoxification or by a change in cell membrane permeability, in which case the site of action is not reached, but very often it is due to a mutation leading to a change at the active site of the toxicant in the metabolism of the fungal cell. This means that by comparing sensitive and resistant strains, often elucidation of the mechanism of resistance involves elucidation of the mechanism of action of the toxicant. Therefore, characterization of the mutants is not only important from a genetic but also from a biochemical point of view.

Resistant mutants are characterized by their degree of resistance. In order to obtain complete information about the sensitivity of both the mutant and the wild-type strain dosage response curves were made (VOMVOYANNI and GEORGOPOULOS, 1966). When the slopes of the sensitive and the resistant strains are compared, it often happens that the degree of resistance is under- or overestimated depending on whether minimal inhibitory concentrations or ED_{50} values are used as a criterion. Another important condition for the determination of dosage response relationships is the use of a well-defined, suitable medium with a constant quality. In all experiments, unless otherwise stated, CM was used. The CM was Oxoid malt extract agar medium, which has a constant composition; moreover it could be used for all fungi and all strains. A disadvantage of this medium was the complexity of its composition, which may influence the fungitoxicity. Therefore, in some cases toxicity was also examined using a synthetic medium.

Cross-resistance has been defined as resistance to two or more toxicants caused by the same mutation. Strictly speaking only genetic recombination analysis can show whether resistance to several compounds is controlled by the same locus. The patterns of cross-resistance of the resistant mutants to chemically related and unrelated toxicants were studied, in order to establish possible similarity of action of toxicants. Such experiments can result in the discovery of a negatively correlated cross-resistance (GEORGOPOULOS and SISLER, 1970; VAN TUYL et al., 1974) which can be very useful in biochemical studies (DAVIDSE, 1974, 1975, 1976) and studies on genetic recombination (6.6.).

Finally, some changes in morphological characteristics due to the action of the fungicide or to the mutation to resistance are described.

5.2. Patterns of cross-resistance

Some special cases in the patterns of cross-resistance, observed in the resistance to benomyl and thiabendazole and in cycloheximide and imazalil resistance are described in sections 5.2.1. and 5.2.2..

The carboxin-resistant strains isolated in the fungi *Aspergillus nidulans*, *Cladosporium cucumerinum*, *Rhodotorula rubra* and *Ustilago maydis* (Table 6) were tested for resistance to oxycarboxin, a related oxathiin fungicide. The former two fungi appeared to be insensitive, whereas the mutants of the latter two were also found resistant to oxycarboxin. Georgopoulos and Sisler (1970) reported a class of carboxin-resistant mutants (*ants*) of *U. maydis*, which showed an extremely high sensitivity to antimycin-A. This negative correlation between the sensitivities to these compounds was found in strains that were only slightly more resistant to carboxin than the wild type. This type of mutants appeared to occur more frequently than that characterized by a high level of carboxin resistance together with an antimycin-A sensitivity equal to that of the wild-type strain. All eight mutants of *U. maydis* described here (3.3.2.) were of the latter type. In *A. nidulans* 200 carboxin-resistant strains were tested for antimycin-A sensitivity. However, in this fungus the negatively correlated cross-resistance could not be demonstrated. This might be due to the acetate medium from which the mutants were isolated, because the resistance of the *ants* mutants of *U. maydis* was observed with glucose but not with acetate as substrate.

Tillman and Sisler (1973) found that strains of *U. maydis* resistant to the aromatic hydrocarbon fungicide chloroneb showed cross-resistance to related compounds, viz. 2,6 dichloro-4-nitroalinine, diphenyl, hexachlorobenzene, naphthalene, p-dichlorobenzene, pentachloronitrobenzene (PCNB) and sodium-o-phenyl-phenate. Similar observations were made in experiments with chloroneb and the fungi *A. nidulans*, *A. niger*, *P. expansum* and *U. maydis* for PCNB (5.4., Fig. 22). In addition, it appeared that cross-resistance also occurred to 3-phenylindole (Dekker et al., 1975; Hoppe et al., 1976). The cross-resistance to this compound, which was much more fungitoxic to the fungi described here than the other aromatic hydrocarbon fungicides was only of a low degree. The ED_{50} and MIC values of the sensitive *A. nidulans* strain were about 0.5 and 1.5 μg/ml, respectively, while these values for 3-phenylindole-resistant mutants were 2 and 5 μg/ml. Threlfall (1968, 1972) described PCNB resistance in *A. nidulans*. In view of this and through genetic analysis (6.4.; 6.5.) it is likely that resistance to PCNB, chloroneb and other aromatic hydrocarbons is defined by the same genetic factor.

5.2.1. *Negatively correlated cross-resistance between benomyl and thiabendazole*

Cross-resistance of benomyl-resistant strains to thiabendazole was found almost generally in all ten fungal species investigated (3.3., Table 6), except for some strains of *A. nidulans* and *A. niger*, where about 1% of the benomyl-

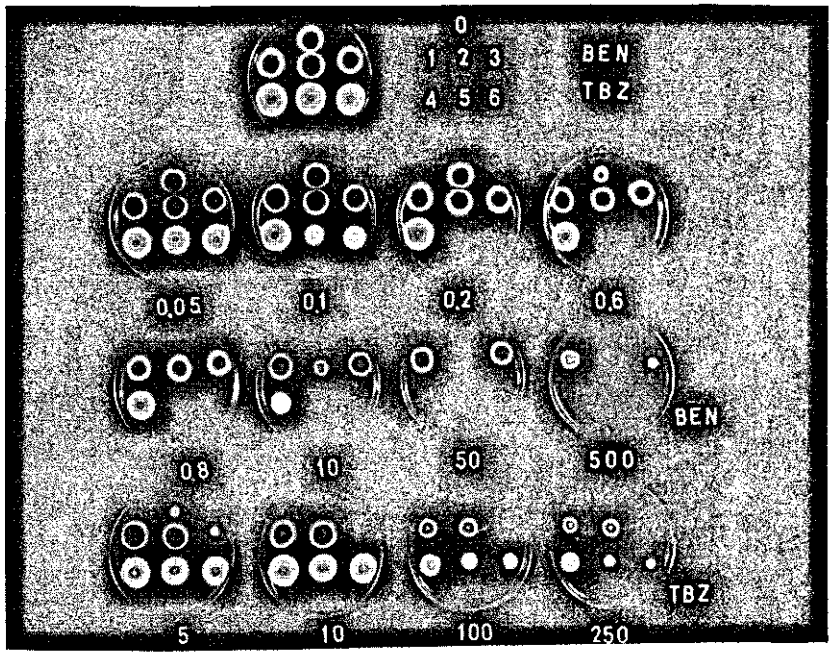


FIG. 16. Different types of benomyl and thiabendazole resistance in strains of *Aspergillus niger* grown on CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml); strain 0 is the wild type, 1, 2 and 3 were isolated from a benomyl-, 4, 5 and 6 from a thiabendazole-containing medium.

resistant strains were as sensitive to thiabendazole as the wild type (Fig. 16, strain No. 3; 5.3., Fig. 20). On the other hand sometimes strains with a high benomyl resistance showed a relatively low thiabendazole resistance and vice versa (chapter 4, Figs 5, 9, 14).

Thiabendazole resistance was induced in *A. nidulans*, *A. niger*, *P. expansum*, *R. rubra* and *U. maydis* (3.3., Table 6). In these cases exceptions with respect to cross-resistance to benomyl were found more frequently. This was observed

TABLE 10. Negative cross-resistance of thiabendazole-resistant strains to benomyl in five fungi; ED_{50} values for growth inhibition of the wild type (W) and the resistant strain (R) (in μg/ml).

	Benomyl		Thiabendazole	
	ED_{50} values		ED_{50} values	
	W	R	W	R
Aspergillus nidulans	0.7	0.15	8	60
Aspergillus niger	0.6	0.12	5	150
Penicillium expansum	0.15	0.02	0.5	5
Rhodotorula rubra	2.0	0.5	15	100
Ustilago maydis	1.5	0.5	20	150

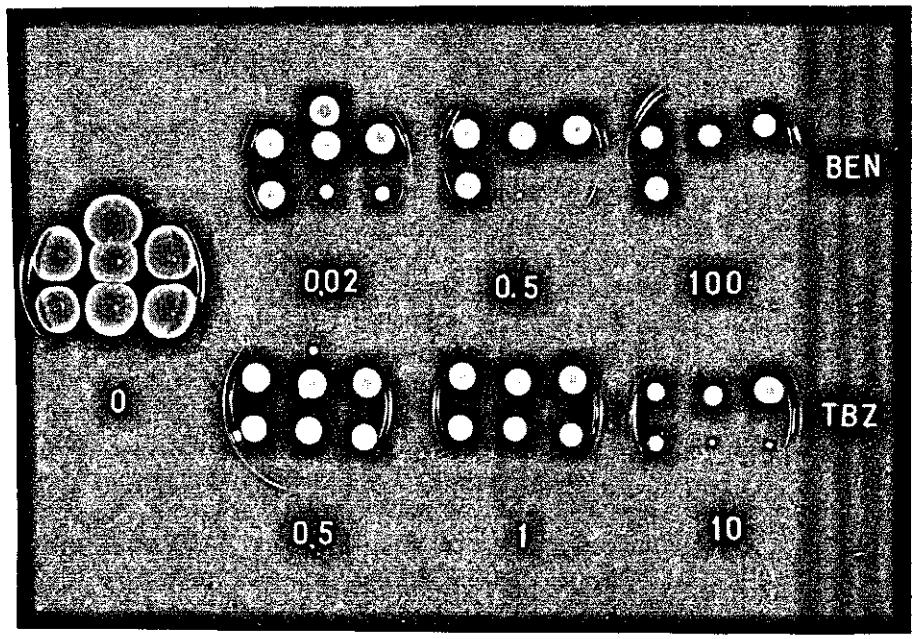


FIG. 17. Negative cross-resistance between benomyl and thiabendazole in *Penicillium expansum*. Seven different strains were replicated on to CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml). At the top of each plate the wild type, in the middle 3 benomyl- and at the bottom 3 thiabendazole-resistant strains (from left to right No. 1, 2, 3).

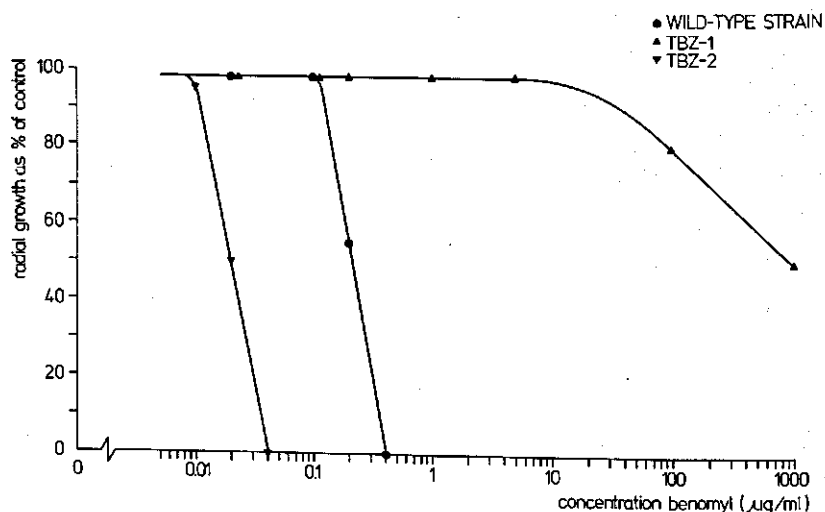


FIG. 18. Dosage response curves of two thiabendazole-resistant strains (No. 1, 2 in Fig. 17) and the wild type of *Penicillium expansum*, with respect to benomyl, showing negative cross-resistance between benomyl and thiabendazole.

in *A. nidulans* and *U. maydis for* 2–4% and in *A. niger*, *P. expansum* and *R. rubra* for 15–20% of the thiabendazole-resistant strains. These strains were not only sensitive to benomyl but they even showed an increased sensitivity to it as compared with the wild type (Table 10; Fig. 16, strain No. 5, 6; Figs 17, 18; 4.4., Fig. 13; 5.3., Fig. 20; 7.2., Fig. 49). This phenomenon was indicated as negative cross-resistance (VAN TUYL, 1975b). In other reports the term collateral sensitivity (RANK and BECK-HANSEN, 1973) or negatively correlated cross-resistance (UESUGI et al., 1974) has been used. In section 6.6. recombination experiments are described in which strains of *A. nidulans* resistant to benomyl and sensitive to thiabendazole, and resistant to thiabendazole and extra-sensitive to benomyl were used, in order to determine the size of the site within the gene that conferred the different types of resistance to benzimidazole fungicides.

5.2.2. *Pleiotropic effects in imazalil- and cycloheximide-resistant strains*

A pleiotropic mutation is defined as a single gene mutation affecting more than one characteristic. Such a mutation often affects a general cell property, which for instance results in positively or negatively correlated cross-resistance to unrelated compounds. This is in contrast with a usual cross-resistance which is only found between compounds, which are chemically related or have a common mechanism of action. In this study several pleiotropic mutations in imazalil- and cycloheximide-resistant mutants were found. More than thirty of these mutants in *A. nidulans* conferred mutations at a number of loci which were allocated to six different linkage groups as is shown in Table 11 and described in detail in chapter 6. On the basis of their pleiotropic properties the mutants could be classified in groups representing mutations at one or two loci. In this way it was established that out of 202 imazalil-resistant strains, 113 were of the type of *imaA*, which was the gene conferring the highest level of imazalil resistance and in addition a slight resistance to acriflavin, fenarimol and neomycin (IMA-4 in 5.3., Figs 19, 23); 52 were of the type of *imaB*, a gene causing a number of pleiotropic effects, viz. resistance to chloramphenicol and fenarimol, and hypersensitivity to acriflavin, cycloheximide and neomycin (IMA-9 in 5.3., Figs 19, 23). Of the remaining 37, 18 showed fenarimol resistance and cycloheximide and neomycin hypersensitivity like those of the type of *imaD* and *imaF* and 19 were cycloheximide-, chloramphenicol- and fenarimol-resistant (*imaC*, *imaE*, *imaG*, *imaH*). In addition to the pleiotropic effects mentioned strain IMA-18 was cold-sensitive. Cold-sensitive strains as described by WALDRON and ROBERTS (1974a, b) were unable to grow at 20°C. Unlike the results obtained with chloramphenicol, cycloheximide and neomycin no effect was found in tests with three other inhibitors of protein synthesis, viz. bacitracin, oxytetracyclin and streptomycin.

The ten cycloheximide-resistant strains of *A. nidulans* used for further genetical investigations could be classified into four groups, representing different genes (Table 11). Three of the five genes conferring cycloheximide resistance also gave rise to imazalil resistance. In order to investigate the fre-

TABLE 11. Pleiotropic effects of the imazalil- and cycloheximide-resistant mutants of *Aspergillus nidulans*.

Mutant numbers of the resistant strains*	Locus involved	Linkage group	Pleiotropic effects**		
			Resistance	Hypersensitivity	Various data
IMA-1, 2, 3,4, 5, 5, 6, 7, 8, 16, 20, 21	*imaA*	VII	neo, acr, fen		IMA-4 see Fig. 23
IMA-9, 11, 12, 17	*imaB*	V	cam, fen	acr, act, neo	allelic with *camD* IMA-9 see Fig. 23
IMA-10	*imaC*	II	cam, act, fen		
IMA-13	*imaD*	VIII	fen	act, neo	IMA-13 see Fig. 23
IMA-14	*imaE*	II	cam, act, neo, fen		IMA-14 see Fig. 23
IMA-15	*imaF*	I	fen	act, neo	
IMA-18	*imaG*	III	cam, act neo, fen		cold-sensitive, closely linked with *actA*
IMA-19	*imaH*	III	cam, act neo, fen		allelic with *actC*
ACT-6, 10	*actA*	III	neo		
ACT-1, 9	*actB*	VII			ACT-1 see Fig. 23
ACT-2, 4, 5, 8	*actC*	III	ima, cam, neo, fen		ACT-4 see Fig. 23 *actC* is allelic with *imaH* ACT-4 is not cam-resistant
ACT-3, 7	*actD, E*	II	ima, cam, fen, neo		

* IMA = imazalil-resistant strain (Table 13.5.).
ACT = cycloheximide-resistant strain (Table 13.4.).

** acr = acriflavin; act = cycloheximide; cam = chloramphenicol; fen = fenarimol; ima = imazalil; neo = neomycin.

TABLE 12. Pleiotropic effects of some imazalil- and cycloheximide-resistant strains of *Aspergillus niger*, expressed as the ratio between the ED_{50} values of the resistant and of the wild-type strain, with respect to imazalil (ima), cycloheximide (act), fenarimol (fen) and acriflavin (acr).

Strains resistant to	Number of strains	Ratio between ED_{50} value resistant strain and ED_{50} value wild type with respect to			
		ima	act	fen	acr
ima	3	10	1.5	4	0.5
ima	1	5	0.5	5	0.4
act	5	1.5	1.5	1	2
act	3	1	3	1	2
act	4	2	10	1.5	0.6

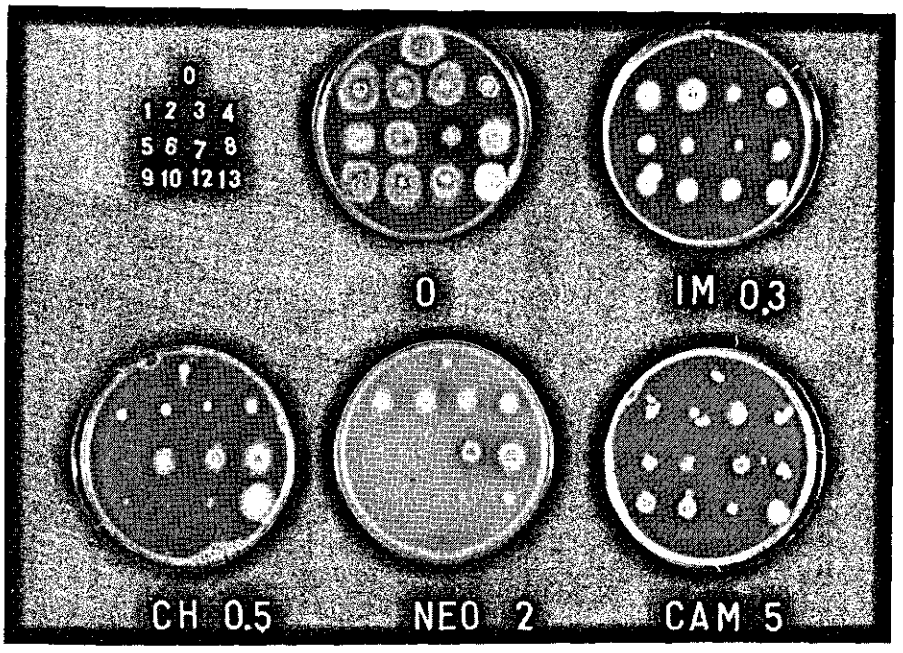


FIG. 19. The wild type and 12 imazalil-resistant strains of *Aspergillus nidulans* grown on SM containing different toxicants: imazalil 0.3 μg/ml (IM), cycloheximide 0.5 mg/ml (CH), neomycin 2 mg/ml (NEO) and chloramphenicol 5 mg/ml (CAM) showing some pleiotropic effects of these strains. Strain 0 is the wild type; 4 and 9 are IMA-4 and IMA-9 in Fig. 23, respectively.

quency with which cross-resistance between imazalil and cycloheximide occurred, 120 cycloheximide-resistant strains were isolated. It appeared that 98 of them were also imazalil-resistant. Fig. 23 (5.3.) shows dosage response curves of two of these strains, namely ACT-1 and ACT-4: one (ACT-4) is cross-resistant to imazalil, the other (ACT-1) is not.

Imazalil resistance in *C. cucumerinum* and *Phialophora cinerescens* was not accompanied by any of the pleiotropic effects detected in *A. nidulans*. With *A. niger*, however, similar results were obtained. Table 12 presents the results of tests on cross-resistance of four imazalil- and twelve cycloheximide-resistant strains of *A. niger*. The degree of cross-resistance is expressed as the ratio between the ED_{50} values of the resistant and of the wild-type strain. All four imazalil-resistant strains showed cross-resistance to fenarimol and hypersensitivity to acriflavin; one of these strains was hypersensitive and the other three were slightly resistant to cycloheximide. Nine of the twelve cycloheximide-resistant strains also showed some resistance to imazalil, and eight were cross-resistant to acriflavin.

5.3. Dosage response relationships of fungicide-resistant strains of *Aspergillus nidulans* grown on agar medium

Since most of the genetical investigations were carried out with resistant mutants of *A. nidulans* (chapter 6) the sensitivity of this fungus to the fungicides was accurately determined, by making dosage response curves. In Figs 20–24 the strains used are indicated as 'WILD-TYPE STRAIN' and 'MASTER STRAIN', while symbols are employed for the mutant strains. The wild type was the initial strain from which the mutants were derived (Table 13) and the master strain was strain No. 1 of 11 (Table 1), with which the mutants were combined in a

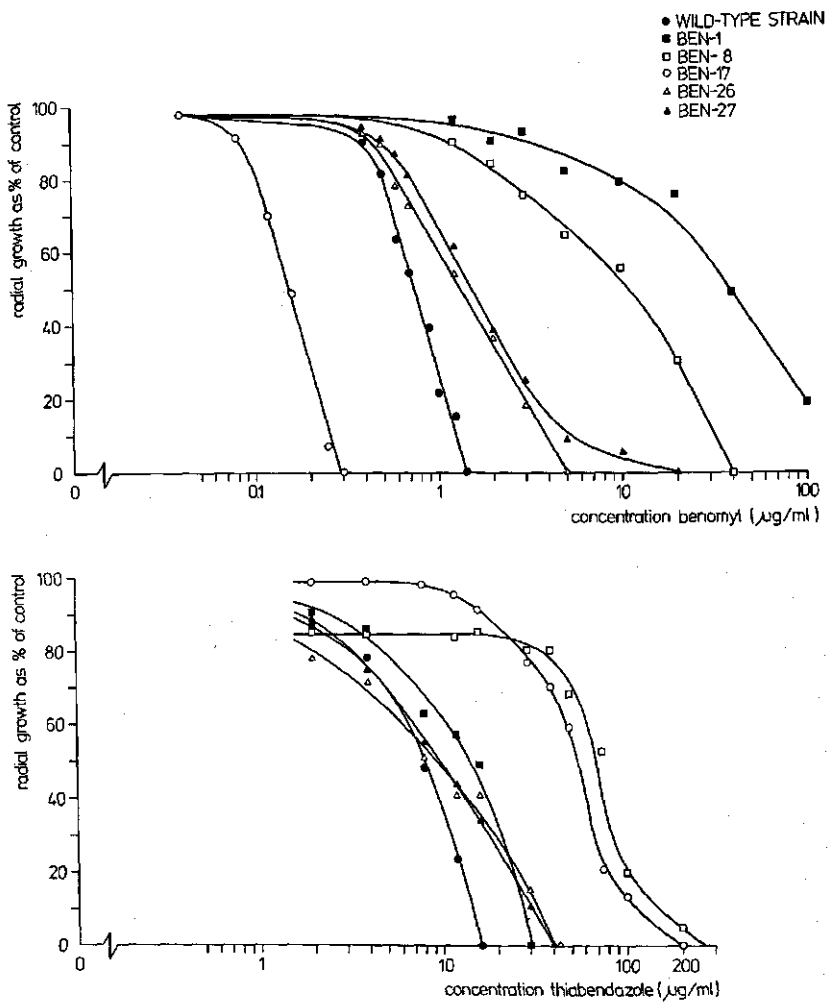


FIG. 20. Dosage response curves of four benomyl-resistant strains, one thiabendazole-resistant strain (BEN-17) and the wild type of *Aspergillus nidulans* (Table 13.1.) with respect to benomyl and thiabendazole.

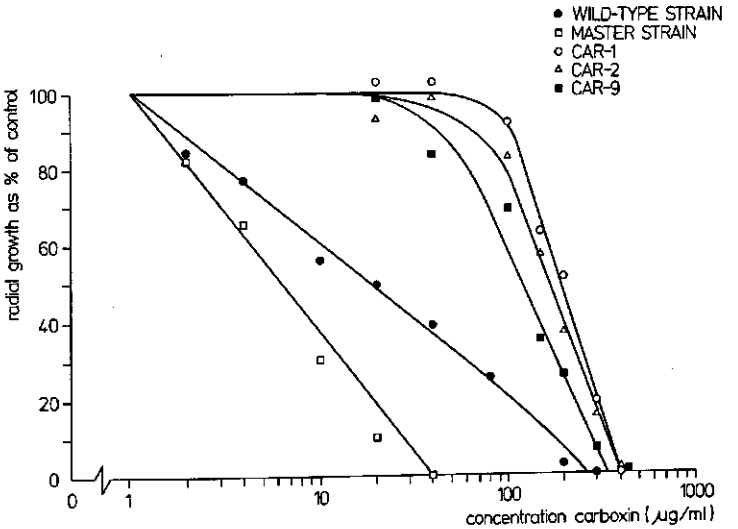


FIG. 21. Dosage response curves of three carboxin-resistant strains, the master strain and the wild type of *Aspergillus nidulans* (Table 13.2.) with respect to carboxin.

heterozygous diploid necessary for further genetic analysis (6.4., 6.7.). The mutant strains are indicated with a three capital letter symbol derived from the name of the fungicide and a mutant number as listed in Table 13.1–13.7 (6.2.).

Dosage response curves of the wild type, four benomyl-resistant strains (BEN-1, 8, 26, 27) and one thiabendazole-resistant strain (BEN-17) are presented in Fig. 20. Strain BEN-17 is extra-sensitive to benomyl. As distinct from BEN-17, BEN-1 displays a high benomyl resistance and an almost wild-type sensitivity to thiabendazole.

Fig 21 depicts the dosage response curves with respect to carboxin of three carboxin-resistant mutants, the master strain and the wild type. The curves of the resistant mutants are characterized by steep slopes. Usually, those of the sensitive strains have the steepest slopes (cf. Fig. 20).

The dosage response curves of three chloroneb-resistant mutants and of two sensitive strains (Fig. 22) demonstrate the close correlation between chloroneb and PCNB with respect to resistance.

The pleiotropic effects of four imazalil- and two cycloheximide-resistant strains (5.2.2.; Table 11) can be derived from the dosage response curves of these strains and the wild type with respect to imazalil, cycloheximide, neomycin, acriflavin and fenarimol (Fig. 23).

A low level of resistance in three pimaricin-resistant strains is revealed by comparing their dosage response curves with that of the wild type (Fig. 24).

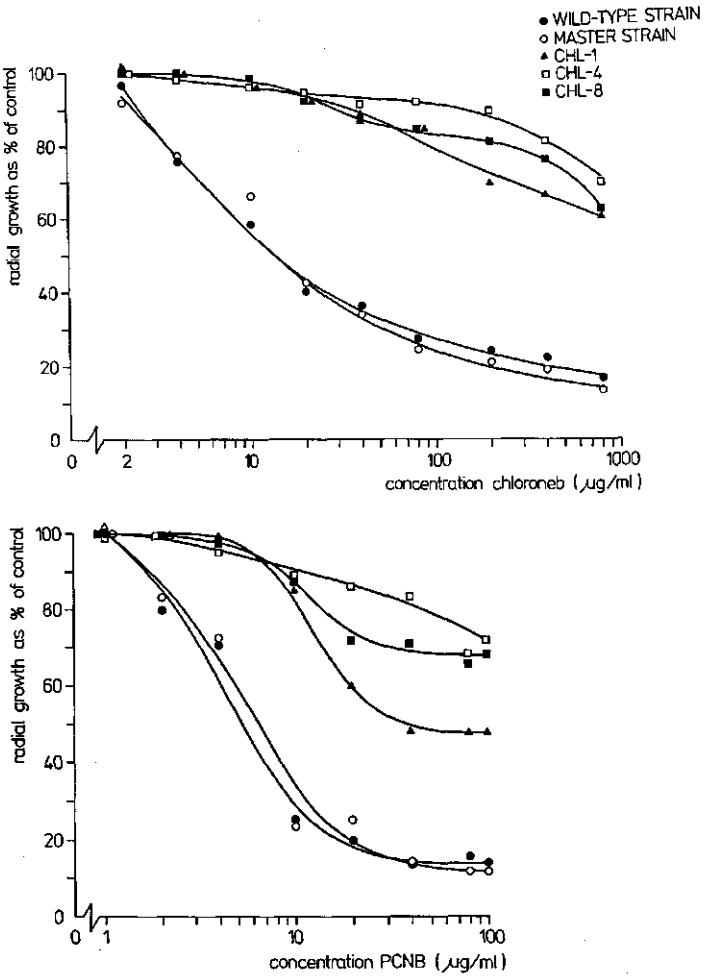


FIG. 22. Dosage response curves of three chloroneb-resistant strains, the master strain and the wild type of *Aspergillus nidulans* (Table 13.3.) with respect to chloroneb and pentachloronitrobenzene (PCNB).

5.4. DOSAGE RESPONSE RELATIONSHIPS OF FUNGICIDE-RESISTANT STRAINS OF *USTILAGO MAYDIS* GROWN IN LIQUID MEDIUM

In addition to the fungitoxicity tests carried out on agar medium (3.3., Table 6), fungicide resistance in *U. maydis* was also examined in liquid medium. The fungicide concentrations causing 50% inhibition (ED_{50}) appeared to be very similar to those obtained in agar tests, when growth was measured after 36–48 hours of incubation. The ED_{50} values of benomyl, carboxin, chloroneb and pimaricin for growth inhibition of the wild type were 2, 0.2, 5 and 1.5 µg/ml, respectively. Growth of the resistant strains was not inhibited at the

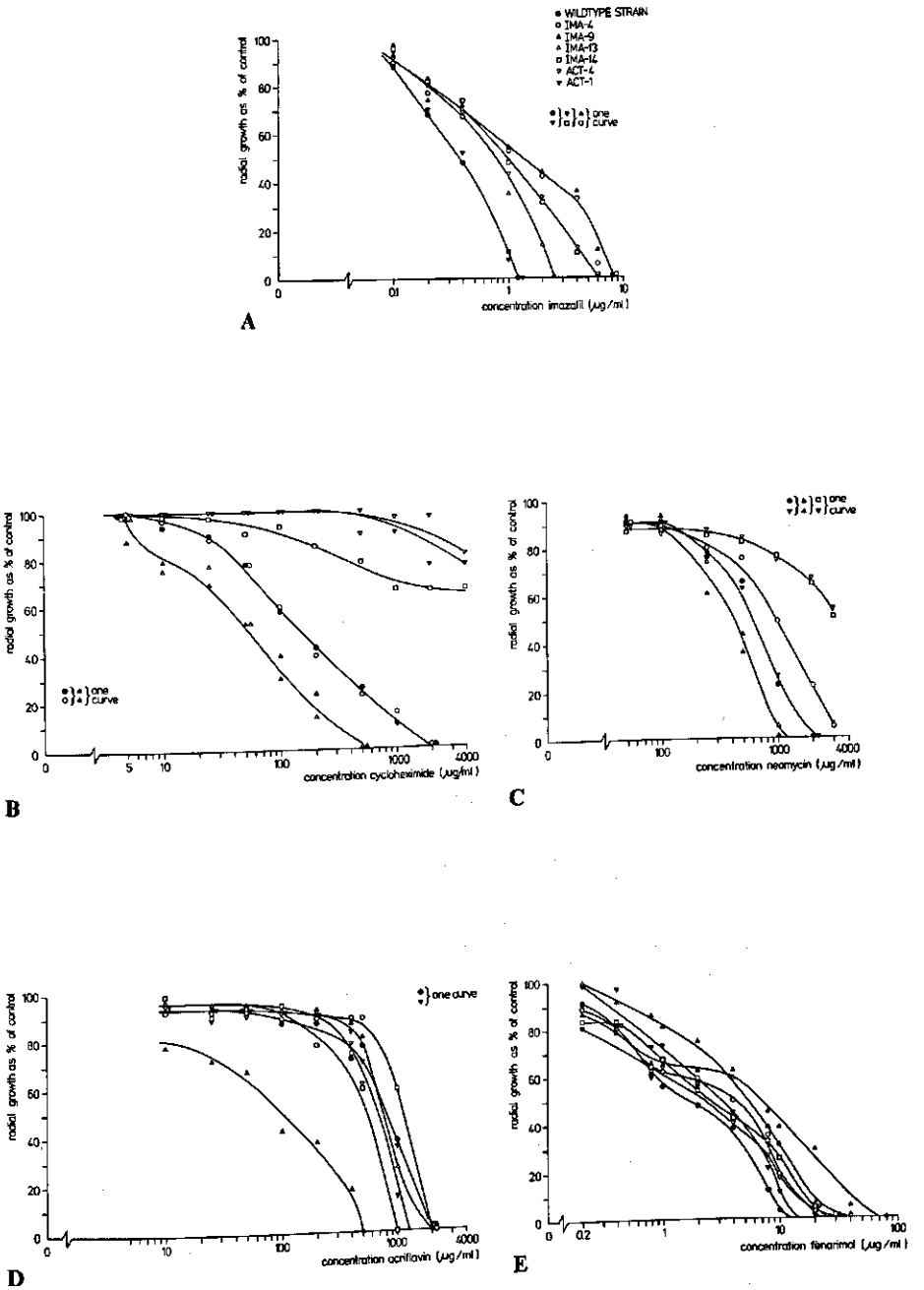


FIG. 23. Dosage response curves of four imazalil-resistant, two cycloheximide-resistant strains and the wild type of *Aspergillus nidulans* (Tables 13.4, 13.5) with respect to imazalil (A) and the pleiotropic effects of these strains (cf. 5.2.2., Fig. 19).

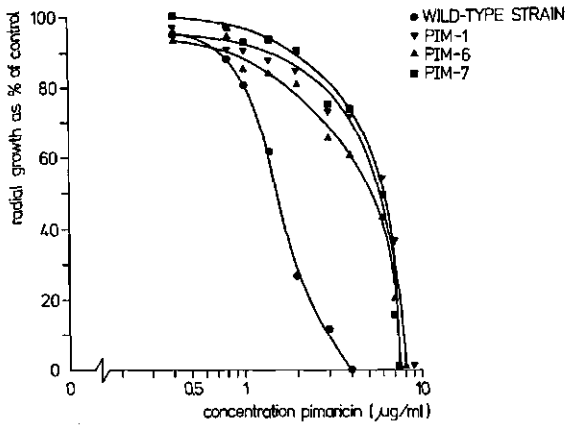


FIG. 24. Dosage response curves of three pimaricin-resistant strains and the wild type of *Aspergillus nidulans* (Table 13.7.) with respect to pimaricin.

following concentrations of the fungicides: benomyl 10, carboxin 2, chloroneb 50 and pimaricin 5 µg/ml.

Two of the three carboxin-resistant strains tested, showed a reduced growth rate in the absence of carboxin, a property which was not observed on agar medium. GEORGOPOULOS and SISLER (1970) reported the same phenomenon of an oxathiin-resistant strain of *U. maydis*. However, in a later publication GEORGOPOULOS et al. (1972) found in recombination analysis that reduced growth rate inherited independently of oxathiin resistance. Nevertheless, it is remarkable that in two independent studies carboxin-resistant strains were found, which required carboxin for maximal growth. If in all instances this would be due to a second mutation, then it might be concluded that the mutation to reduced growth rate promoted the detection of the mutation to carboxin resistance. This hypothesis would be in agreement with the observations on the low frequency in which this mutation occurred as reported by GEORGOPOULOS and SISLER (1970) and as shown in Table 6 (3.3.).

5.5. INFLUENCE OF EXPERIMENTAL CONDITIONS ON TOXICITY

Various factors may influence the toxicity of a fungicide. Composition of medium and temperature influence the level of growth inhibition considerably. Some examples dealing with these factors, are described here.

5.5.1. *Composition of medium*

In experiments with *U. maydis*, *Neurospora crassa* and *Saccharomyces cerevisiae* RAGSDALE and SISLER (1970) showed that carboxin is ten times more fungitoxic in a medium with acetate as sole carbon source than with glucose. This phenomenon was also observed with the carboxin-sensitive strains of *A.*

nidulans. When the wild type (strain No. 8) was grown on SM supplied with different carbon sources, viz. acetate, citrate, fumarate, glucose, malate or succinate, the following ED_{50} values for growth inhibition by carboxin (in μg/ml) were found: acetate 1.2, malate 2, citrate and succinate 5 and fumarate and glucose 9 μg/ml. In addition, it was observed that growth on a medium with the different carbon sources became less dense in the sequence glucose, acetate, succinate, malate, fumarate, citrate. It appeared that carboxin was more than seven times more toxic to the wild type of *A. nidulans* on a medium with acetate as sole carbon source than on a medium with glucose. For the carboxin-resistant strains, however, only small differences were found (Fig. 25). From the data presented it can be concluded that the more resistant the strain is the smaller the difference is between toxicity of carboxin on acetate and that in glucose medium. Since the mutations to carboxin resistance (6.3.), present in these strains, probably determine sensitivity of mitochondrial succinate oxidation to carboxin (GUNATILLEKE et al., 1975a), this means that the elimination of the increased toxicity effect of carboxin in acetate medium is related to the primary action of carboxin.

By comparing the toxicity of the different fungicides to the wild type of *A. nidulans* in SM and CM it was found that benomyl, carboxin, chloroneb, cycloheximide and pimaricin were slightly more toxic in SM than in CM. Imazalil, however, completely inhibited *A. nidulans* at a concentration of 0.4 μg/ml in SM and at 1.2 μg/ml in CM. The effect of all nutrients listed in Table 5 (2.6.4.) on the decreased toxicity of imazalil in SM, was tested at constant

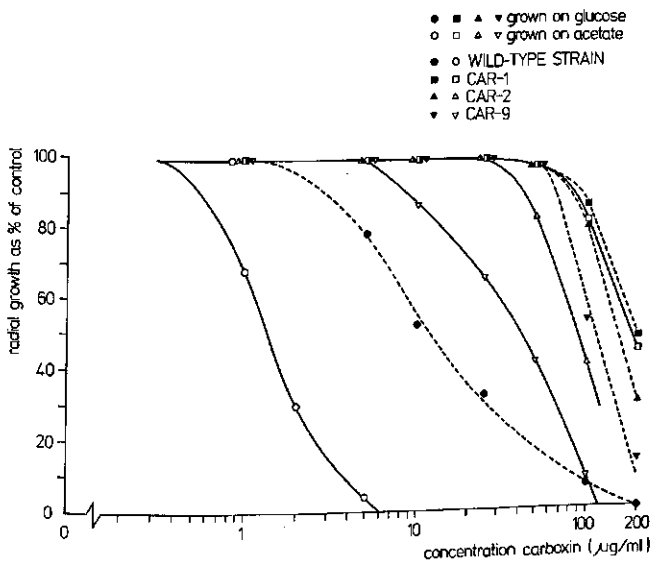


FIG. 25. Dosage response curves of three carboxin-resistant strains and the wild type of *Aspergillus nidulans* with respect to carboxin, grown on SM with glucose (dotted lines) or acetate (solid lines) as carbon source, respectively.

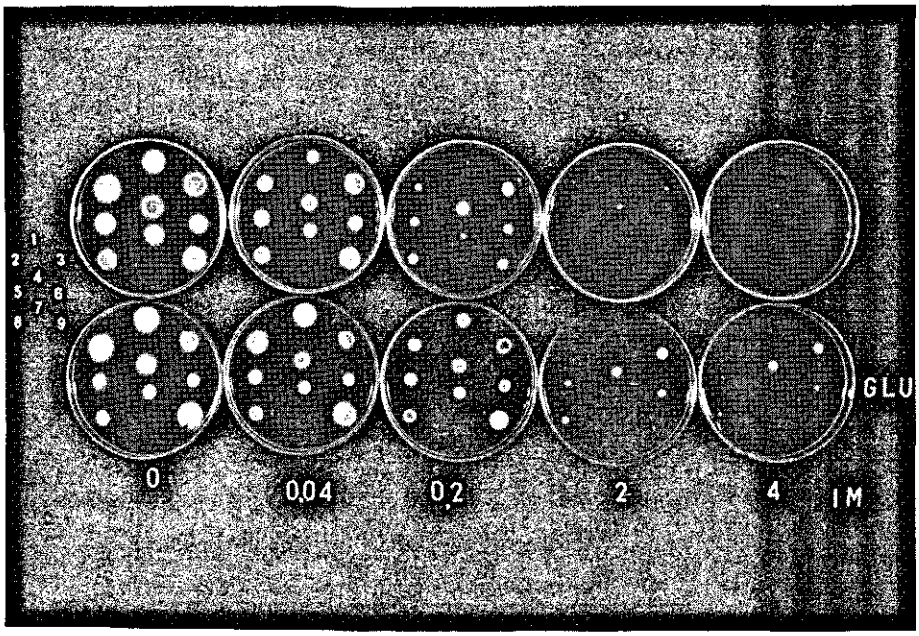


FIG. 26. Effect of glutaric acid on toxicity of imazalil to the master strain (1), the wild type (2), five imazalil-resistant (3–7) and two cycloheximide-resistant strains (8, 9) of *Aspergillus nidulans*, grown on SM containing various concentrations of imazalil (in μg/ml), with or without glutaric acid (400 μg/ml) (GLU).

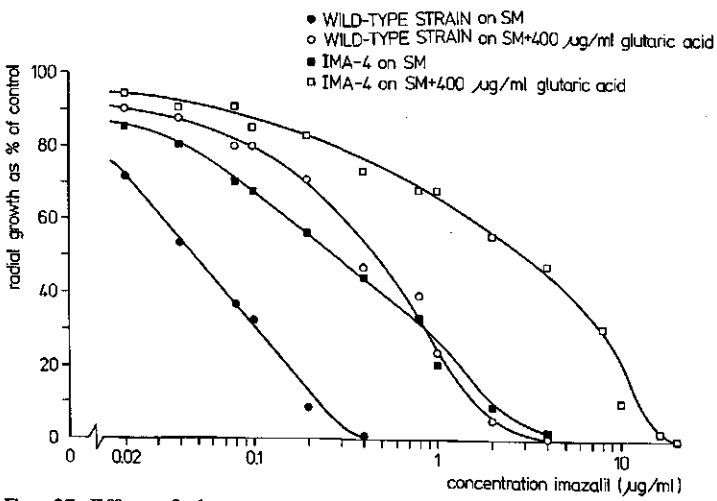


FIG. 27. Effect of glutaric acid on toxicity of imazalil to the wild type and the imazalil-resistant strain IMA-4 (strains No. 2, 3 in Fig. 26, respectively) of *Aspergillus nidulans*, as shown in dosage response curves of these strains with respect to imazalil, grown on SM with or without glutaric acid.

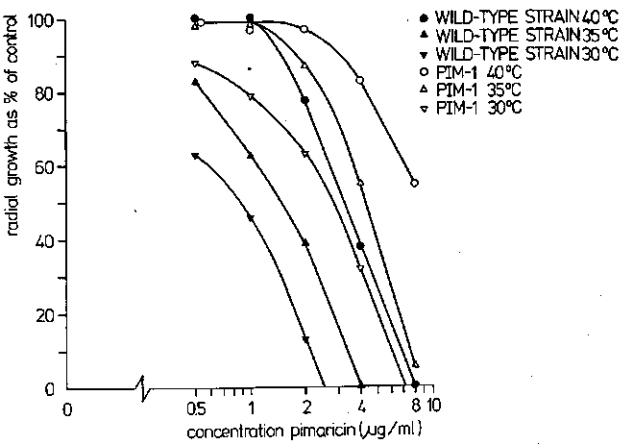


FIG. 28. Effect of temperature on toxicity of pimaricin to the wild type and the pimaricin-resistant strain PIM-1 of *Aspergillus nidulans*, as shown in dosage response curves of these strains with respect to pimaricin, grown at temperatures of 30, 35 or 40°C, respectively.

pH.L-glutamic acid caused a decrease in fungitoxicity in SM. Of the other amino acids only L-cysteïne gave a slight effect. Compounds related to L-glutamic acid, viz. D-glutamic acid, glutaric acid and L-glutamine caused a comparable effect. The influence of glutaric acid on the toxicity of imazalil to the wild type and imazalil-resistant strains is presented in Figs 26 and 27. Fig. 27 shows that in the wild type as well as in the resistant strain glutaric acid caused a decrease in imazalil toxicity by a factor six. A similar phenomenon was observed by VAN DEN BOSSCHE (1974) in studies with miconazole, a compound related to imazalil. Interference with uptake and/or utilization of glutamine was supposed to be involved in the mode of action of miconazole.

5.5.2. *Temperature*

A. nidulans can grow over a wide temperature range from 20° to over 40°C. The level of toxicity to *A. nidulans* of benomyl, carboxin, chloroneb, cycloheximide and imazalil is rather similar at these temperatures. However, pimaricin sensitivity appeared to depend on incubation temperature. The higher the temperature the more pimaricin resistant the fungus is. This effect was observed for the wild type as well as for the pimaricin-resistant strains (Figs 28, 29).

5.6. MORPHOLOGICAL ALTERATIONS OF THE RESISTANT STRAINS

In studying the effects of fungicides, sometimes alterations in growth and morphology of fungi were observed, on the one hand caused by the mutation to resistance, on the other hand as a direct effect of the fungicide itself. Some examples of both types of alterations are described here.

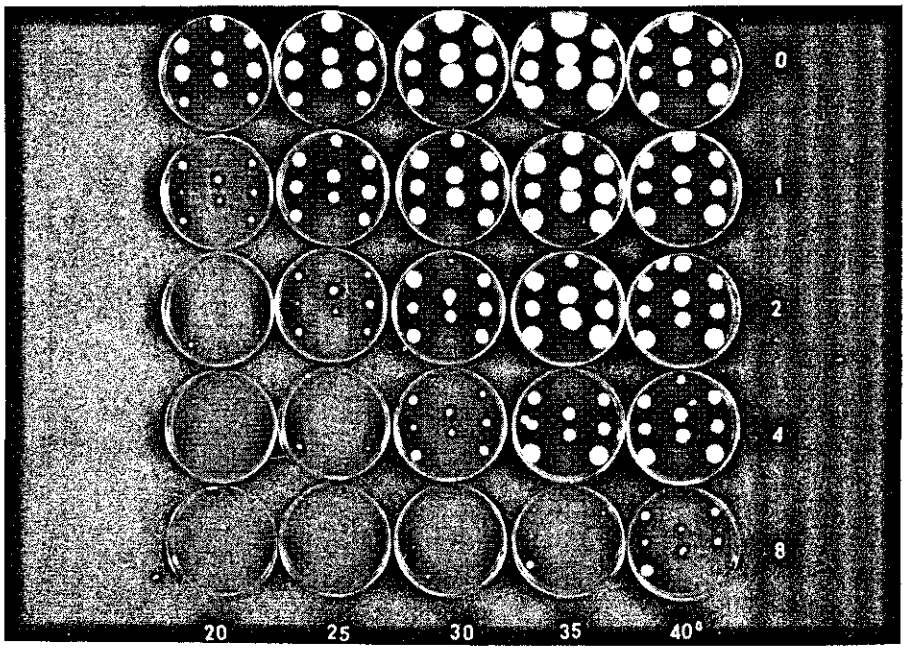


FIG. 29. Effect of temperature on toxicity of pimaricin to the wild type (at the top of each plate) and eight pimaricin-resistant strains of *Aspergillus nidulans* grown on CM containing (from top to bottom) various concentrations of pimaricin (in μg/ml) and incubated (from left to right) at temperatures of 20, 25, 30, 35 or 40°C.

5.6.1. *Alterations due to the mutations to resistance*

Chloroneb-resistant mutants of *A. niger* and *P. expansum* appeared to form colonies which sporulated poorly on CM (4.4., Fig. 11). The colonies were phenotypically almost white in contrast to the wild type, the colonies of which reflected the color of their conidia (black, green). When the resistant strains grew on a medium containing chloroneb or a related compound, the colonies, however, were sporulating abundantly. Thus, it seemed that the resistant mutants required the fungicide for sporulation. Chloroneb-resistant strains of *A. nidulans* sporulated profusely, but here the mutants appeared to be partially cold-sensitive. At 20°C the growth of the mutants was only 25% of that of the wild type at the same temperature.

A reduced colonial growth was observed of all pimaricin-resistant mutants of *A. nidulans* (6.8., Figs 47, 48). It is evident that a mutation to pimaricin resistance, implies a reduction of the colonial growth.

5.6.2. *Alterations due to the fungicides*

In *A. nidulans* benomyl-resistant mutants did no longer sporulate at a con-

centration exceeding 5 μg/ml benomyl, although colonies did grow at a concentration over 100 μg/ml. The colonies had a brownish color, probably due to a melanin-like pigment secreted in the medium. Sensitive strains did not show this behaviour. BULL and FAULKNER (1965) reported that the wild type of *A. nidulans* produces a large amount of melanin. Benomyl-resistant strains of the other fungi studied (3.3., Table 6) did sporulate at all toxicant concentrations tested.

Another type of inhibition of sporulation in *A. nidulans* was found with the imazalil-resistant strains, when grown at a concentration exceeding 2 μg/ml imazalil. The colonies were white indicating that mycelium but no conida were formed. With the wild-type strain growing on imazalil-containing medium this effect was only observed at concentrations which caused maximal inhibition.

The two types of inhibition of sporulation in *A. nidulans* due to a toxicant suggest that sporulation in *A. nidulans* in contrast to *A. niger*, can rather easily be disturbed.

5.7. DISCUSSION

Mutants resistant to benomyl, thiabendazole, carboxin, chloroneb, cycloheximide, imazalil and pimaricin were studied intensively with *Aspergillus nidulans*. Of the latter fungus dosage response relationships of fungicide-resistant strains were examined.

Chloroneb-resistant strains of all fungi investigated, viz. *A. nidulans*, *Aspergillus niger*, *Penicillium expansum* and *Ustilago maydis* showed cross-resistance to PCNB, a related fungicide of the aromatic hydrocarbon group. In addition to cross-resistance of a chloroneb-resistant strain of *U. maydis* to the aromatic hydrocarbons which was reported by TILLMAN and SISLER (1973), it was here shown to 3-phenylindole.

Usually resistance to benomyl or thiabendazole resulted in cross-resistance to the other compound but in rare cases there was only resistance to benomyl or more frequently only to thiabendazole with increased sensitivity to benomyl. The latter phenomenon was indicated as negative cross-resistance. The rare occurrence of a lack of cross-resistance in some benomyl- and thiabendazole-resistant mutants of *A. nidulans* has been reported earlier (VAN TUYL et al., 1974). Negative cross-resistance was also found in *A. niger* and *P. expansum* (VAN TUYL, 1975b) and in *Rhodotorula rubra* and *U. maydis*. Thus, negative cross-resistance appears to occur rather generally in fungi sensitive to benzimidazole fungicides. This phenomenon proved to be very useful in the elucidation of the mechanism of action of the benzimidazole fungicides (DAVIDSE, 1974, 1975, 1976), which was shown to be based on binding to tubulin, the subunit of microtubuli, causing inhibition of mitosis.

MATHRE (1971) and WHITE (1971) revealed that carboxin inhibits mitochonchondrial respiration of *U. maydis* at or close to the site of action of succinate

oxidase. GUNATILLEKE et al. (1975a) obtained a similar result in studies with resistant mutants of *A. nidulans*.

Observations in this study on carboxin-resistant strains of *A. nidulans* support the view that carboxin acts primarily on reactions in the tricarboxylic acid cycle. Carboxin inhibited growth of the wild type but not in the most resistant strains, seven times more strongly on medium with acetate than with glucose as sole carbon source.

Pleiotropic effects of mutations to imazalil resistance in *A. nidulans*, as described by VAN TUYL (1977) were also found in resistant strains of *A. niger*. However, with imazalil-resistant strains of *Cladosporium cucumerinum* and *Phialophora cinerescens* these effects were not found. Some of these pleiotropic effects, viz. hypersensitivity and cross-resistance to cycloheximide in *A. nidulans* were also reported by WALDRON and ROBERTS (1974a, b) and GUNATILLEKE et al. (1975b) in cold-sensitive and chloramphenicol-resistant mutants, respectively. In addition to cycloheximide which inhibits cytoplasmic protein synthesis in *A. nidulans* (TURNER, 1973; WALDRON and ROBERTS, 1974b); chloramphenicol is another well-known inhibitor of protein synthesis. These results, however, do not necessarily imply that imazalil interferes with protein synthesis. On the other hand, pleiotropic effects of a single nuclear mutation, viz. to increased resistance to eight inhibitors of mitochondrial functions and to one inhibitor of cytoplasmic protein synthesis (cycloheximide), could be explained by a reduced permeability of the plasma membrane (RANK et al., 1975). Moreover, in studies with miconazole, a compound structurally related to imazalil, it was assumed that miconazole induced permeability changes of the cell membrane (VAN DEN BOSSCHE et al., 1975). The complete cross-resistance between imazalil and fenarimol in *A. nidulans* and *A. niger* suggests interference with reactions in the sterol biosynthetic pathway, which are believed to be the primary site of action of triarimol (RAGSDALE and SISLER, 1973), a compound closely related to fenarimol. These data indicate that the action of imazalil may be an interference with cell membrane permeability, and that resistance to this compound can be associated with a modified composition of sterols in these membranes.

Finally, it can be concluded that by characterizing genetically well-defined mutants resistant to a certain toxicant with regard to patterns of cross-resistance, influence of medium on toxicity, etc., much information can be gathered, which can contribute to the elucidation of the mechanism of action of and the mechanism of resistance to a toxicant.

6. GENETIC ANALYSIS OF FUNGICIDE RESISTANCE IN *ASPERGILLUS NIDULANS*

6.1. INTRODUCTION

Aspergillus nidulans was explored genetically by PONTECORVO et al., (1953b); by using this organism they discovered and elucidated the parasexual cycle. The advantages of *A. nidulans* as an experimental organism for genetic studies can be summarized as follows: it is a haploid eukaryote growing rapidly on a minimal medium of mineral salts and glucose; it produces abundant, darkly pigmented, uninucleate conidia; the colonial growth form on agar makes it suitable for examination of many discrete colonies; mutant strains can be isolated easily and include types differing from the wild type in color of conidia, nutritional requirements, ability to grow on various sugars and resistance to inhibitory agents; it is homothallic so any strain can be crossed to any other one for genetic analysis in the sexual cycle; strains with relatively stable diploid nuclei can be selected in the parasexual cycle and used in tests on gene action, in genetic analysis through mitotic crossing-over and for assigning a new mutant to its linkage group.

Mutants of *A. nidulans* resistant to various inhibitors (ROPER, 1971) have been obtained, for instance to acriflavin (ROPER and KÄFER, 1957); actidione (WARR and ROPER, 1965); and p-fluorophenylalanine (SINHA, 1967, 1972); methylammonium (ARST and COVE, 1969); fluoropyrimidines (PALMER et al., 1975). Interactions between resistance and nutritional requirements have been observed in several cases offering the possibility of two-way selection and of studying interlocus specificity of mutagenic activity (ALDERSON and HARTLEY, 1969). Such a two-way selection has been applied to fluoroacetate-resistant strains unable to utilize acetate as sole carbon source (APIRION, 1962, 1965). Recently, extranuclear genes in *A. nidulans* have been reported by ROWLANDS and TURNER (1973) among mutations to oligomycin resistance and by GUNATILLEKE et al. (1975b) to chloramphenicol resistance. Only a few genetic studies on resistance to agriculturally important fungicides in *A. nidulans* have been reported. THRELFALL (1968, 1972) described mutants resistant to pentachloronitrobenzene and HASTIE and GEORGOPOULOS (1971) reported resistance to benzimidazole fungicides. In this study seven fungicides were chosen for a genetic study in which the frequency of mutation to resistance (chapter 3), the characteristics of the mutants such as the levels of resistance (chapter 5) and the genetic basis of the resistant mutants (this chapter) were compared. Using the sexual and parasexual cycles (chapter 2), the number of genes involved, their map position on the linkage groups, dominance relationships and additive effects of the genes conferring resistance were determined.

6.2. Nomenclature and properties of the resistant strains

Mutant strains resistant to the following toxicants were selected: benomyl, carboxin, chloroneb, cycloheximide, imazalil, oligomycin, pimaricin and thiabendazole. In Table 13 strains used for further genetic investigations are listed according to the selective agent. This table presents the most important data of these mutants, viz. the original strain and the mutagens through which they were obtained. In some cases the degree of resistance is indicated. Investigations carried out with each mutant are listed. In the nomenclature used a distinction is made between the mutant strains and the mutations they carry. The notation is derived from the name of the toxicant in the presence of which the resistant mutants were selected except in the case of the thiabendazole-resistant strains, which are included in one group with the benomyl-resistant strains (Table 13.1). Mutant strains are indicated with three capital letters followed by the mutant number, and the mutations they carry are indicated with the same three letters in italics as the respective mutant strain, followed by a capital locus-specific letter and the mutation number according to Clutterbuck (1973, 1974). Mutant and mutation number are identical in the cases where no reports of resistant strains were known from the literature; otherwise, the mutation numbers continue the series of numbers mentioned in the literature.

6.3. Determination of the number of loci involved in fungicide resistance

The genetic basis of the inheritance of fungicide resistance was investigated in crosses of each resistant strain (Table 13) with a sensitive one. In all crosses resistance and sensitivity segregated in about 1:1 ratio, indicating inheritance of single gene mutations. In the case of imazalil- and cycloheximide-resistant mutants the pleiotropic effects (5.2.2.) appeared to segregate with the resistance to the toxicant, on the presence of which the mutant was selected, except in strain IMA-17 where cold sensitivity was determined by an independent single gene. Some results with the imazalil-resistant mutants were at first sight contradictory to this. When an imazalil-resistant strain, which also carried the mutation to acriflavin resistance *acrA*, was crossed with an imazalil- and acriflavin-sensitive strain it always yielded four classes of progeny with respect to imazalil sensitivity. This appeared to be due to the presence of the mutation to acriflavin resistance (*acrA*) which also caused a certain degree of imazalil resistance (6.7., Fig. 41). This phenomenon is understandable in view of the many pleiotropic effects detected in the imazalil-resistant mutants (5.2.2., Table 11).

By crossing different mutants resistant to a toxicant it was examined whether more than one gene was involved. About hundred colonies from every cross were generally tested for resistance; if no sensitive recombinants were found

TABLES 13.1–13.7. Data about the resistant mutants of *Aspergillus nidulans*.

The data are ordered in 7 Tables according to the toxicants to which resistant mutants were selected. The general terms used are:

Mutant: mutant strains are indicated with capitals.

Initial strain: strain of origin, numbers of which are listed in Table 1.

Mutagen: UV = ultraviolet light, NG = N-methyl-N′-nitro-N-nitrosoguanidine; SP = spontaneous mutation.

Mutation: mutations are indicated with three letters followed by a capital locus specific letter and a mutation number, continuing numbers known from the literature.

Investigations concerning the mutants: mutants used for detailed investigations:
add. = additive effects in fungicide resistance (6.8.); *alloc.* = allocation to linkage groups (6.4.); *cross.* = patterns of cross-resistance (5.2.); *dom.* = dominance relationships (6.7.); *dos.* = dosage response graphs (5.3.); *infl.* = influence of medium, temperature on toxicity (5.5.); *map.* = mapping of mutant loci on linkage groups (6.5.); *rec.* = intragenic recombination analysis (6.6.).

TABLE 13.1. The benomyl- and thiabendazole-resistant mutants.

Mutant*	Initial strain	Mutagens	Mutation	ED_{50} (μg/ml)		Investigations concerning the mutants
				benomyl	thiaben-dazole	
Initial strain	3	–	–	0.8	8	
BEN-1	3	UV	*benA3***	24	12	cross.; dos.; rec.;
BEN-2	3	UV	*benA4*	6	90	
BEN-3	3	UV	*benA5*	15	85	map.;
BEN-4	3	UV	*benA6*	7	90	
BEN-5	3	UV	*benA7*	8	90	alloc.;
BEN-6	3	UV	*benA8*	8	90	
BEN-7	3	UV	*benA9*	6	90	
BEN-8	3	UV	*benA10*	6	90	alloc.; dom.; dos.; map.;
BEN-9	3	UV	*benA11*	8	100	alloc.;
BEN-10	3	UV	*benA12*	8	100	
BEN-11	3	UV	*benA13*	8	80	
BEN-12	3	UV	*benA14*	8	90	
BEN-13	3	UV	*benA15*	30	125	R } ***
BEN-14	3	UV	*benA16*	0.2	60	186 }
BEN-15	3	UV	*benA17*	8	75	
BEN-16	3	UV	*benA18*	24	12	
BEN-17	3	UV	*benA19*	0.2	60	add.; cross.; dom.; rec.;
BEN-18	3	UV	*benA20*	5	75	
BEN-19	3	UV	*benA21*	20	65	
BEN-20	3	UV	*benA22*	6	90	
BEN-21	3	UV	*benA23*	6	100	
BEN-22	3	UV	*benA24*	15	60	
BEN-23	3	UV	*benA25*	10	65	
BEN-24	3	UV	*benA26*	6	90	
BEN-25	3	UV	*benA27*	6	100	
BEN-26	3	UV	*benC28*	2.2	13	alloc.; dos.; map.;
BEN-27	1	UV	*benB29*	1.7	13	add.; alloc.; dom.; dos.; map.;
BEN-28	1	UV	*benA30*	5	80	alloc.;

* BEN-13, 14 and 17 were isolated on thiabendazole (25 μg/ml), the other strains on benomyl (2 μg/ml).

** *benA1*, *benA2* are mentioned by HASTIE and GEORGOPOULOS (1971).

*** Notations used by DAVIDSE (1974, 1975, 1976).

TABLE 13.2. The carboxin-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Investigations concerning the mutants
CAR-1	9	UV	*carA1*	alloc.; dom.; dos.; infl.; map.;
CAR-2	8	UV	*carB2*	alloc.; dom.; dos.; map.;
CAR-3	8	UV	*carB3*	
CAR-4	8	UV	*carB4*	
CAR-5	8	UV	*carB5*	
CAR-6	8	UV	*carA6*	
CAR-7	8	UV	*carA7*	
CAR-8	8	UV	*carB8*	
CAR-9	8	UV	*carC9*	alloc.; dom.; dos.; map.;
CAR-10	3	UV	*carA10*	

TABLE 13.3. The chloroneb-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Investigations concerning the mutants
CHL-1	3	SP	*chlA1*	dos.;
CHL-2	8	SP	*chlA2*	
CHL-3	8	UV	*chlA3*	
CHL-4	8	UV	*chlA4*	dos.;
CHL-5	8	UV	*chlA5*	
CHL-6	8	UV	*chlA6*	
CHL-7	8	UV	*chlA7*	
CHL-8	2	UV	*chlA8*	alloc.; dom.; dos.;
CHL-9	2	UV	*chlA9*	alloc.;
CHL-10	IMA-19*	UV	*chlA10*	map.;

* IMA-19 is listed in Table 13.5.

TABLE 13.4. The cycloheximide-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Investigations concerning the mutants
ACT-1	10	SP	*actB2**	alloc.; dos.; dom.; map.;
ACT-2	3	NG	*actC3*	
ACT-3	3	NG	*actD4*	alloc.; cross.;
ACT-4	3	NG	*actC5*	alloc.; cross.; dos.; dom.; map.;
ACT-5	3	NG	*actC6*	
ACT-6	3	NG	*actA7*	alloc.;
ACT-7	3	NG	*actE8*	alloc.; cross.; map;
ACT-8	3	SP	*actC9*	
ACT-9	3	SP	*actB10*	
ACT-10	3	SP	*actA11*	

* *actA1* is reported by WARR and ROPER (1965) and present in strain No. 20 (Table 1).

TABLE 13.5. The imazalil-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Degree of resistance*	Investigations concerning the mutants
Initial strain	3	–	–	–	
IMA-1	3	UV	*imaA1*	++	
IMA-2	3	UV	*imaA2*	+	
IMA-3	3	UV	*imaA3*	++	
IMA-4	3	UV	*imaA4*	++	alloc.; cross.; dos.; dom.; map.;
IMA-5	3	UV	*imaA5*	+	
IMA-6	2	UV	*imaA6*	++	
IMA-7	2	UV	*imaA7*	++	
IMA-8	2	UV	*imaA8*	++	
IMA-9	3	SP	*imaB9*	+++	alloc.; cross.; dos.; dom.; map;
IMA-10	3	SP	*imaC10*	+	alloc.; cross.; map;
IMA-11	3	SP	*imaB11*	++	
IMA-12	3	SP	*imaB12*	++	
IMA-13	3	SP	*imaD13*	+	alloc.; cross.; dos.; map.;
IMA-14	3	SP	*imaE14*	+	alloc.; cross.; dos.;
IMA-15	3	SP	*imaF15*	+	alloc.; cross.;
IMA-16	3	NG	*imaA16*	+++	add.;
IMA-17	3	NG	*imaB17*	++	
IMA-18	3	NG	*imaG18*	+	alloc.; cross.; map.;
IMA-19	3	NG	*imaH19*	+	alloc.; cross.; map.;
IMA-20	3	NG	*imaA20*	+	
IMA-21	3	NG	*imaA21*	++	

* The degree of imazalil resistance is expressed as the minimal inhibitory concentration on complete medium in μg/ml.

– = 1
\+ = 2.5–5
++ = 5–8
+++ = 8–12

TABLE 13.6. The oligomycin-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Investigations concerning the mutants
OLI-1	10	UV	*oliC13**	dos.; map.;
OLI-2	10	UV	*oliC14*	
OLI-3	10	UV	*oliC15*	
OLI-4	10	UV	*oliC16*	
OLI-5	10	UV	*oliC17*	alloc.; dos.;
OLI-6	10	UV	*oliC18*	
OLI-7	10	UV	*oliC19*	

* *oliA1*,*oliB2* and *oliC3*-12 are reported by ROWLANDS and TURNER (1973) and WATSON and TURNER (1976); *oliC3* is present in strain No. 29 (Table 1).

TABLE 13.7. The pimaricin-resistant mutants.

Mutant	Initial strain	Mutagens	Mutation	Investigations concerning the mutants
PIM-1	3	UV	*pimA1*	alloc.; dos.; dom.; infl.; map.;
PIM-2	3	UV	*pimA2*	
PIM-3	3	UV	*pimA3*	
PIM-4	3	UV	*pimB4*	
PIM-5	3	UV	*pimA5*	
PIM-6	3	UV	*pimA6*	add.; alloc.; dos.;
PIM-7	3	SP	*pimB7*	add.; alloc.; dos.;
PIM-8	3	SP	*pimB8*	
PIM-9	3	SP	*pimB9*	
PIM-10	3	SP	*pimB10*	map.;

among them, it was assumed that one locus was responsible for the two mutations (2.7.4.). In this way in 26 strains showing a high degree of benomyl and/or thiabendazole resistance one locus (*benA*) was found to be responsible for the different mutations (Table 13.1) and in two mutants showing a relatively low degree of benomyl and thiabendazole resistance another two loci were established (*benB* and *benC*). The *ben-1* mutation (present in strain No. 19) obtained by HASTIE and GEORGOPOULOS (1971) also appeared to be allelic to *benA*. The strain carrying the *ben-2* mutation to resistance at a second locus obtained by these authors got lost, but in view of the map position of this mutation on linkage group II (6.4., 6.5.), it was probably allelic to *benB*.

In the ten mutations to carboxin resistance (Table 13.2) three different genes were involved (*carA*, *carB* and *carC*). GUNATILLEKE et al. (1975a) have independently determined three genes conferring carboxin resistance, namely *cbxA*, *cbxB* and *cbxC* (present in strains No. 24, 25, 26), which proved to be allelic to *carC*, *carA* and *carB*, respectively.

In all ten chloroneb-resistant strains (Table 13.3) mutations at one gene (*chlA*) appeared to be responsible for chloroneb resistance. These mutations were presumably alleles of *pcnbA*, described by THRELFALL (1968), which conferred resistance to pentachloronitrobenzene, because of the complete cross-resistance to chloroneb and pentachloronitrobenzene and the similarity in map position of the genes on linkage group III (6.4., 6.5.).

The seven mutations to oligomycin resistance (Table 13.6) proved to be alleles of *oliC* (present in strain No. 29) already described by ROWLANDS and TURNER (1973) and TURNER and WATSON (1976) as *oli-2*.

The resistance of ten mutants to pimaricin (Table 13.7) was determined by two freely recombining loci, called *pimA* and *pimB*.

Both cycloheximide and imazalil resistance proved to be defined by a multigenic system. WALDRON and ROBERTS (1974b) showed the existence of at least eight genes conferring cycloheximide resistance. The ten mutations to cycloheximide (actidione) resistance (Table 13.4) were found to be distributed among five loci. Two of these mutations appeared to be alleles of *actA* (present

in strain No. 20) described by WARR and ROPER (1965). Three of the loci (*actC*, *actD* and *actE*) also conferred imazalil resistance (Table 11), but only one of them (*actC*) was picked up among the mutants selected directly for imazalil resistance. Among 21 mutations to imazalil resistance (Table 13.5) eight genes were established. Eleven mutations were alleles of *imaA*, a gene causing the highest level of resistance to imazalil. Four mutations were allelic to *imaB*. This gene, also leading to extra-sensitivity to cycloheximide and resistance to chloramphenicol and fenarimol (Fig. 23), appeared to be allelic to *camD* (present in strain No. 23), a mutation to chloramphenicol resistance identified by GUNATILLEKE et al. (1975b) and to a gene determining fenarimol resistance (DE WAARD and GIESKES, 1977; DE WAARD and SISLER, 1976). Two other nuclear mutations to chloramphenicol resistance described by the former authors as *camB* and *camC* (present in strains No. 21, 22), did confer also a low degree of imazalil resistance. The six remaining mutations to imazalil resistance of which four conferred also cycloheximide resistance were identified as mutations at different loci (*imaC*, *D*, *E*, *F*, *G*, *H*). *ImaH* was found to be allelic to *actC*. A mutation to imazalil resistance which also conferred cold sensitivity and cycloheximide resistance *imaG18* was found to be allelic or closely linked to *actA*, the known alleles of which, however, did not confer resistance to imazalil. In a cross of strains carrying *imaG* and *actA* one out of 400 colonies proved to be sensitive to cycloheximide. In total, the analysis of the different imazalil- and cycloheximide-resistant mutations resulted in the identification of at least seven and ten different genes determining resistance to cycloheximide and imazalil, respectively.

6.4. ALLOCATION OF THE MUTATIONS TO LINKAGE GROUPS

The loci discovered in the recombination analysis (6.3.) were allocated to linkage groups by mitotic haploidization of heterozygous diploids; the results are presented in Table 14. Benomyl was generally used as the haploidizing agent. Because of the higher sensitivity to benomyl of a diploid compared with a haploid strain (6.7. Figs 32, 33), the segregation of haploids from the diploids is favoured so that haploids can easily be selected (Fig. 30). To induce haploidization of a diploid without a mutation to benomyl resistance in the parental strains, a concentration of 0.7 μg/ml was used; however, in most of the diploids a mutation to benomyl resistance was present and linked with the marker for fawn-colored conidia (strains No. 8, 11), with these diploids 1 μg/ml benomyl was used, resulting in only fawn-colored, benomyl-resistant haploids. Haploid strains obtained in this way were classified on the basis of the segregation of at least one nutritional marker, which should be on another chromosome than the conidial-color marker (2.7.2.); this is necessary to distinguish haploidization from mitotic crossing-over. In the allocation of *benC28* the fawn-colored haploids carrying the *benA10* mutation could not be used; therefore, griseofulvin was used as the haploidizing agent at a con-

TABLE 14. Allocation of mutations to resistance to linkage groups in *Aspergillus nidulans*.

Mutation to allocate	Diploid combination: Initial strain	Diploid combination: Master strain	total no. of haploids: *res.*	total no. of haploids: +	Number of recombinant haploids between the mutation to allocate and each linkage group-marker of the mutant- and master strain*															
					biA +	+ *yA*	*wA* +	*acrA* +	+ *chlA*	+ *phenA*	+ *pyroA*		+ *lysB*		+ *sB*		+ *nicB*	*carA* +	+ *riboB*	+ *benA, fwA* *ornB* +
benA10	3	1	47	0	0	30			0	11	0	23	0	8			0	23		
benA10	3	2	21	0	0	15									0	6			0	0
benA7	3	1	33	0	0	19			0	10	0	17	0	10			0	13		
benA11	3	2	60	0	0	41									0	26			0	0
benB29	12	2'	21	0	0	19	0	0	0	10			0	4	0	7			0	13
benC28	3	11	7	8	2	5	3	6	5	3	2	4	7	3			0	0	7	0
carA1	9	1	35	43	23	21	18	16	22	23	21	4	18	7			0	0	12	31
carB2	8	1	20	0**	0	10	0	9	0	8	0	6	0	4			0	7	0	0
carC9	8	1	11	10	3	5	4	6	0	0	6	2	3	3			6	0	5	4
chlA8	2	11	26	3			5	2	0	0	0	12	2	13	16	1	3	14	0	26
chlA9	2	12	11	10	1	10	0	10	0	0			8	2	8	4			5	4
actA7	3	11	3	17	6	0	7	2	0	0	7	1	13	0			15	2	0	3
actB2	10	1	51	43			32	11									0	0	0	51
actC5	3	11	5	22	8	2	11	2	0	0	16	3	14	4			12	2	0	3
actD4	3	11	7	41	13	3	0	0	19	2	17	5	12	3			17	3	0	7
actE8	3	11	6	25	14	2	0	0	11	2	8	6	15	1			14	5	0	6
imaA4	3	11	25	16	10	14	20	9	6	12	5	15	6	11			0	1	0	25
imaA4	3	2	25	19	3	16									3	17				
imaB9	3	11	16	16	5	11	10	11	10	6	7	5	0	0			4	9	0	16
imaC10	3	11	6	26	17	4	0	0	19	3	7	4	17	2			16	1	0	6
imaD13	3	11	0	18**	12	0	9	0	13	0	10	0	10	0			9	0	0	0
imaE14	3	11	14	28	15	4	2	0	12	5	14	6	7	3			10	5	0	14
imaF15	3	11	6	25	2	0	10	5	12	2	9	1	16	4			15	2	0	6
imaG18	3	11	7	24	9	3	13	4	0	0	11	4	8	2			11	3	0	7
imaH19	3	11	14	4	4	3	3	8	0	0	2	10	2	6			1	9	0	14
oliC17	10	1	16	8	9	9	8	5	4	4	2	10	8	11			0	0	0	16
pimA1	3	11	9	20	7	4	6	3	6	5	0	0	10	3			8	3	0	9
pimA6	3	11	3	14	4	2	12	0	7	2	6	1	6	1			11	1		
pimB7	3	11	3	12	0	0	7	1	5	2			4	2			7	2	0	3
linkage group					I		II		III		IV		V		VI		VII		VIII	
master strains			1(1')		*yA2*		*acrA1*		*phenA2*		*pyroA4*		*lysB5*		*sB3*		*nicB8*		*(benA30)* *riboB2*	
			2(2')		*yA2*		*acrA1*		*(chlA9)*											
			11		*yA2*				*phenA2*		*pyroA4*		*lysB5*				*nicB8*		*benA10, fwA1*	
			12		*biA1*		*benB29*		*phenA2*				*lysB5*							
initial strains			3		*biA1*		*acrA1*												*benA10, fwA1*	
			8		*biA1*														*ornB7*	
			9		*biA1*		*wA2*										*carA1*			
			10		*biA1*		*wA2*													

* The relevant markers (cf. Table 1) of the mutant and master strains are arranged in their linkage groups as follows.
res = mutation to resistance.
** Only one type of resistance was found, because the mutation involved appeared to be located to the same linkage group as the marker selected for *(benA)*

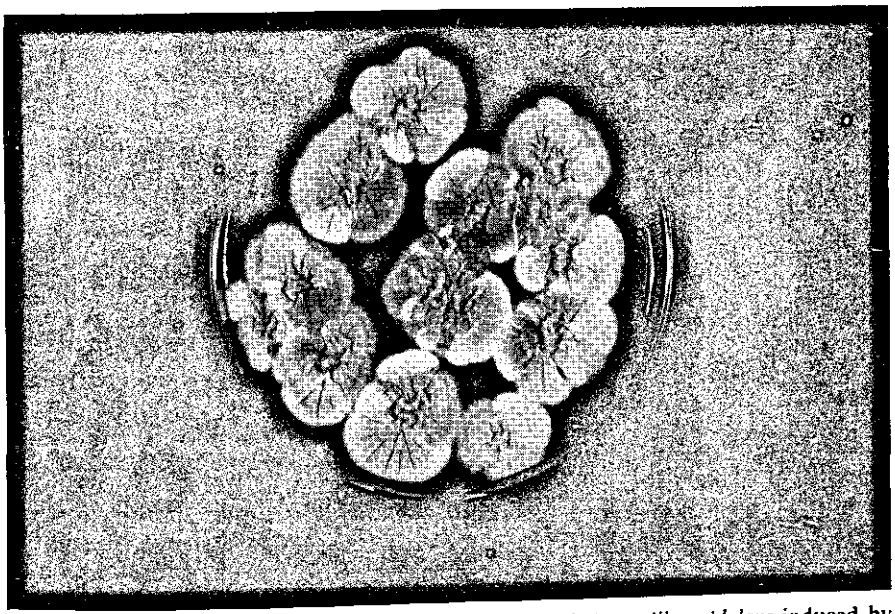


FIG. 30. The haploidization of a heterozygous diploid of *Aspergillus nidulans* induced by growing the diploid on benomyl-containing CM (BEN 1 μg/ml).

centration of 25 μg/ml (KAPPAS and GEORGOPOULOS, 1974). In this case only the yellow and green segregants were isolated and tested.

Table 14 lists the numbers of recombinants between the mutation to resistance to be located and each linkage-group marker of the master strain. Thus, within 28 heterozygous diploids at least 21 different loci, underlined in Table 14, were allocated to seven different linkage groups, by elimination of the other groups.

6.5. MAPPING OF THE MUTANT LOCI ON THEIR LINKAGE GROUP

Meitotic mapping of a number of different loci conferring fungicide resistance was carried out by crossing the mutants with meitotic mapping strains, carrying a number of markers on the relevant linkage group (MATHER, 1937). The crosses made are summarized in Table 15. The meitotic mapping data of each cross are subdivided in Tables 15.1–15.10. The most probable maps are given (some results gave no definite answer on the exact map position).

On linkage group VIII *benA* was located 34 units from *tsD*, 5 units from *ornB* and 28 units from *fwA* (Table 15.1); *carB* was mapped between *cnxB* and *fpaD* (Table 15.3) and *imaD* 3 units from *chaA* and 5 from *palB* (Table 15.8).

For mapping five loci conferring resistance on linkage group VII the respective mutant loci *actB*, *benC*, *carA*, *imaA* and *oliC* were recombined in one strain together with *choA* (originating from strain No. 13); the strain was cros-

TABLE 15. Meiotic mapping of different loci conferring resistance in *Aspergillus nidulans*.

Cross No.	Strains involved*	Loci considered**	Progeny number	Linkage group	Table No.
1	BEN-3 × 7	*benA* × *tsD, ornB*	420	VIII	15.1
2	BEN-8 × 4	*benA* × *ornB, fwA*	398	VIII	15.1
3	12 × 16, 17	*benB* × *adD, acrB*	629	II	15.2
4	CAR-2 × 5	*benA, fwA, carB* × *cnxB, fpaD*	366	VIII	15.3
5	CHL-10 × 24	*chlA, imaG* (= *actA*) × *phenA, carC*	370	III	15.4
6	ACT-4 × 24	*act C* (= *imaH*) × *phenA, carC*	260	III	15.4
7	ACT-1, BEN-26, OLI-1, IMA-4, 13 × 14, 15	*actB, oliC, imaA, benC, choA, carA* × *pantoB, malA, wetA, methE, nicB, palD*	585	VII	15.5
8	PIM-1 × 31	*pimA* × *palC, pabaB, pyroA*	284	IV	15.6
9	PIM-10 × 18	*pimB, biA* × *proA, pabaA*	284	I	15.7
10	IMA-13 × CAR-2,6	*imaD* × *palB, chaA*	229	VIII	15.8
11	IMA-9 × 30	*imaB* × *lysB, pA, riboD*	287	V	15.9
12	IMA-10 × 27	*imaC* × *abA, cnxE, adD*	249	II	15.10

* The strains are listed with their genotype in Tables 1, 13; *actD*, *actE*, *imaE* and *imaF* are not in this Table, because no definite linkages were found.

** Loci conferring fungicide resistance are underlined; in Tables 15.1–15.10 the parental genotypes are underlined.

TABLE 15.1. Meiotic mapping of *benA*.

Cross 1: *biA1; acr A1; benA5* × *pabaA1; wA2; tsD15, ornB7, galC7, facB101, riboB2*

Progeny genotype	Progeny number
ts ben orn	3
+ + +	6
ts + +	7
+ *ben orn*	4
+ *ben* +	163
ts + *orn*	103
+ + *orn*	63
ts ben +	71
	420

recombination (%)

tsD – *benA*	34.0 ± 2.3
benA – *ornB*	4.8 ± 1.0
ornB – *tsD*	34.5 ± 2.3

Cross 2: *biA1; acrA1; benA10* × *ornB7, fwA1*

Progeny genotype	Progeny number
ben orn fw	4
+ + +	7
ben + +	151
+ *orn fw*	125
+ *orn* +	45
ben + *fw*	56
+ + *fw*	5
ben orn +	5
	398

benA – *ornB*	5.3 ± 1.1
benA – *fwA*	28.1 ± 2.2
ornB – *fwA*	27.9 ± 2.2

map (linkage group VIII)

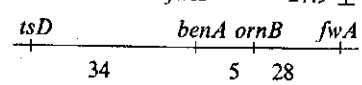


TABLE 15.2. Meiotic mapping of *benB*.

Cross 3: *yA2; adD3, benB29* × *proA1, biA1; wA3, acrB2*

progeny genotype	number
ad ben acr	2
ad ben +	283
ad + *acr*	7
+ *ben acr*	0
ad + +	1
+ *ben* +	7
+ + *acr*	329
+ + +	0
	629

recombination (%)

adD – benB	2.4 ± 0.5
benB – acrB	0.5 ± 0.3
adD – acrB	2.5 ± 0.5

map (linkage group II)

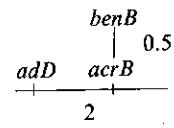


TABLE 15.3. Meiotic mapping of *carB*

Cross 4: *biA1; benA10, fwA1, carB2* × *yA2; cnxB11, fpaD43*

progeny genotype	number	genotype	number
ben fw cnx car fpa	0	*ben fw* + + +	1
ben fw cnx car +	1	*ben* + *cnx* + +	2
ben fw cnx + *fpa*	39	*ben* + + *car* +	15
ben fw + *car fpa*	5	*ben* + + + *fpa*	1
ben + *cnx car fpa*	0	+ *fw cnx* + +	1
+ *fw cnx car fpa*	0	+ *fw* + *car* +	23
ben fw cnx + +	1	+ + *cnx car* +	5
ben fw + *car* +	79	+ + + *car fpa*	4
ben + *cnx car* +	1	+ *fw* + + *fpa*	4
+ *fw cnx car* +	0	+ + *cnx* + *fpa*	68
ben fw + + *fpa*	5	*ben* + + + +	0
ben + *cnx* + *fpa*	30	+ *fw* + + +	0
+ *fw cnx* + *fpa*	14	+ + *cnx* + +	6
ben + + *car fpa*	1	+ + + *car* +	51
+ *fw* + *car fpa*	1	+ + + + *fpa*	8
+ + *cnx car fpa*	0	+ + + + +	0
			366

recombination (%)

benA – fwA	25.4 ± 2.3	*benA – cnxB*	45.1 ± 2.6
fwA – cnxB	37.1 ± 2.5	*fwA – carB*	38.8 ± 2.5
cnxB – carB	7.1 ± 1.3	*fwA – fpaD*	40.4 ± 2.6
carB – fpaD	6.0 ± 1.2	*cnxB – fpaD*	12.6 ± 1.7

map (linkage group VIII)

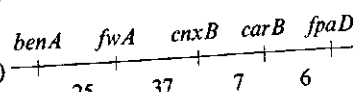


TABLE 15.4. Meiotic mapping of *chlA*, *imaG*, *carC* and *actC*.

Cross 5: *biA1; acrA1; chlA10, imaG18* × *yA2, adE20; phenA2, cbxA17*		Cross 6: *biA1; acrA1; actC5* × *yA2, adE20; phenA2, cbxA17*	
progeny genotype	number	progeny genotype	number
chl ima phen car	1	*phen car act*	26
chl ima phen +	0	*phen car* +	72
chl ima + *car*	19	*phen* + *act*	11
chl + *phen car*	48	+ *car act*	12
+ *ima phen car*	0	*phen* + +	9
chl ima + +	83	+ + *act*	78
chl + + *car*	2	+ *car* +	15
chl + *phen* +	6	+ + +	37
+ + *phen car*	115		—
+ *ima phen* +	2		260
+ *ima* + *car*	18		
chl + + +	1		
+ *ima* + +	54		
+ + *phen* +	19		
+ + + *car*	1		
+ + + +	1		
	—		
	370		

recombination (%)			
chlA – *imaG*	35.4 ± 2.5	*phenA* – *carC*	18.8 ± 2.6
imaG – *phenA*	2.2 ± 0.8	*carC* – *actC*	32.3 ± 2.9
phenA – *carC*	18.1 ± 2.0	*phenA* – *actC*	34.2 ± 2.9
chlA – *carC*	39.5 ± 2.6		
chlA – *phenA*	34.9 ± 2.5		
imaG – *carC*	17.8 ± 2.0		

map (linkage group III) — *chlA* — 35 — *phenA* — 2 — *imaG* — 18 — *carC* — 32 — *actC*

TABLE 15.5.1. Meiotic mapping of *actB, benC, carA, imaA* and *oliC*.

Cross 7: *yA2; phenB6, pantoB100, malA1, wetA6, methE6, nicB8, palD8*
×
biA1; acrA1; actB2, oliC13, imaA4, benC28, choA1, carA1

Progeny genotype	number	genotype	number	genotype	number	genotype	number
+ + + +	22	+ + + +	16	+ + + +	20	+ + +	120
+ + + *panto*	14	+ + + *ben*	4	+ + + *car*	46	+ + *pal*	23
+ + *oli* +	22	+ + *ima* +	107	+ + *meth* +	191	+ *nic* +	38
+ + *oli panto*	10	+ + *ima ben*	117	+ + *meth car*	37	+ *nic pal*	152
+ *phen* + +	37	+ *wet* + +	22	+ *cho* + +	2	*car* + +	111
+ *phen* + *panto*	90	+ *wet* + *ben*	13	+ *cho* + *car*	7	*car* + *pal*	32
+ *phen oli* +	42	+ *wet ima* +	9	+ *cho meth* +	5	*car nic* +	29
+ *phen oli panto*	26	+ *wet ima ben*	8	+ *cho meth car*	1	*car nic pal*	80
act + + +	48	*mal* + + +	1	*ben* + + +	3		
act + + *panto*	38	*mal* + + *ben*	1	*ben* + + *car*	7		585
act + *oli* +	117	*mal* + *ima* +	17	*ben* + *meth* +	7		
act + *oli panto*	34	*mal* + *ima ben*	21	*ben* + *meth car*	1		
act phen + +	17	*mal wet* + +	130	*ben cho* + +	38	+ +	192
act phen + *panto*	40	*mal wet* + *ben*	85	*ben cho* + *car*	140	+ *mal*	128
act phen oli +	15	*mal wet ima* +	11	*ben cho meth* +	67	*panto* +	120
act phen oli panto	13	*mal wet ima ben*	23	*ben cho meth car*	13	*panto mal*	145
	585		585		585		585

recombination (%)

actB-phenB	26.2 ± 1.8	*pantoB-malA*	42.4 ± 2.0	*benC-choA*	5.6 ± 1.0	*carA-nicB*	43.0 ± 2.0
phenB-oliC	37.3 ± 2.0	*malA-wetA*	15.7 ± 1.5	*choA-methE*	27.7 ± 1.9	*nicB-palD*	20.9 ± 1.7
oliC-pantoB	35.0 ± 2.0	*wetA-imaA*	12.5 ± 1.4	*methE-carA*	19.7 ± 1.6		
		imaA-benC	42.2 ± 2.0				

map (linkage group VII):

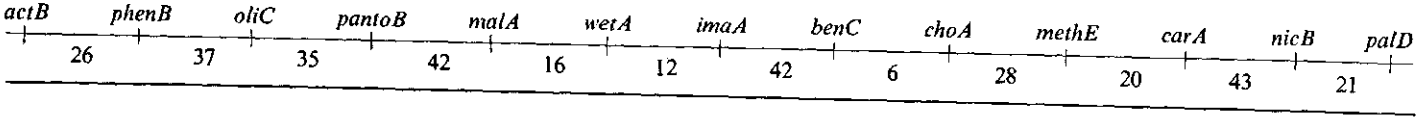


TABLE 15.5.2. Recombination frequencies between all markers of Cross 7.

	actB	*phen B*	*oliC*	*pantoB*	*malA*	*wetA*	*imaA*	*benC*	*choA*	*methE*	*curA*	*nicB*	*palD*
actB													
phenB	26.2												
oliC	41.5	36.7											
pantoB	44.1	35.4	35.0										
malA	48.4	50.8	45.8	42.4									
wetA	46.0	48.2	43.2	43.1	15.7								
imaA	49.2	48.5	41.7	39.8	24.6	12.5							
benC	50.8	48.5	47.2	45.9	48.5	46.0	42.2						
choA	49.2	49.1	48.4	46.8	45.8	47.2	43.0	5.6					
methE	48.6	48.5	45.3	42.6	48.5	47.8	45.5	27.9	27.7				
carA	47.9	46.8	45.6	39.0	45.3	44.8	44.2	35.2	34.7	19.7			
nicB	52.1	50.1	47.9	46.8	45.4	48.5	44.4	47.2	50.1	43.9	43.0		
palD	50.0	47.4	45.1	41.9	44.1	43.8	44.6	47.5	47.7	45.5	46.2	20.9	

TABLE 15.6. Meiotic mapping of *pimA*.

Cross 8: *biA1; acrA1; pimA1*
×
riboA1, yA2, adE20; methG1, suC1adE20, palC4, pabaB22, pyroA4

progeny genotype	number
pim pal paba pyro	3
pim pal paba +	0
pim pal + *pyro*	1
pim + *paba pyro*	16
+ *pal paba pyro*	87
pim pal + +	2
pim + *paba* +	4
pim + + *pyro*	10
+ *pal paba* +	17
+ *pal* + *pyro*	17
+ + *paba pyro*	7
pim + + +	65
+ *pal* + +	43
+ + *paba* +	5
+ + + *pyro*	1
+ + + +	6
	284

recombination (%)

pimA – *palC*	8.8 ± 1.7
palC – *pabaB*	33.5 ± 2.8
pabaB – *pyroA*	19.4 ± 2.3
pimA – *pabaB*	31.7 ± 2.8
pimA – *pyroA*	35.6 ± 2.8
palC – *pyroA*	33.8 ± 2.8

map (linkage group IV):

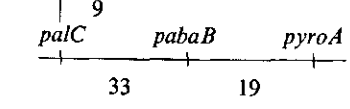


TABLE 15.7. Meiotic mapping of *pimB*.

Cross 9: *pimB10, biA1; acrA1* × *proA1, pabaA1, yA2; wA3*

progeny genotype	number
pim pro paba bi	14
pim pro paba +	23
pim pro + *bi*	3
pim + *paba bi*	1
+ *pro paba bi*	12
pim pro + +	1
pim + + *bi*	80
+ + *paba bi*	0
+ *pro* + *bi*	9
+ *pro paba* +	94
pim + *paba* +	3
pim + + +	12
+ *pro* + +	1
+ + *paba* +	5
+ + + *bi*	19
+ + + +	7
	284

recombination (%)

pimB – *proA*	25.4 ± 2.6
proA – *pabaA*	8.1 ± 1.6
pabaA – *biA*	16.9 ± 2.3
pimB – *pabaA*	27.1 ± 2.6
pimB – *biA*	27.8 ± 2.6
proA – *biA*	22.9 ± 2.5

map (linkage group I):

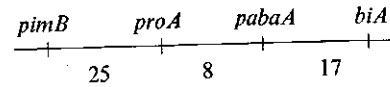


TABLE 15.8. Meiotic mapping of *imaD*.

Cross 10: *biA1; acrA1; imaD13* × *benA10, galC7, carB2, palB7, chaA1*

progeny genotype	number
pal cha ima	5
pal cha +	100
pal + *ima*	4
pal + +	0
+ *cha ima*	2
+ *cha* +	1
+ + *ima*	116
+ + +	1
	229

recombination (%)

palB – *chaA*	3.1 ± 1.1
chaA – *imaD*	3.5 ± 1.2
palB – *imaD*	4.8 ± 1.4

map (linkage group VIII):

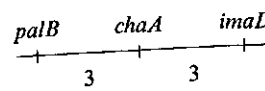


TABLE 15.9. Meiotic mapping of *imaB*.

Cross 11: *biA1; acrA1; imaB9* × *acrA1; lysB5, pA2, riboD5*

progeny genotype	number
lys p ribo ima	12
lys p ribo +	48
lys p + *ima*	8
lys + *ribo ima*	9
+ *p ribo ima*	8
lys p + +	8
lys + *ribo* +	10
lys + + *ima*	17
+ *p ribo* +	9
+ *p* + *ima*	7
+ + *ribo ima*	20
lys + + +	22
+ *p* + +	8
+ + *ribo* +	26
+ + + *ima*	33
+ + + +	45
	287

recombination (%)

lysB – pA	31.4 ± 2.7
pA – riboD	33.5 ± 2.8
lysB – imaB	46.7 ± 2.9
pA – imaB	48.1 ± 2.9
riboD – imaB	46.0 ± 2.9

map (linkage group V):

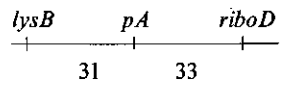


TABLE 15.10. Meiotic mapping of *imaC*.

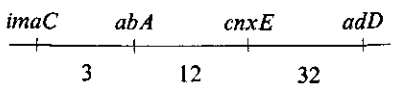
Cross 12: *biA1; acrA1, wA3, abA1, cnxE16, adD3* × *biA1; acrA1, imaC10*

progeny genotype	number
act ab cnx ad	0
act ab cnx +	0
act ab + *ad*	0
act + *cnx ad*	10
+ *ab cnx ad*	63
act ab + +	0
act + *cnx* +	5
act + + *ad*	27
+ *ab cnx* +	38
+ *ab* + *ad*	5
+ + *cnx ad*	0
act + + +	84
+ *ab* + +	9
+ + *cnx* +	1
+ + + *ad*	5
+ + + +	2
	249

recombination (%)

imaC – abA	3.2 ± 1.1
imaC – cnxE	14.5 ± 2.2
abA – cnxE	12.1 ± 2.1
cnxE – adD	32.5 ± 3.0
abA – adD	35.7 ± 3.0
imaC – adD	34.9 ± 3.0

map (linkage group II):



sed with a strain possessing another seven loci on this linkage group obtained from a cross of strains No. 14 and 15. The final result was the composition of a map of linkage group VII in which all 13 loci were involved (Tables 15.5.1, 15.5.2).

Four loci on linkage group III (*actC*, *carC*, *chlA* and *imaG*) were found to be linked to each other (Table 15.4). The location of *chlA*, 35 units from *actA* (= *imaG*) agrees with the supposed allelism to *pcnbA* (6.3.) which THRELFALL (1968) mapped 15 units from *argB* and 17 units from *galA* (Fig. 31). This confirms the map position of *chlA* according to the known linkage maps (CLUTTERBUCK, 1974). *CarC* was already known to map 18 units from *phenA* (VAN TUYL, 1975a) and an allele *cbxA* isolated by GUNATILLEKE et al. (1975a) gave the same results (Table 15.4). In cross 5 *imaG*, allelic or closely linked to *actA* (6.3.), was mapped 2 units from *phenA*. In this cross *imaG* was located right from *phenA*, while it is considered to be left from it (CLUTTERBUCK, 1974); the latter position is given in Fig. 31. In this cross the cycloheximide resistance of IMA-17 (carrying *imaG*) was used as a marker in testing the progeny.

The meiotic mapping of *imaB* on linkage group V, using two meiotic-mapping strains (strains No. 28, 30), which together carry six markers on this linkage group, did not result in assigning of linkage (Table 15.9).

The loci conferring pimaricin resistance, viz. *pimA* and *pimB*, were mapped on linkage group IV and I, respectively (Tables 15.6, 15.7). In cross 8 irregularities in recombination percentages were found. However, using the mutations *palC*, *pabaB* and *pyroA* often irregular map distances have been observed (CLUTTERBUCK, 1974).

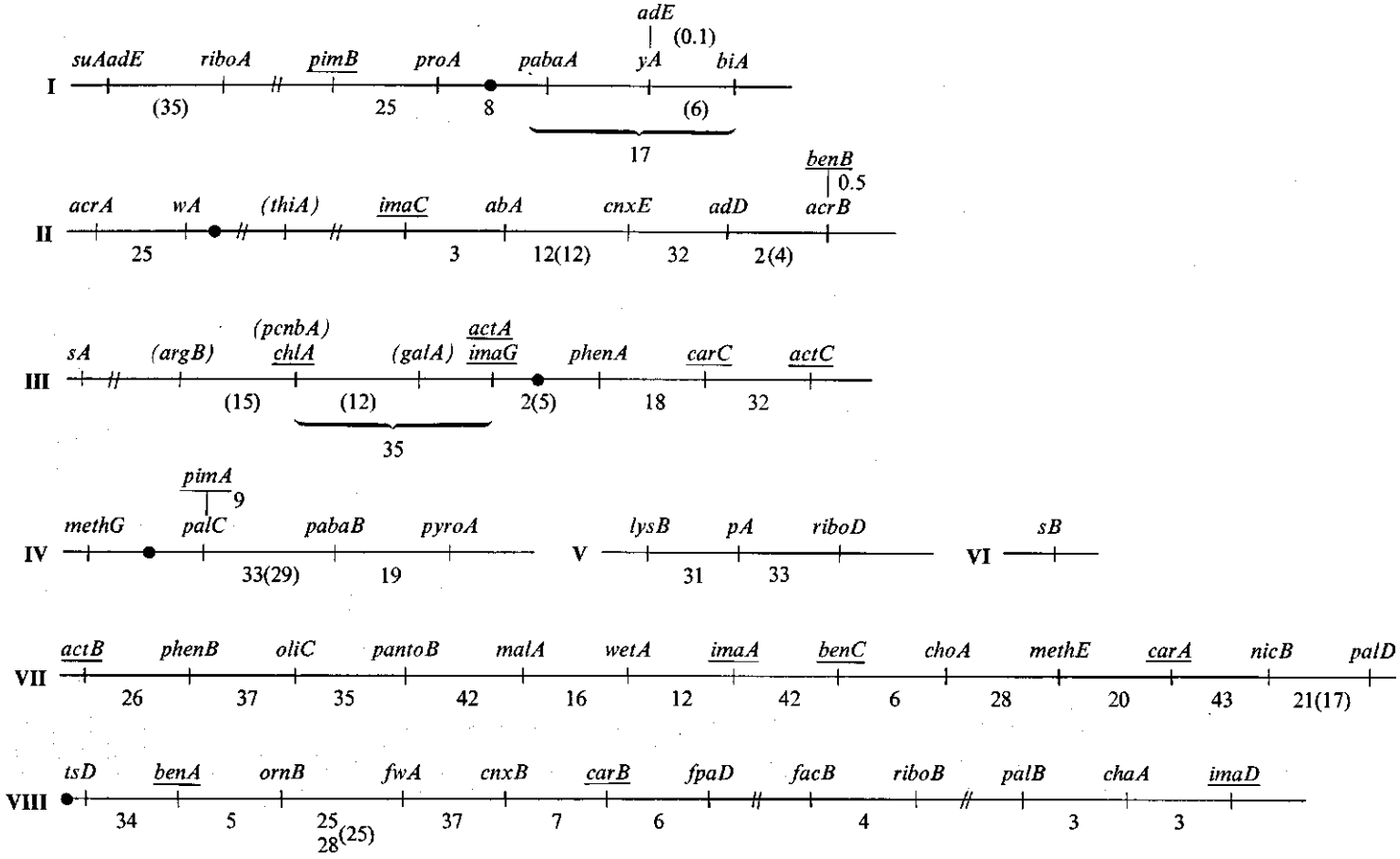
The *benB* locus on linkage group II was mapped 2 units from *adD* and a 0.5 unit from *acrB* (Table 15.2). HASTIE and GEORGOPOULOS (1971) mentioned for *ben-2*, a possible allele of *benB*, a distance of 14 units from *adD*. Another anomaly in this cross is the distance of *adD* to *acrB*, considered to be 29 units in the known linkage map (CLUTTERBUCK, 1974). The cause of this anomaly might be sought in the strains No. 12, 16 or 17 used in the mapping analysis here. Recently, the position of the *acrB* marker has been questioned (CASE and ROPER, 1975). These authors found in mitotic crossing-over analysis of a diploid carrying an extra chromosome segment in a translocated position a linkage relationship of the mutant alleles *thiA* and *acrB*. *ThiA*, however, is mapped at a large distance from *adD* near to the centromere (Fig. 31).

Using strain No. 27 *imaC* was located 3 units from *abA* and 12 units from *cnxE* on linkage group II (Table 15.10). In similar crosses of strain No. 27 no definite linkages were assigned with *imaE*, *actD* and *actE*.

ImaF, located to linkage group I, was not found to be linked with *yA2* and *biA1*. All meiotic mapping data are summarized in Fig. 31.

The meiotic analyses gave in some cases (*pimA*, *palC*, *pA*) allele ratios which did not fit a 1:1 ratio of mutant and wild-type alleles using the χ^2-test with $p = 0.05$. This can be due to a selection for one of the two alleles, caused by a smaller growth rate of the mutant allele (*pimA*, Fig. 47) or by difficulties in testing a marker (*palC*).

FIG. 31. Mapping of the loci conferring resistance (symbols underlined) in *Aspergillus nidulans*. The loci which were mapped meiotically on one of the eight linkage groups (I–VIII) are shown in relation to other markers used. Figures are percentages recombination, those in parenthesis are from the literature (CLUTTERBUCK, 1974). Loci shown in parenthesis were not used in this study. The mapping data are represented in Table 15: the locus symbols are listed in Table 2.



6.6. RECOMBINATION ANALYSIS OF *BENA* MUTATIONS

The occurrence of mutants carrying a *benA* mutation showing an opposite response to benomyl and thiabendazole, namely BEN-1, BEN-16 and BEN-14, BEN-17 made it possible to study intragenic recombination of the *benA* gene by selection for cross-resistant recombinants. Therefore, strains were selected from crosses of BEN-1 and BEN-17 with strain No. 4 in such a way that each of the two *benA* mutations were combined with one of the markers linked to *benA*, viz. *ornB* and *fwA*. Table 16 shows the results of two crosses in which the two *benA* mutations are arranged in coupling and repulsion with *fwA* and *ornB*. By plating many ascospores of these crosses on CM containing 0.4 μg/ml benomyl and 25 μg/ml thiabendazole from both crosses three cross-resistant recombinants were isolated, the genotype of which is given in Table 16. The mutation frequencies in which each of the two mutations gave spontaneously rise to cross-resistance was less than 2.10^{-8}. Supposing there were six recombinants of the type without any resistance, this gives a recombination percentage of 0.0004. A calculation using the estimation of PONTECORVO (1958) of 4.10^4 nucleotides per map unit shows that the distance between the two *benA* mutations might be about 16 nucleotides. From the genotypes of the recombinants the sequence of the two *benA* mutations was determined from the known map position of *ornB* and *fwA*. The sequence *benA19–benA3–ornB7–fwA1* could be established.

In addition to intragenic (reciprocal) recombination this phenomenon can be explained by gene conversion, being non-reciprocal recombination. When this process is supposed to take place, the position of the *benA* mutations will be identical, however.

TABLE 16. Intragenic recombination of the *benA* gene in *Aspergillus nidulans*.

Cross	*biA1; benA3, fwA1* × *biA1; benA19, ornB7*	*acrA1; benA3, ornB7* × *biA1; acrA1; benA19, fwA1*
Progeny number	2.10^6	9.10^5
Number of cross-resistant recombinants	3	3
Genotype	*biA1; benA3,19, fwA1*	*biA1; acrA1; benA3,19, ornB7* *acrA1; benA3,19, ornB7, fwA1*** *biA1; acrA1; benA3,19, ornB7, fwA1***
map*	*benA19* — 0.0004 — *benA3* — 5 — *ornB7* — 28 — *fwA1*	

* In Table 15.1 the recombination % of *benA – ornB – fwA* are given.
** Two crossing-overs had taken place to give these genotypes.

6.7. Dominance relationships of resistant mutants in diploids

Most types of mutants such as conidial color, nutritional requirement, morphological shape, utilization of carbon and nitrogen sources, temperature and cold sensitivity show mainly a recessive character when observed in a heterozygous diploid. However, mutations to resistance often have a semi-dominant character as known for the mutations to acriflavin resistance *acrA1* (Roper and Käfer, 1957; Ball and Roper, 1966) and to cycloheximide resistance *actA1* (Warr and Roper, 1965).

In dominance studies it is often difficult to decide which criterion should be used, because there is a range of dominance degrees. In this study dosage responses of sensitive and resistant haploids and of sensitive and heterozygous resistant diploids to the inhibiting agents were compared with each other. Thus, the degree of dominance was estimated through absence of the homozygous resistant diploid. For an arbitrary quantitation of the degree of dominance the ED_{50} values of the sensitive strains for growth inhibition by the toxicants were taken as a standard. Thus, the resistance factor was defined as the ratio between the ED_{50} value of the resistant haploid (or heterozygous diploid) minus that of the sensitive haploid (or diploid) and the ED_{50} value of the sensitive haploid (or diploid) with respect to growth inhibition by a toxicant.

$$\textit{Resistance factor} = \frac{ED_{50}\ \textit{value resistant haploid (heterozygous diploid)}}{ED_{50}\ \textit{value sensitive haploid (diploid)}} - 1$$

From this, the percentage dominance was calculated as the ratio between the resistance factor of the heterozygous resistant diploid and that of the resistant haploid multiplied by 100%. According to these calculations the dominance percentages of sixteen different mutations to resistance were obtained and listed in Table 17. For the calculation of the ED_{50} values the dosage response curves of the haploid and diploid strains were used (Figs 32–43). The figures of Table 17 show that the degree of dominance can vary from almost recessive in the case of mutations to chloroneb resistance to completely dominant in some mutations to cycloheximide resistance.

The dosage response curves of the sensitive and resistant haploid and diploid strains to the respective inhibiting agents as shown in Figs 32, 33, 36, 37, 40, 41, 42 and 43 were made using comparable data as obtained in experiments like portrayed in Figs 34 and 38 with respect to mutations to benomyl and carboxin at three different loci, respectively. Using the replica technique the haploid and diploid strains were tested on CM containing various concentrations of fungicide. After 2–3 days of incubation growth was measured as a percentage of the control. Another method of determining the degree of dominance, as shown in Figs 35 and 39, is based on the growth of single conidia on a fungicide-containing medium. This method which was carried out for strains carrying mutations at three different loci conferring benomyl and carboxin resistance in Figs 35 and 39, respectively, proved to give results similar to the general procedure used here.

TABLE 17. Dominance relationships of mutations to resistance in *Aspergillus nidulans*.

Mutation	Resistance to	Resistance factor*		Percentage** dominance	Reference figure
		Resistant haploid	Heterozygous diploid		
benA10	benomyl	13.0	0.8	6.1	Figs 32, 34, 35
	thiabendazole	8.2	3.1	37.8	
benB29	benomyl	0.7	0.6	85.7	Figs 33, 34, 35
	thiabendazole	0.4	0.15	37.5	
benC28	benomyl	1.1	0.5	45.4	
carA1	carboxin	11.1	9.5	85.6	Figs 36, 37, 38, 39
carB2	carboxin	8.6	3.3	38.4	
carC9	carboxin	6.5	1.5	23.1	
chlA8	chloroneb	153	0.7	0.05	Fig. 40
	PCNB	79	0.2	0.03	
actA7	cycloheximide	4.0	1.0	26.3	
actB2	cycloheximide	152	152	100	Fig. 42
actC5	cycloheximide	152	152	100	
	imazalil	1.9	0.8	42.1	
imaA4	imazalil	4.1	1.7	42.5	Fig. 41
imaB9	imazalil	4.1	1.8	43.9	
imaD13	imazalil	1.4	0.8	57.1	
imaE14	imazalil	1.9	0.8	42.1	
	cycloheximide	29.8	4.2	14.1	Fig. 42
pimA1	pimaricin	3.4	0.2	5.9	Fig. 43
acrA1	acriflavin	56	29.7	53.0	Fig. 41

* The resistance factor was defined as the ratio between the ED_{50} value of the resistant haploid or heterozygous diploid minus that of the sensitive one, and the ED_{50} value of the sensitive haploid or diploid, respectively.

** The percentage dominance was calculated as the ratio between the resistance factor of the heterozygous diploid and that of the resistant haploid × 100%.

The dosage response curves in Figs 32 and 33 of two strains (carrying the *benA19* and *benB29* mutations to benomyl and/or thiabendazole resistance) show that each mutation may have a different degree of dominance with respect to each of the two fungicides. HASTIE and GEORGOPOULOS (1971) called their mutations to benomyl resistance recessive. Here it is shown that there is a considerable variation in the degree of dominance depending on the mutations, the fungicide and the concentrations tested.

The three loci identified in carboxin resistance appeared to have a different degree of dominance (Figs 36, 37). The degree of dominance tends to increase with the degree of resistance determined by the mutations to carboxin resistance in the sequence *carC*, *carB*, *carA*. In a former report (VAN TUYL, 1975a) the *carA* mutation was described as dominant; here, it is proved to be nearly dominant. The method of expressing dominance in quantitative terms differs from that of GUNATILLEKE et al. (1975a); their way of describing the alleles of *carA*, *carB* and *carC* as partially dominant is only qualitative.

From Fig. 40 it can be seen that the degree of dominance of the mutation to chloroneb resistance tends to decrease with increasing concentration. This

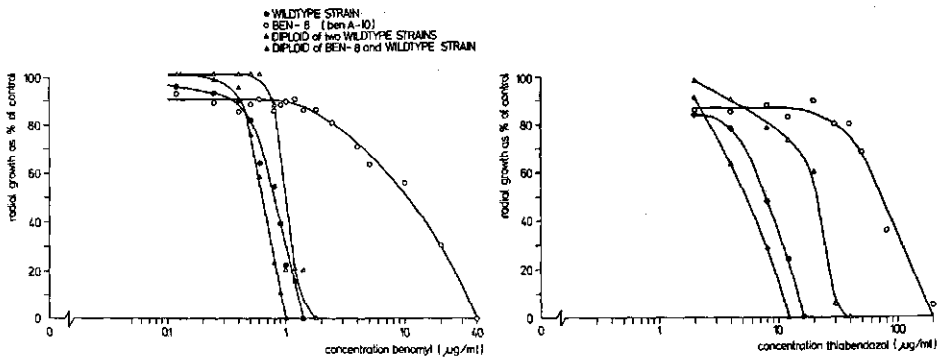


FIG. 32. Dosage response curves of the benomyl-resistant strain BEN-8 (carrying *benA10*), the wild type and two diploid strains (one of which is heterozygous for *benA10*) of *Aspergillus nidulans* with respect to benomyl and thiabendazole, showing the higher sensitivity of a diploid strain as compared with a haploid one.

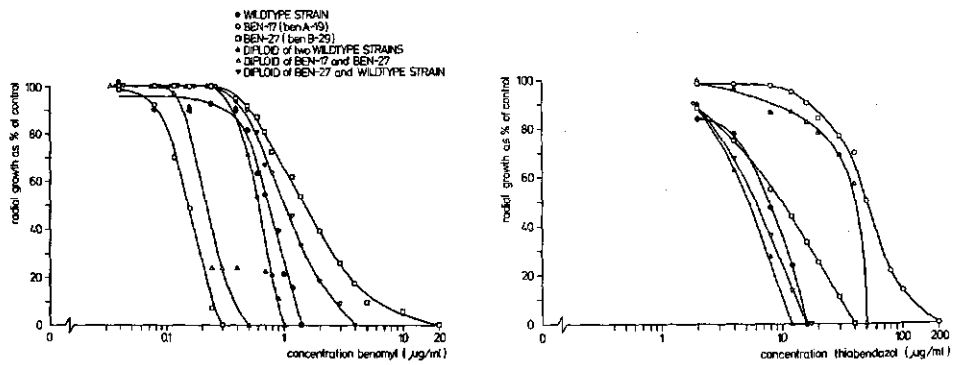


FIG. 33. Dosage response curves of two thiabendazole-resistant strains BEN-17 and BEN-27 (carrying *benA19* and *benB29*, respectively) and three diploid strains (carrying no benomyl resistance, *benB29*, and *benA19* and *benB29* in heterozygous condition) of *Aspergillus nidulans*, with respect to benomyl and thiabendazole.

tendency is reflected in the relatively low percentage of dominance given in Table 17.

Table 17 shows that of four loci conferring imazalil resistance the percentage dominance with respect to imazalil resistance is about 50, so they can be called semi-dominant. This is represented in Fig. 41A for the loci *imaA* and *imaB*. *ImaA4* and *imaB9* were (like most of the other mutations to resistance) induced in strain No. 3 with the genotype (*biA1*; *acrA1*), this being considered as the wild type. Fig. 41B shows that, with respect to acriflavin resistance *acrA1* is semi-dominant. Relative to the wild type (*biA1*; *acrA1*), IMA-9 (*biA1*; *acrA1*; *imaB9*) is hypersensitive to acriflavin; so the heterozygous diploid of the strain with the master strain causes a phenomenon, which can be described as over-dominance.

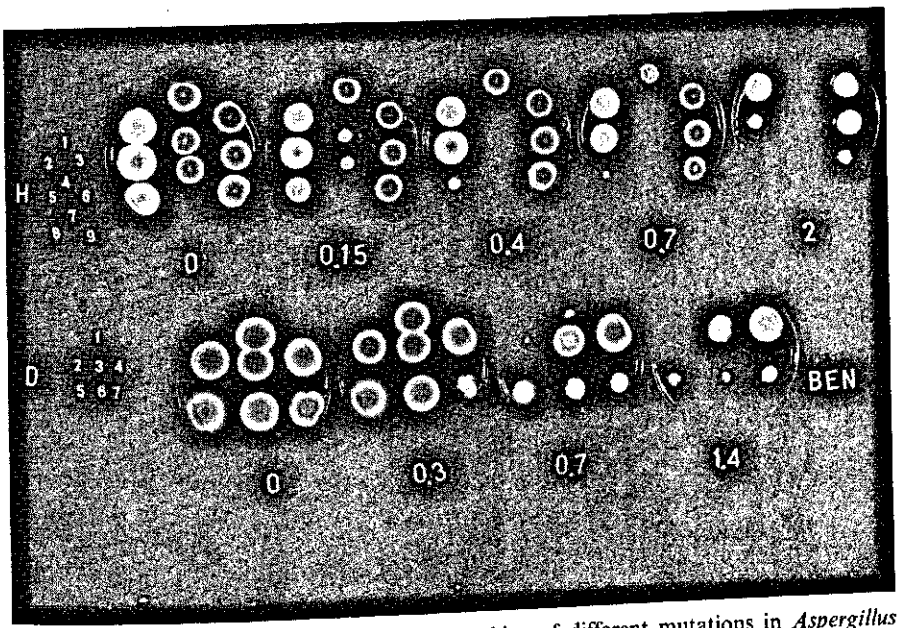


FIG. 34. Determination of dominance relationships of different mutations in *Aspergillus nidulans* using the replica technique. A plate with haploid (H) and one with diploid strains (D) were replicated on to CM containing various concentrations of benomyl. The data for the dosage response curves of Figs 32 and 44 were derived from this experiment. The mutations to benomyl resistance in the different strains are: H: 1 – no; 2 – *benA10*; 3 – *benA5*; 4 – *benA16*; 5 – *benB29*; 6 – *benA3*; 7 – *benA19*; 8 – *benC28*; 9 – *benA19* and *benB29*; D: 1 – no: 2 – no; 3 – *benA10*; 4 – *benA3* and *benA5*; 5 – *benB29*; 6 – *benC28*; 7 – *benA19* and *benB29*.

Because of the high level of resistance caused by the two mutations to cycloheximide resistance, shown in Fig. 42, the ED_{50} values of the strains, carrying these mutations and their respective heterozygous diploids could be obtained only by extrapolation. However, in view of the curves presented, it is acceptable to ascribe a completely dominant character to these mutations.

Pimaricin sensitivity (Fig. 43) offered an unusual situation in which the wild-type diploid was less sensitive than the wild-type haploid. This was the opposite of the case with benomyl (Fig. 32). The diploid heterozygous with respect to pimaricin resistance seemed semi-dominant relative to the sensitive and resistant haploid, but was in reality almost recessive relative to the sensitive diploid strain.

6.8. ADDITIVE EFFECTS IN FUNGICIDE RESISTANCE IN RECOMBINANT STRAINS CARRYING MORE THAN ONE MUTATION TO RESISTANCE

When more than one locus conferring resistance is identified with respect to a particular resistance, additive effects may be observed in recombinant strains carrying more than one mutation to that particular resistance. Such a

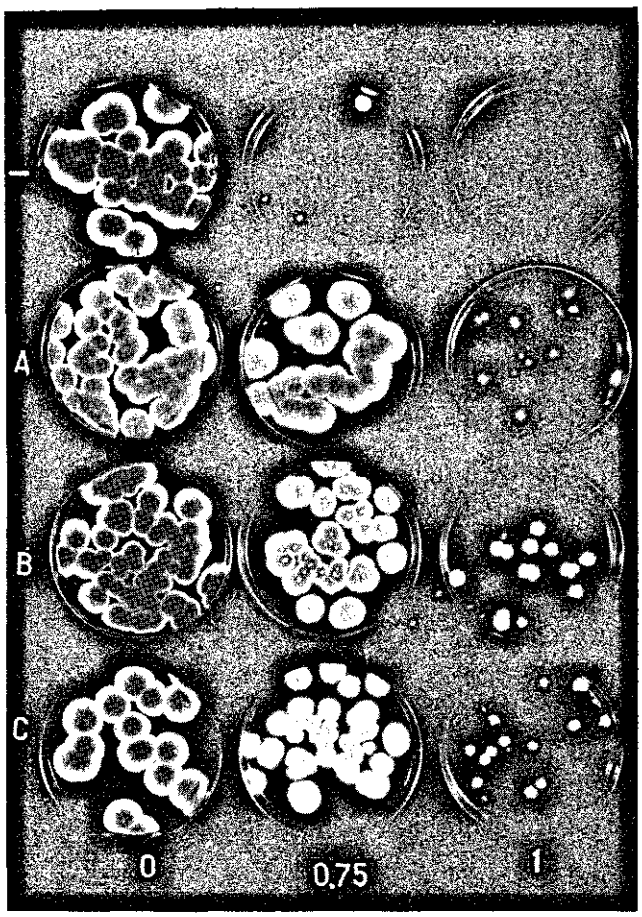


FIG. 35. Dominance relationships of three mutations to benomyl resistance at different loci in *Aspergillus nidulans* as shown in an experiment in which conidia of four diploid strains (carrying no benomyl resistance (–), *benA* (A), *benB* (B) or *benC* (C) in heterozygous condition) were plated on CM containing various concentrations of benomyl (in μg/ml).

positive interaction in recombinants is known in the multigenic determined resistance to cycloheximide in *Saccharomyces cerevisiae* (WILKIE and LEE, 1965) and *Neurospora crassa* (HSU, 1963, VOMVOYANNI, 1974) and in dodine resistance in *Nectria haematococca* (KAPPAS and GEORGOPOULOS, 1970).

In the cases of chloroneb and oligomycin resistance no additive effects could be detected, because only one locus was identified and moreover, in these cases of high level of resistance a still higher level could not be tested.

Although three loci conferring carboxin resistance were discovered, no distinctly higher level of resistance could be observed in recombinants with more than one mutation to carboxin. This is not surprising in view of the shapes of the dosage response curves of CAR-1 compared with that of the wild type (5.3., Fig. 21).

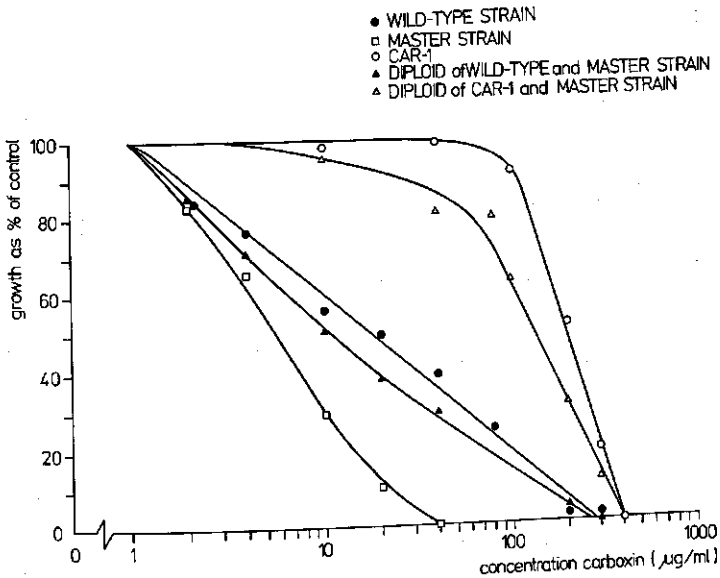


FIG. 36. Dosage response curves of the carboxin-resistant strain CAR-1 (carrying *carA1*), the wild type and the master strain, and the corresponding diploid strains of CAR-1 and the wild type with the master strain of *Aspergillus nidulans*, with respect to carboxin, showing the nearly dominant character of *carA1*.

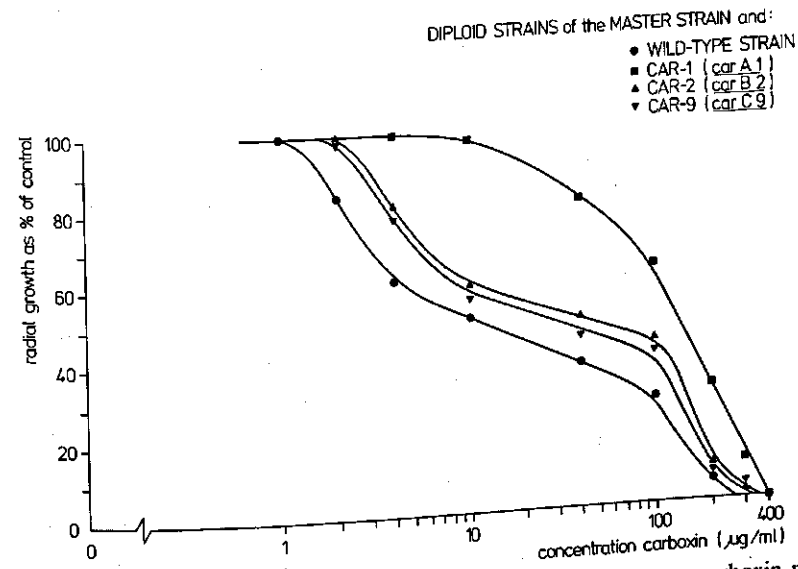


FIG. 37. Differences in degree of dominance of three mutations to carboxin resistance at different loci in *Aspergillus nidulans* as shown in dosage response curves of four diploid strains (carrying no mutation to carboxin resistance, *carA1*, *carB2* or *carC9* in heterozygous condition), with respect to carboxin.

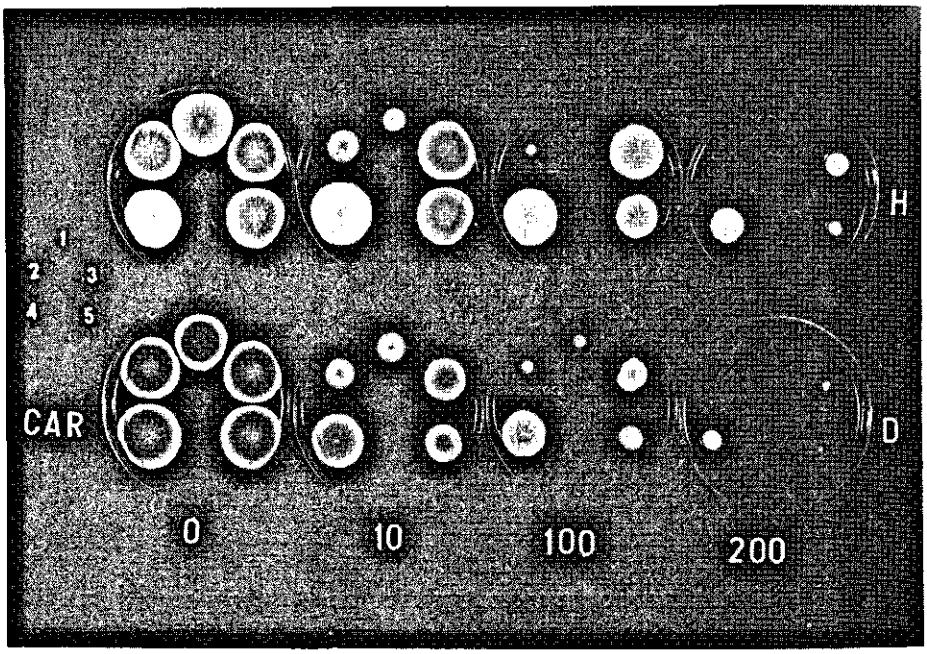


FIG. 38. Determination of dominance relationships of three mutations to carboxin resistance at different loci in *Aspergillus nidulans*. A plate with haploid (H) and one with diploid strains (D) were replicated on to CM containing various concentrations of carboxin (in μg/ml). The data for the dosage response curves of Figs. 36, 37, were derived from this experiment. The mutations to carboxin resistance in the different strains are in H as well as in D: 1 – no; 2 – no; 3 – *carB2;* 4 – *carA1;* 5 – *carC9.*

In the case of benomyl resistance one important gene *benA*, determining high level resistance and two genes *benB* and *benC* causing a less pronounced resistance were detected. In a cross of the negatively cross-resistant strain BEN-17 (carrying *benA19*), showing resistance to thiabendazole along with extra-sensitivity to benomyl and strain BEN-27 (carrying *benB29*), the recombinants with both the mutations showed an intermediate type of resistance to benomyl (Figs 44, 45) and a somewhat higher resistance to thiabendazole. The same results were obtained when instead of *benB*, *benC* was used, but when *benB* and *benC* were recombined in one strain only a slight increase in resistance was observed.

Both cycloheximide and imazalil resistance appeared to be determined by a multigenic system (6.3). Because of the relatively low sensitivity of the wild type to cycloheximide and an almost maximal resistance of the resistant strains, no additive effects could be studied. This is contrary to the situation with imazalil. Fig. 46 shows the pronounced additive effects when different genes conferring imazalil resistance did cooperate. All single gene mutations to imazalil resistance caused a relatively low level of resistance with a maximum increase of the minimal inhibitory concentration (MIC) by a factor 10. The

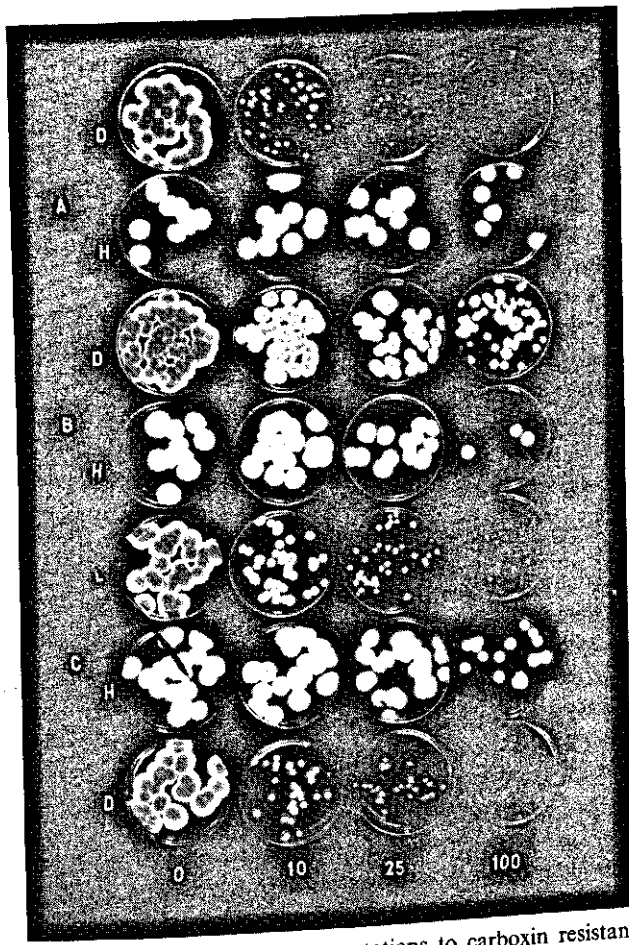


FIG. 39. Dominance relationships of three mutations to carboxin resistance at different loci in *Aspergillus nidulans* as shown in an experiment in which conidia of four diploid strains (D) (carrying no mutation to carboxin resistance, *carA* (A), *carB* (B) or *carC* (C) in heterozygous condition), and three haploid strains (H) (carrying *carA*, *carB* and *carC*) were plated on CM containing various concentrations of carboxin (in μg/ml).

positive interaction of three genes *imaA*, *imaB* and a modifier *M*, recombined in one strain, resulted in a MIC up to 200 μg/ml, which is 100 times the MIC of the wild type. The mutation at a modifier gene was induced by UV irradiation in strain IMA-7; it did not confer resistance separately but increased the resistance of IMA-7 considerably.

When the two genes determining pimaricin resistance *pimA* and *pimB* were recombined in one strain, together with a higher level of resistance (Figs 47, 48) an effect on colony growth was observed (Fig. 47B). It appeared that pimaricin resistance involved a reduction of the normal colonial growth and consequently two mutations to pimaricin resistance caused a further reduction of colonial growth.

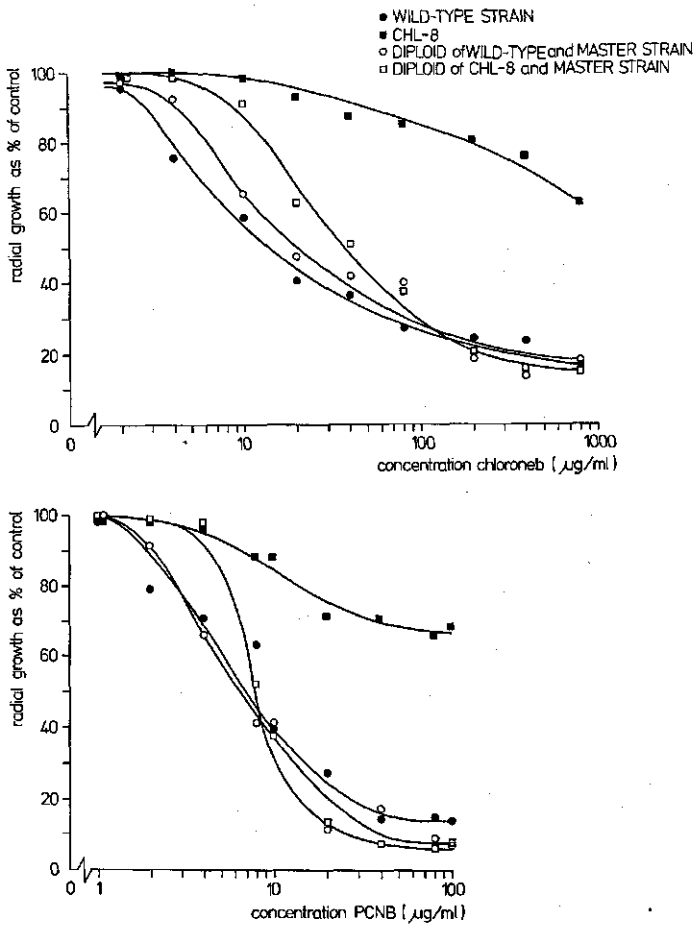


FIG. 40. Dosage response curves of the chloroneb-resistant strain CHL-8, the wild type and the corresponding diploid strains of CHL-8 and the wild type with the master strain of *Aspergillus nidulans*, with respect to chloroneb and pentachloronitrobenzene (PCNB).

6.9. DISCUSSION

Mutant strains of *Aspergillus nidulans* resistant to the fungicides benomyl, chloroneb, carboxin, cycloheximide, imazalil, oligomycin, pimaricin and thiabendazole were isolated from CM containing the inhibiting agents. Analysis of the resistant strains indicated that the resistance was determined in all cases by a single nuclear mutation. Recombination analysis of the mutations to resistance provided an insight into the number of loci at which mutations to resistance can occur. Both oligomycin and chloroneb resistance were determined by single loci *oliC* and *chlA* which were very likely allelic to *oli-2* (ROWLANDS and TURNER, 1973) and to *pcnbA* (THRELFALL, 1968), respectively. Two

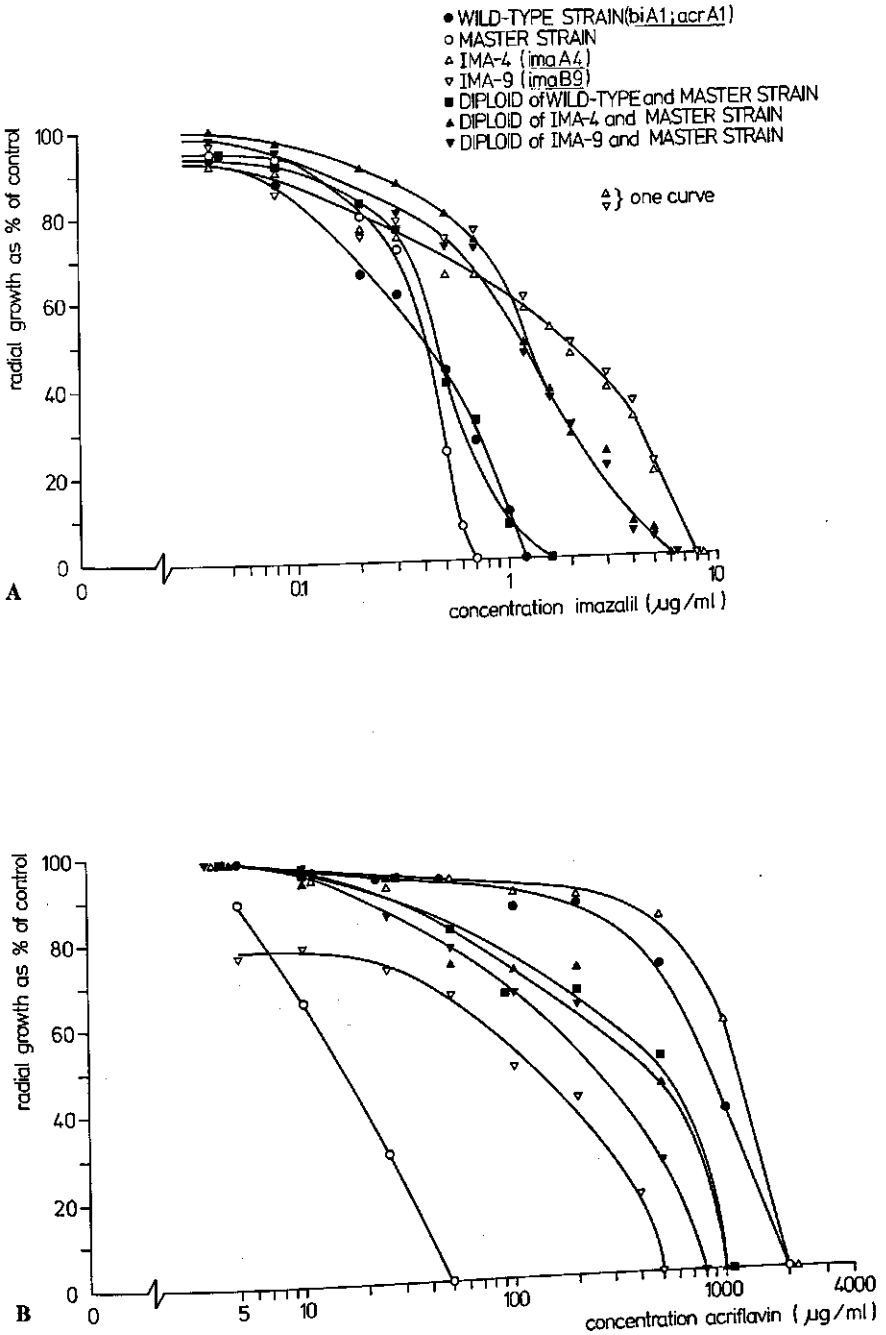


FIG. 41. Dosage response curves of two imazalil-resistant strains IMA-4 and IMA-9, the wild type and the diploid strains of these three strains with the master strain of *Aspergillus nidulans*, with respect to imazalil (A) and acriflavin (B).

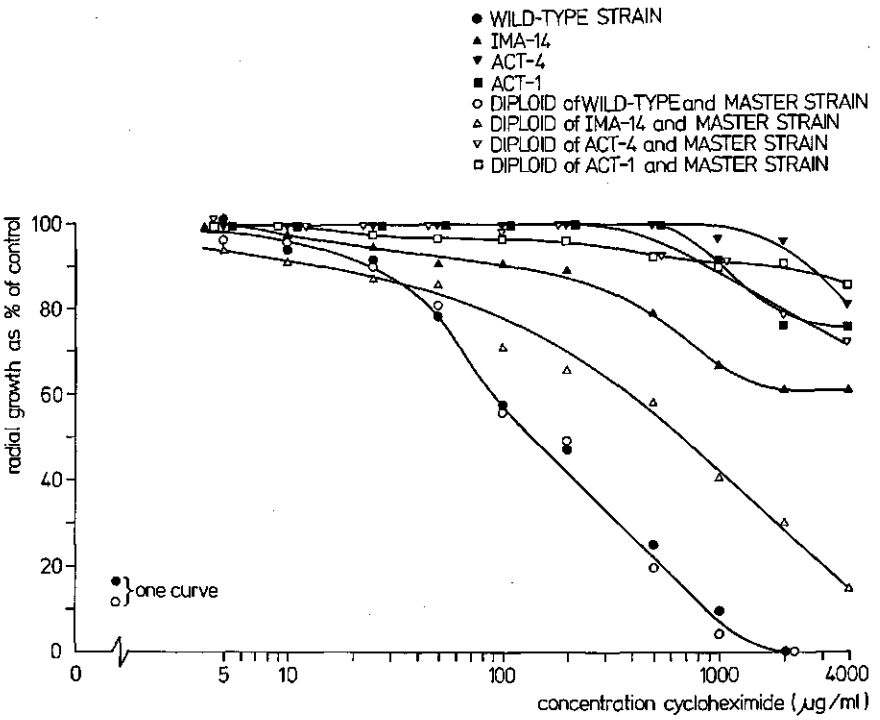


FIG. 42. Dosage response curves of three cycloheximide-resistant strains IMA-14, ACT-4 and ACT-1, the wild type and the diploid strains of these four strains with the master strain of *Aspergillus nidulans*, with respect to cycloheximide, showing the dominant character of ACT-4 and ACT-1 and the semi-dominance of IMA-14.

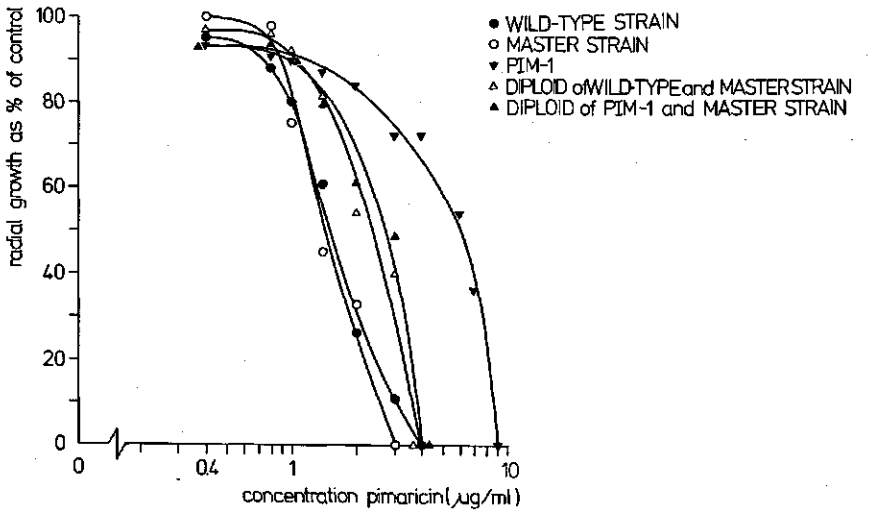


FIG. 43. Dosage response curves of the pimaricin-resistant strain PIM-1, the wild type, the master strain and the corresponding diploid strains of PIM-1 and the wild type with the master strain of *Aspergillus nidulans*, with respect to pimaricin.

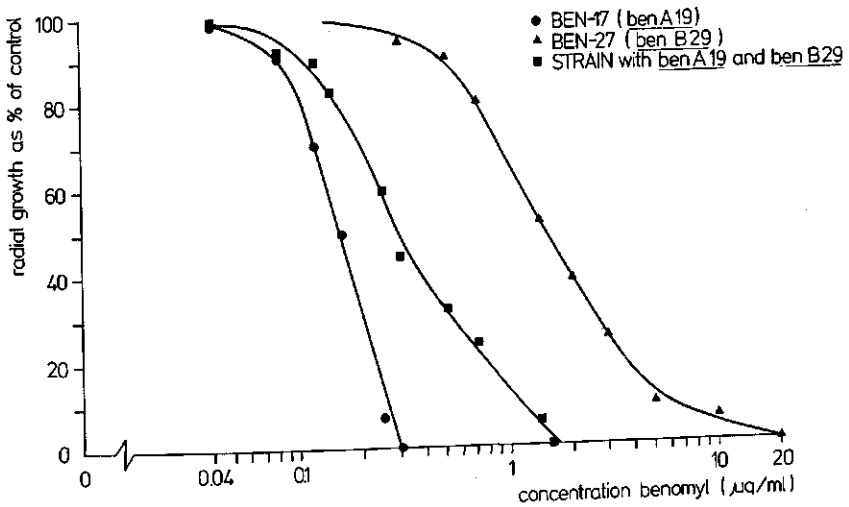


FIG. 44. Dosage response curves of two benomyl-resistant strains BEN-17 and BEN-27 (carrying *benA19* and *benB29*, respectively) and a recombinant strain (carrying both the mutations *benA19* and *benB29*) (strain No. 9 in Fig. 34) of *Aspergillus nidulans*, with respect to benomyl.

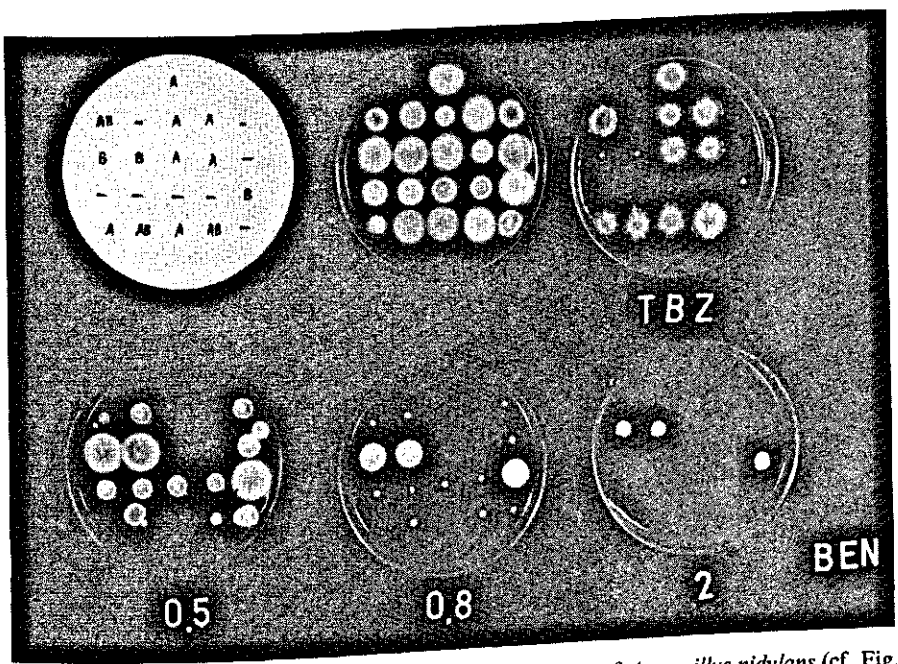


FIG. 45. Progeny of a cross of strains BEN-17 and BEN-27 of *Aspergillus nidulans* (cf. Fig. 44) as shown in a test plate (top middle) replicated on to CM containing either thiabendazole 25 µg/ml (TBZ) or benomyl 0.5, 0.8 and 2 µg/ml (BEN), in this way the parental and recombinant types can be distinguished from each other: *A-benA19*, *B-benB29*, – no resistance, AB- *benA19* and *benB29*.

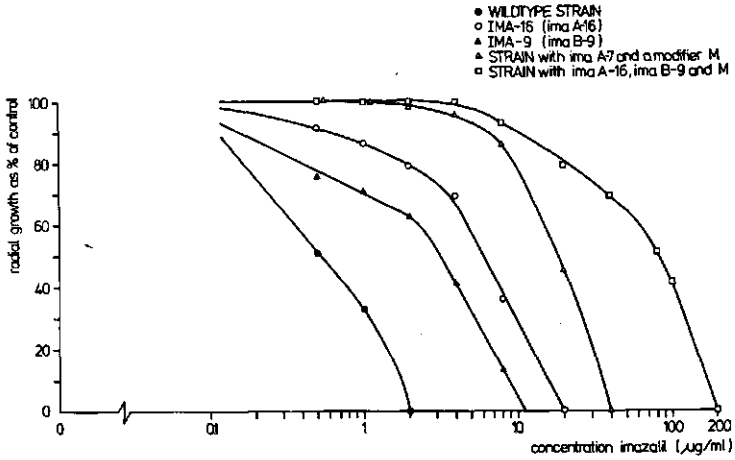


FIG. 46. Additive effects of combining three different genes conferring imazalil resistance in one strain of *Aspergillus nidulans*, as shown in dosage response curves of the wild type, IMA-16 (carrying *imaA16*), IMA-9 (carrying *imaB9*), a strain derived from IMA-7 (carrying a modifier gene *M* increasing the resistance of IMA-7) and a recombinant strain (carrying the three genes together), with respect to imazalil.

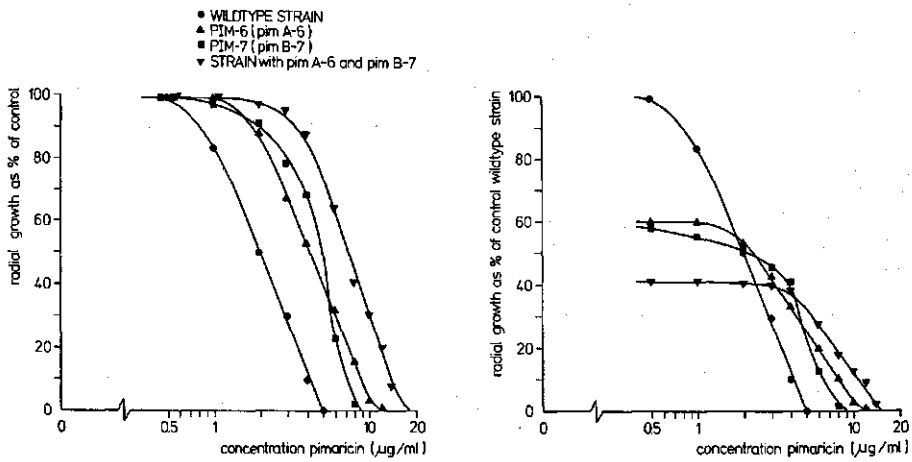


FIG. 47. Additive effects of combining the two genes conferring pimaricin resistance *(pimA* and *pimB)* in one strain and effects of pimaricin resistance on colony diameter in *Aspergillus nidulans*. Dosage response curves are given of the wild type, two pimaricin-resistant strains, PIM-6 and PIM-7 (carrying *pimA6* and *pimB7*, respectively) and a recombinant strain (carrying both the mutations *pimA6* and *pimB7*) with respect to pimaricin. Growth is expressed as percentage of control (A), and as percentage of the control of the wild type (B) (cf. Fig. 48).

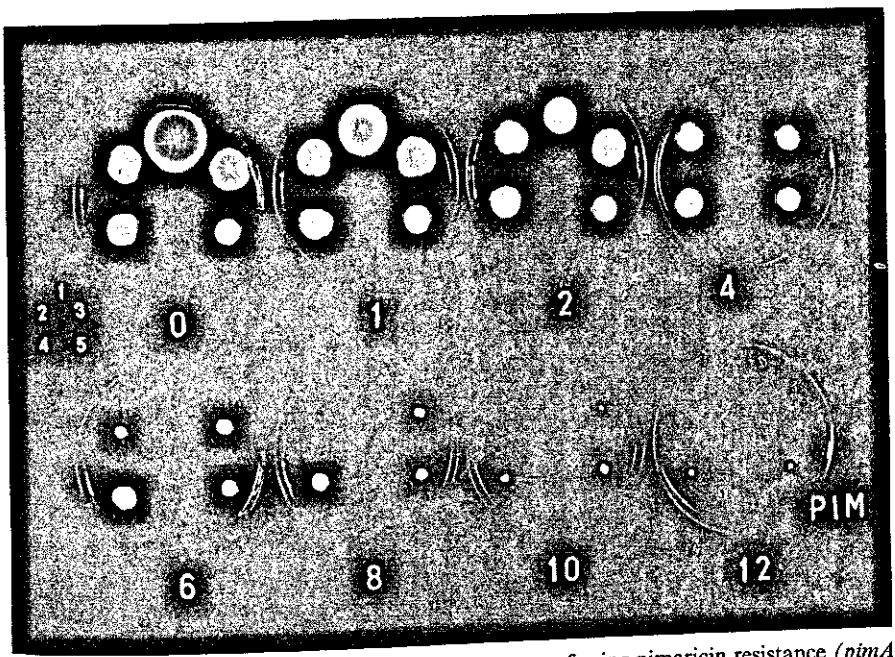


FIG. 48. Additive effects of combining the two genes conferring pimaricin resistance *(pimA* and *pimB)* in one strain of *Aspergillus nidulans*. The data for the dosage response curves of Fig. 47 were derived from this experiment. A plate with the wild type (1), two pimaricin-resistant strains PIM-6 (2) and PIM-7 (3) (carrying *pimA6* and *pimB7*, respectively) and two recombinant strains (carrying both the mutations *pimA6* and *pimB7*) was replicated on to CM containing various concentrations of pimaricin (in μg/ml).

loci conferring pimaricin resistance were identified, viz. *pimA* and *pimB*. Carboxin resistance was due to mutation at three loci called *carA*, *carB* and *carC*, and this has been confirmed by an independent study (GUNATILLEKE et al., 1975a) in which also three genes have been identified. These genes appeared to be allelic to the former genes concerned. Benomyl and thiabendazole resistance were shown to be determined by one main gene *benA* conferring a high level of benomyl and/or thiabendazole resistance and by at least two genes giving less pronounced benomyl and thiabendazole resistance *benB* and *benC*. Both cycloheximide and imazalil resistance, which proved to be determined in some cases by the same genes, were governed by a multigenic system. Seven loci were involved in cycloheximide resistance and ten loci conferred imazalil resistance.

An interesting mutation to imazalil resistance was found in *imaB*, which pleiotropically gave rise to hypersensitivity to acriflavin, cycloheximide and neomycin and to resistance to chloramphenicol and fenarimol. Allelic mutations at this locus have been described in two other studies, namely a mutation to chloramphenicol resistance (GUNATILLEKE et al., 1975b) and one to fenarimol resistance (DE WAARD and GIESKES, 1977; DE WAARD and SISLER, 1976).

The cause of the pleiotropic effects of this gene was not yet elucidated but might be sought in a reduced membrane permeability, as has been proved for pleiotropic effects of a single nuclear mutation in *Saccharomyces cerevisiae* (RANK et al., 1975). Moreover, in *Escherichia coli* it has been found that changes in membrane permeability were associated with acriflavin sensitivity (NAKAMURA and SUGANUMA, 1972).

Recombination within the *benA* gene, performed with a benomyl- and a thiabendazole-resistant strain showing an opposite response to these compounds, did suggest that the mutations, responsible for this response, were situated very close together at the *benA* locus. This knowledge contributed to the elucidation of the mechanism of action of benomyl and related compounds, DAVIDSE (1974, 1975, 1976) has shown that carbendazim the active principle of benomyl, is bound to tubulin, the protein building block of microtubules.

In mapping the mutations to resistance it appeared that the genes conferring resistance were not clustered but distributed over the fungal genome (Fig. 31), except perhaps in the case of cycloheximide and imazalil resistance in which four genes were located to linkage group II. Seventeen genes determining resistance were mapped on six of the eight linkage groups; to V a mutation (*imaB*) was located but not found to be linked to any of six markers on the same linkage group (Table 15.9) and to VI no resistant mutation was assigned. Using five loci conferring a different type of resistance, allocated to linkage group VII, a new map of this linkage group with the location of 13 markers was composed.

Detailed studies about dominance relationships of resistant mutants in *A. nidulans* are not yet known. Therefore, dosage response curves of sensitive and resistant haploid strains and sensitive and heterozygous resistant diploid strains were compared. It appeared that a range of degrees of dominance could be distinguished, viz. from the practically recessive condition of mutations to chloroneb resistance, through a low degree of dominance of those to pimaricin and benomyl resistance, and semi-dominance of those to imazalil resistance to almost complete dominance of one of the mutations to carboxin resistance and of some to cycloheximide resistance (Table 17).

In some cases a positive interaction of genes resulting in a higher degree of resistance in recombinant strains was observed. A pronounced effect was found in multigenic determined imazalil resistance. Here, different mutations each conferring a low degree of resistance, gave rise to a relatively high resistance when recombined in one strain. SISLER and SIEGEL (1967) argued that positive interaction of genes might conceivably involve a lowered cell membrane permeability combined with increased resistance at the site of action.

Finally, this comparative study of genetic analysis of fungicide resistance in the non-pathogen *A. nidulans* demonstrated the different behaviour of mutations to resistance to various fungicides, which is reflected in the number of genes involved, the expression of mutations in diploid strains and the effects of combining mutations to resistance in one strain. Further, it emphasizes the advantages of working with an organism, which is genetically well-defined

and which is easy to handle. Moreover, as proved in chapters 3, 5 and 7 with respect to mutation frequencies, patterns of cross-resistance and other characteristics of the resistant strains, the results obtained with *A. nidulans* could also be applied to other plant-pathogenic as well as non-pathogenic fungi.

7. GENETIC ANALYSIS OF FUNGICIDE RESISTANCE IN *ASPERGILLUS NIGER*, *CLADOSPORIUM CUCUMERINUM* AND *USTILAGO MAYDIS*

7.1. INTRODUCTION

In addition to the genetic studies on fungicide resistance with the very suitable but non-pathogenic fungus *Aspergillus nidulans* (chapter 6), also genetic experiments with pathogenic fungi were carried out, not only to compare the results but also to examine whether the host-parasite relationship was affected. Knowledge of the genetics of plant-pathogenic fungi, which can be analysed in vitro by means of the sexual cycle, is limited to only a few pathogens, e.g. *Ustilago maydis* (HOLLIDAY, 1961), *Venturia inaequalis* (BOONE, 1971), *Nectria galligena* (KRÜGER, 1974) and *Hypomyces solani f. sp. cucurbitae* (SNYDER et al., 1975). Since PONTECORVO et al. (1953b) discovered the parasexual cycle in *A. nidulans*, it has been applied in the genetic analysis of many imperfect fungi. PONTECORVO (1958) described the steps of the cycle as follows:

a) fusion of two unlike haploid nuclei in a heterokaryon;
b) multiplication of the resulting heterozygous diploid nucleus;
c) mitotic crossing-over within the diploid nucleus;
d) vegetative haploidization of the diploid nucleus.

Recombination outside the sexual cycle has since been demonstrated in a number of fungi, viz. *Aspergillus niger* (PONTECORVO et al., 1953a); *Penicillium chrysogenum* (PONTECORVO and SERMONTI, 1953); *Fusarium oxysporum f. pisi* (BUXTON, 1956); *Aspergillus oryzae* and *A. sojae* (ISHITANI et al., 1956); *U. maydis* (HOLLIDAY, 1961b; PUHALLA, 1969); *Cephalosporium mycophilum* (TUVESON and COY, 1961); *Penicillium expansum* (BARRON, 1962; GARBER and BERAHA, 1965); *Verticillium albo-atrum* (HASTIE, 1962, 1964); *Aspergillus fumigatus* (STRØMNAES and GARBER, 1962); *Cochliobolus sativus* (TINLINE, 1962); *Penicillium digitatum* and *P. italicum* (STRØMNAES et al., 1964); *Aspergillus amstelodami* (LEWIS and BARRON, 1965); *Ascochyta imperfecta* (SANDERSON and SRB, 1965); *Phymatotrichum omnivorum* (HOSFORD and GRIES, 1966); *Ustilago violacea* (DAY and JONES, 1969); *Dictyostelium discoideum* (KATZ and SUSSMAN, 1972); *Penicicillium patulum* (CALAM et al., 1973); *Cephalosporium acremonium* (NÜESCH et al., 1973); a *Humicola* spp. (DE BERTOLDI and CATEN, 1975); *Aspergillus flavus* (LEAICH and PAPA, 1975) and *Pyricularia oryzae* (GENOVESI and MAGILL, 1976).

Several reviews on parasexual genetics have been appeared (BRADLEY, 1962; ROPER, 1966), while more recently work on parasexual genetics in plant-pathogenic fungi has been reviewed (TINLINE and MACNEILL, 1969; WEBSTER, 1974).

This chapter deals with the genetic analysis of fungicide resistance in *U. maydis*, by means of the sexual cycle while in the imperfect fungi *A. niger* and *Cladosporium cucumerinum* parasexual genetics was employed. Therefore, in the latter two fungi auxotrophic mutants differing in conidial color had first

to be isolated, in order to make use of the parasexual cycle.

A. niger causes post-harvest diseases (MULLER, 1974; HALLOIN, 1975) and oxalosis in man (NIME and HUTCHINS, 1974); in industry it is used for the production of citric acid (SEICHERTOVÁ and LEOPOLD, 1969). The parasexual cycle of *A. niger* has been described (PONTECORVO et al., 1953a; LHOAS, 1961, 1967), while recently the occurrence of a diploid strain was found in nature (NGA et al., 1975).

C. cucumerinum is the pathogen that causes cucumber scab (VAN DEN MUYZENBERG, 1932). In phytopathology, this fungus has been used extensively as an experimental organism in plant chemotherapy (VAN ANDEL, 1958, 1966a, b, 1968, 1969; DEKHUYZEN, 1964; DEKKER, 1968; KAARS SIJPESTEIJN and SISLER, 1968; SHERALD et al., 1973; FUCHS and VIETS-VERWEY, 1975). The host-parasite relationship has been studied by Raa and co-workers (PAUS and RAA, 1973; SKARE et al., 1974). Because of this frequent use of *C. cucumerinum* it seemed worthwhile to attempt to demonstrate the parasexual cycle in order to gather genetic information on this species.

7.2. CROSSING ANALYSIS IN *USTILAGO MAYDIS*

Genetic analysis of oxathiin resistance in *U. maydis* has been examined by GEORGOPOULOS and SISLER (1970) and GEORGOPOULOS et al. (1972) who proved that the mutations to resistance *ant* and *oxr* were single gene mutations. TILLMAN and SISLER (1973) showed that chloroneb resistance in *U. maydis* was determined by a single gene.

In a crossing experiment of a chloroneb-resistant strain (parental strain 220) and a benomyl-resistant strain (parental strain 213) it appeared that benomyl and chloroneb resistance segregated independently. No close linkage was observed with the nutritional markers *ad1*, *me15* and *pan1*.

In Fig. 49 a thiabendazole-resistant strain (T) is represented which shows negative cross-resistance to benomyl as described in 5.2.1. In *A. nidulans* it was proved (6.3.) that this type of thiabendazole resistance as well as every high-level resistance to benomyl was determined by one single gene. The possible allelism of mutations to benomyl and thiabendazole resistance in *U. maydis* was examined in five resistant strains. Strain (*ad1*; *ben2*) was crossed with three strains: (*pan1*; *me15*; *ben5*), (*pan1*; *me15*; *ben6*) and (*pan1*; *me15*; *ben7*) in each case resulting in benomyl-resistant progeny only (Table 18). The negative cross-resistant strain (*ad1*; *ben8*) was crossed with (*pan1*; *me15*; *ben5*) and (*pan1*; *me15*; *ben6*) and gave a progeny consisting of only benomyl-resistant and benomyl-extra-sensitive sporidia (Table 18). The considerable deviation from the 1:1 ratio in the segregation of the parental types in the last two crosses can be explained by a selective advantage of one parental type and by the inadequacy of the random analysis for obtaining accurate genotype ratios. The results of the crosses in Table 18 show that no recombinants were detected among a 650 sporidia progeny, so probably benomyl resistance is also determined by one locus in *U. maydis*.

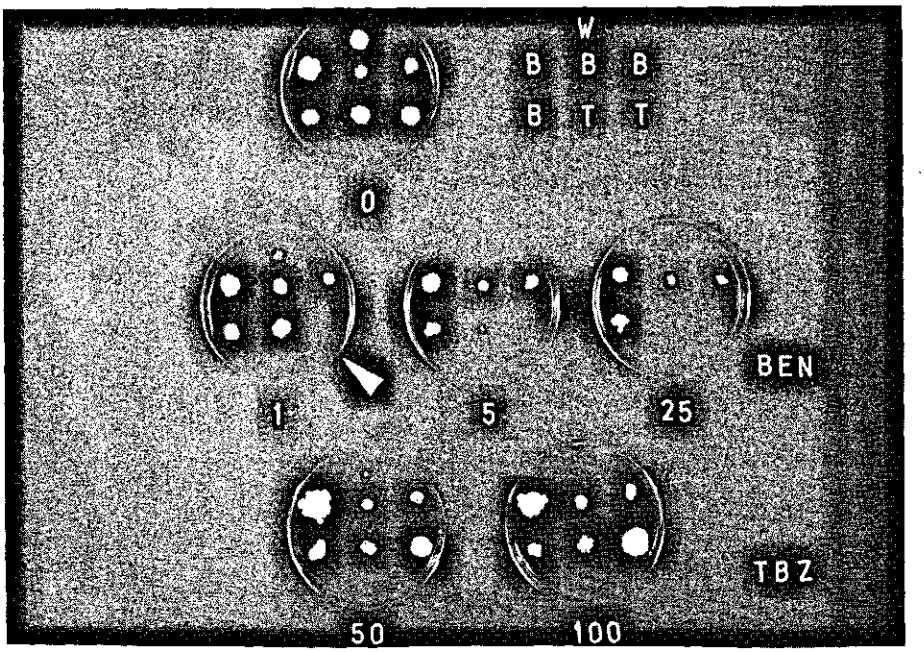


FIG. 49. Negative cross-resistance between benomyl and thiabendazole in *Ustilago maydis* (cf. 5.2.1.; Figs 17, 18). The wild type (W), four benomyl- (B) and two thiabendazole-resistant (T) strains were replicated on to CM containing various concentrations of benomyl (BEN) or thiabendazole (TBZ) (in μg/ml).

TABLE 18. Crossing analysis of different benomyl- and thiabendazole-resistant mutants of *Ustilago maydis*.

Cross	Progeny number arranged to type of benomyl sensitivity		
	resistant *(ben2, ben5, ben6, ben7)*	sensitive (wild type)	extra-sensitive *(ben8)*
(ad1; ben2) × *(pan1; me15; ben5)*	129	0	0
× *(pan1; me15; ben6)*	129	0	0
× *(pan1; me15; ben7)*	131	0	0
(ad1; ben8) × *(pan1; me15; ben5)*	99	0	32
× *(pan1; me15; ben6)*	81	0	49

7.3. THE PARASEXUAL CYCLE IN *ASPERGILLUS NIGER*

7.3.1. *Induction of mutations and their properties*

All strains of *A. niger* were derived from the same wild type. The number and type of mutations, that were induced and employed in this study are listed in Table 19. The strains in which these mutations were induced are listed in

Table 20. Mutants with different conidial color, viz. fawn, olive, white and yellow, could easily be selected. To obtain auxotrophic mutants different methods were tried out (2.6.3.), viz. total analysis, starvation and pimaricin enrichment. Two mutagenic agents, UV irradiation and NG treatment were used in total analysis. In Table 21 the results obtained with different methods are summarized. For a comparison of the different experiments it must be taken into account that survival percentage and mutation frequency are closely related (3.2., Fig. 2). Although with NG treatment significantly more auxotrophic mutants were obtained than with UV irradiation, many disordered double mutants with reduced growth and sporulation were observed. The starvation method, carried out with a p-aminobenzoic acid requiring strain

TABLE 19. List of mutations employed in *Aspergillus niger* and *Cladosporium cucumerinum*.

Gene symbol	Number of mutations in		phenotype*
	A. niger	*C. cucumerinum*	
acr	2		acriflavin res.
act	13	3	cycloheximide res.
ade	1	5	adenine req.
arg	1	5	arginine req.
asp		1	aspartic acid req.
ben	11	5	benomyl res.
bio	1	1	biotin req.
car		6	carboxin res.
chl	6		chloroneb res.
cho		1	choline req.
dar		1	dark conidia
faw	1	1	fawn conidia
glu		1	glutamine acid req.
his	1	6	histidine req.
ima	8	13	imazalil res.
ino	1		inositol req.
iva		2	isoleucine and valine req.
leu	5	1	leucine req.
lys	3	3	lysine req.
met	6	9	methionine req.
nic		2	nicotinic acid req.
orn		1	ornithine req.
oli	2		oligomycin res.
pab	1		p-aminobenzoic acid req.
pal		7	pale conidia
pim	2	1	pimaricin res.
pur		3	purine req.
rib		2	riboflavin req.
thi	2		thiamine req.
whi	2	1	white conidia
ylo	2		yellow conidia

* res. = resistant.
req. = requiring.

TABLE 20. Origin of strains of *Aspergillus niger*.

Strain No.	Genotype	Mutagenic agent	Mutations induced in each strain
1	wild type	UV	*faw1; pab1; whi1*
2	*pab1*	UV	*ben1; ben2; ben3; ben4; ben5; ben6; leu3; met4; ylo1*
3	*whi1*	UV	*ade1; his1; leu1; lys1; lys2*
4	*faw1*	UV	*leu2*
5	*whi1; leu1*	UV	*ben7; ben8*
6	*faw1; leu2*	UV	*ben9; ben10; ben11;* (selected on thiabendazole)
7	*whi1; his1*	NG	*ima1; ima2; ima3; ima4; act1; act2; act3; act4; act5; act6; act7; act8; act9; act10; act11; act12*
		UV	*chl1; chl2; chl3; chl4; chl5; chl6*
8*	*pab1; leu1; ben3*	UV	*arg1; ino1; lys3; met1; met2; met3; thi1; thi2; whi2*
9	*pab1; leu1; ben3; thi1*	NG	*ima5; ima6; ima7; ima8; pim1; pim2*
10**	*whi1; his1; ben3; arg1*	NG	*acr1; acr2; oli1; oli2; oli3; leu4; leu5; met5; met6*

* origin: recombinant of *(pab1; ben3)* and *(whi1; leu1; ben8)*.
** origin: recombinant of *(pab1; leu1; ben3; arg1)* and *(whi1; leu1)*.

TABLE 21. Isolation of auxotrophic mutants of *Aspergillus niger* with different methods.

Initial* strain	Mutagenic agent	Survival percentage	Method**	Percentage auxotrophs	Number of auxotrophs in Table 20
8	UV	2	tot.	1.2	8
2	NG	0.2	tot.	20	–
10	NG	10	tot.	3.5	3
2	UV	20	stv.***	89	2
3	UV	20	pim.	1.3	5

* genotype of the strains are listed in Table 20.
** tot. = total analysis; stv. = starvation method; pim = pimaricin enrichment, (s. 2.6.3.).
*** the starvation was stopped after 140 h of incubation.

(strain No. 2, Table 20), yielded many auxotrophs, however, 72 of the 75 mutants that proved to be deficient grew when methionine, cysteïne or sulphite was added. The starvation was continued for 140 h, after which 0.1% of the plated prototrophic conidia were still alive. The pimaricin enrichment technique was not very efficient; only a slightly higher percentage of auxotrophs (when corrected for survival percentage) was noticed.

Mutations to resistance were induced to acriflavin (*acr*), benomyl and thiabendazole (*ben*), chloroneb (*chl*), cycloheximide (*act*), imazalil (*ima*), oligomycin (*oli*) and pimaricin (*pim*). The experimental data for these inductions (except for *acr*, *oli*) were given in Table 6 (3.3.) and Table 20. Characterization of the fungicide-resistant mutants was described in chapter 5.

7.3.2. *Location of mutations to linkage groups*

By means of the parasexual cycle nuclear genes can be located to different linkage groups. LHOAS (1967) described six linkage groups in *A. niger* containing 11, 9, 6, 3, 1 and 1 markers, respectively. As far as similarity of markers in this study and that of Lhoas could be established the same linkage-group numbers were chosen.

Heterozygous diploids of different strains (Table 20) were isolated in a frequency of about one in every million plated conidia of the heterokaryon. Haploid segregants were selected readily on CM containing 0.5 μg/ml benomyl. In order to establish single gene segregation of every marker examined and to detect linkage between different markers more than 70 heterozygous diploids were analysed. All 42 mutations analysed showed independent segregation with other markers, unless located to the same linkage group, proving that single nuclear genes were involved. When no or only a few (cf. 2.7.2.) recombinants were found in the segregation of two markers, it was concluded that they belonged to the same linkage group. Applying this technique the following linkage groups were established:

I *acr1, 2, faw1, pab1, whi1, ylo1, 2*
II *his1, ino1, ima5, 6, 7, 8, met1, pim1*
III *ade1, chl3, 5, lys1, 2, 3, whi2*
IV *arg1, met2, thi1*
V *leu1, 2, thi2*
VI *ben1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11*

Oli1, oli2 and *act9* could not be located to any of the six linkage groups.

Table 22 shows an example of a haploidization analysis. Here, it appeared that *pab1* and *whi1*, and *his1* and *ino1* belonged to the same linkage groups,

TABLE 22. Haploidization analysis in *Aspergillus niger* of a diploid constituted from *(whi1; his1)* and *(pab1; ben3; leu1; ino1)*.

Pairs of markers examined	Classification of the haploids; + = wild type, − = mutant			
	+ +	+ −	− +	− −
whi – his	8	10	10	11
whi – pab	1	17	20	1
whi – ino	11	17	11	10
whi – leu	16	2	8	13
whi – ben	16	2	16	5
his – pab	11	7	10	11
his – ino	1	17	21	0
his – leu	10	8	14	7
his – ben	16	2	16	5
pab – ino	11	10	11	7
pab – leu	10	11	14	4
pab – ben	16	5	16	2
ino – leu	14	8	10	7
ino – ben	16	6	16	1
leu – ben	20	4	12	3

whereas other markers segregated independently. In the case of *pab1–whi1* 2 recombinants were found. In the analysis of 574 haploids from different experiments 13 recombinants were observed. Another conidial-color marker *faw1* was, like *whi1*, located to the first linkage group. With respect to *pab1* and *faw1* 9 recombinants were found out of 214 analysed haploids. These color markers were complementary in a heterozygous diploid, giving black conidia. In 75 tested haploids which were derived from such a diploid no recombinants were found, so *faw1* and *whi1* are closely linked.

Comparing these data with those of LHOAS (1967) the following markers were located to similar linkage groups: *acr*, *ade*, *faw*, *his*, *leu*, *lys*, *met2*, *pab*, *whi2* and *ylo*, while *arg1*, *met1*, *thi1* and *whi1* were assigned to different groups. These exceptions are remarkable because Lhoas located *arg*, *met* and *thi* to linkage group I and *whi1* to IV while here the location was found just the other way round. This might be explained by a translocation between chromosomes I and IV, already present in one of the two original wild-type strains, which had probably existed in nature independently from each other for a long time.

7.3.3. *Recombination analysis of benomyl- and thiabendazole-resistant mutants*

In order to establish whether benomyl resistance in *A. niger* is based on mutations at one locus, as already proved for *A. nidulans* (6.3.) and *U. maydis* (7.2.), eleven benomyl- and/or thiabendazole-resistant strains were examined (Table 20). The degrees of resistance of these strains to benomyl and thiabendazole were in many cases so different, that in recombination analysis parental types could easily be distinguished. The ED_{50} values of the wild type and these mutants for inhibition of growth by benomyl (in μg/ml) is given here in parenthesis together with the mutation they carry: no *ben* mutation (0.6), *ben1* (> 500), *ben2* (30), *ben3* (> 500), *ben4* (20), *ben5* (> 500), *ben6* (15), *ben7* (200), *ben8* (15), *ben9* (20), *ben10* (2) and *ben11* (0.2). Strains carrying one of the last three mutations were selected on a thiabendazole-containing medium of which *ben11* determines negative cross-resistance to benomyl

TABLE 23. Recombination analysis of benomyl- and thiabendazole-resistant strains of *Aspergillus niger* (Fig. 50).

Diploid number	*ben* mutations present in the analysed diploid	Number of haploids analysed	Number of recombinants
1	*ben1* × *ben7*	42	0
2	*ben2* × *ben7*	45	0
3	*ben3* × *ben8*	73	0
4	*ben3* × *ben7*	63	0
5	*ben4* × *ben7*	41	0
6	*ben5* × *ben8*	88	0
7	*ben6* × *ben7*	36	0
8	*ben9* × *ben3*	40	0
9	*ben10* × *ben3*	76	0
10	*ben11* × *ben3*	65	0

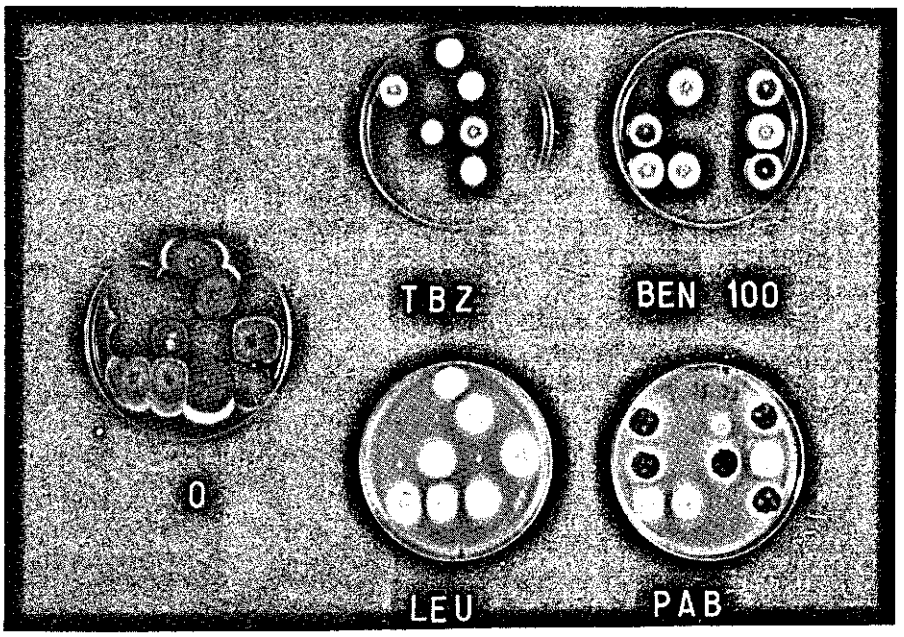


FIG. 50. Diploid analysis in *Aspergillus niger*. Haploids originated from a diploid of a benomyl- (carrying *ben3*, Table 20) and a thiabendazole-resistant strain (carrying *ben10*), were tested for their resistance and deficiencies. Only the parental types with respect to resistance were found, demonstrating the allelism of the mutations (Table 23, diploid No. 9).

(5.2.1., Fig. 16, strains No. 5, 6). *Ben3* (present in strain No. 3, Fig. 16) confers benomyl resistance without cross-resistance to thiabendazole. To examine allelism of the different *ben* mutations, diploids constituting pairs of the *ben* mutations were selected and analysed. Table 23 shows the combinations of *ben* mutations and the number of haploids analysed. Within the 569 haploids analysed no recombinants were detected. Fig. 50 represents a test plate in which *ben3* and *ben10* segregate independently from *faw1*, *leu1* and *pab1* but are completely linked to each other.

Diploid No. 10 (Table 23) was used in a recombination experiment, in which about 8 million haploids were tested for growth on a medium containing benomyl 0.5 μg/ml and thiabendazole 75 μg/ml according to experiments carried out with *A. nidulans* (6.6.). No cross-resistant recombinant was found. This experiment could be carried out because diploid No. 10 proved to be a very instable diploid. When conidia of this diploid were plated on a benomyl- or thiabendazole-containing medium about 5% of the conidia gave rise to haploids. From this experiment and the results with the other *ben* mutations presented in Table 23, it was concluded that in *A. niger* like in *A. nidulans* mutations at one locus are very probably responsible for all types of benomyl and thiabendazole resistance.

7.4.1. *Induction of mutations and their properties*

Types of mutations that were induced in *C. cucumerinum* and employed in this study are listed in Table 19. Table 24 shows in which strain the mutations were induced, originating from the wild type. In this fungus it appeared that NG was a very effective mutagen for the induction of color and auxotrophic mutations in comparison with UV irradiation. In strains No. 3, 4 and 5 (Table 24) eight mutations to auxotrophy were induced with UV (25% survival), as the result of a total analysis of more than 4000 colonies. Thus, a percentage of 0.2 auxotrophs was obtained. Employing NG (1.5% survival) auxotrophs were obtained in strains 1, 5, 7 and 8 at a frequency of 1.0%. In addition, in the latter experiments a wider spectrum of auxotrophs was obtained. However, a disadvantage of the NG treatment was the loss of pathogenicity of many strains as shown in Table 24. This will be the consequence of the induction

TABLE 24. Origin and pathogenicity of strains of *Cladosporium cucumerinum*.

Strain No.	Genotype	Mutagenic agent	Mutations induced in each strain; percentages of pathogenicity of the resulting strains are given in parenthesis
1	wild type (100)	UV	*ben1* (50); *ben2* (80); *ben3* (85); *ben4* (80); *ben5* (100); *car1* (100); *car2* (100); *car3* (100); *car4* (100); *car5* (75); *car6* (75)
		NG	*arg2* (0); *arg3* (0); *his2* (6); *met6* (66); *orn1*(55); *pal6* (0); *pal7* (100); *pur2* (3)
2	*ben2*	UV	*pal1* (100); *pal2* (80)
3	*car1*	UV	*met2* (0); *met3* (80); *met4* (65); *iva1* (90); *iva2* (80)
4	*ben2; pal2*	UV	*whi1* (100); *met1* (70)
5	*ben2; pal2; met1*	NG	*ade2* (0); *ade3* (0); *arg1* (0); *asp1* (0); *leu1* (0); *rib1* (0)
		UV	*pim1* (15)
6	*ben2; pal2; whi1*	UV	*ade1* (0); *met5* (100)
		NG	*his1* (0); *pur1* (0)
7	*car1; iva1*	NG	*arg4, dar1* (0); *arg5* (0); *his5; lys3; met7; met8* (0); *met9* (0); *nic2* (60); *pal3* (40); *pal4* (0)
8	*car1; met3*	NG	*ade4* (3); *ade5* (0); *bio1* (0); *cho1* (50); *faw1* (70); *his3* (3); *his4* (0); *lys1* (0); *lys2* (0); *nic1* (0); *pal5* (0); *rib2* (0); *whi2* (15)
9	*car1; met3; faw1*	NG	*his6; glu1; ima10* (57); *ima 11* (57); *ima12* (47); *ima13* (47)
10	*ben2; pal2; whi1; met5*	NG	*pur2* (0); *ima1* (67); *ima2* (0); *ima3* (33); *ima4* (53); *ima5* (57); *ima6* (63); *ima7* (57); *ima8* (20); *ima9* (47)
11	*ben2; pal2; whi1; met5; pur2*	NG	*act1* (0); *act2* (0); *act3* (0)

of many additional mutations, causing physiological disorders which apparently easily affect pathogenicity. Two out of eight auxotrophs obtained after UV irradiation lost their pathogenicity, while 23 out of 32 mutants obtained after NG treatment were no longer pathogenic whereas the others showed a more or less reduced pathogenicity (cf. 3.2.). No correlation was found between any particular nutritional requirement and loss of pathogenicity as assumed by GARBER (1956) in his nutrition-inhibition hypothesis (4.1.).

In *C. cucumerinum* resistance was induced to the fungicides benomyl, carboxin, cycloheximide, imazalil and pimaricin. Table 6 (3.3.) presented the degree of resistance of the mutants, the mutagenic agents employed and the mutation frequencies. Figs 5 and 7 (4.2.) depicted a fungitoxicity test in vitro of benomyl- and carboxin-resistant strains, respectively. Section 5.2. dealed with the patterns of cross-resistance of the resistant mutants, while in 4.2. pathogenicity was discussed.

7.4.2. *Selection of heterozygous diploids*

The methods for heterozygous diploid selection described for *A. nidulans* and *A. niger* (2.7.1.) were adopted for *C. cucumerinum*. Only the incubation time was altered. Different auxotrophic strains were grown for 2–3 days on CM after which pieces of mixed mycelium were transferred to MM. The experiments, in which 50–100 pieces were used (three per dish) were carried out with more than 30 strain combinations. After 3–6 weeks of incubation no heterokaryon formation was observed. Back mutation of a nutritional requirement in one of the strains was often observed. The back mutation frequency of an auxotrophic mutation can be expressed as the frequency with which one spore plated on SM, from which the required nutrient is omitted, gives rise to a colony. It was found that back mutation of *met1* and *met3* occurred at a frequency of about 8.10^{-8} and 4.10^{-8}, respectively, while in the case of *arg5*, *his1* and *leu1* the back mutation frequency was less than 3.10^{-8}, 1.10^{-7} and 2.10^{-8}, respectively. Nevertheless for three strain combinations diploid strains were recovered almost certainly. The frequency of back mutation in these experiments was about a factor 20 higher than that of the occurrence of diploids. Thus, diploid strains were selected under the most favourable circumstances at a frequency of 5.10^{-9}. The strain combinations in which diploid selection was successful were:

1. *car1*; *met3* × *ben2*; *pal2*; *whi1*;
2. *car1*; *iva1*; *arg5* × *ben2*; *pal2*; *met1*; *leu1*
3. *car1*; *iva1*; *arg4*; *dar1* × *ben2*; *pal2*; *met1*; *leu1*

Spores of *C. cucumerinum* showed a considerable variation in size. Therefore, to be sure whether a strain was really diploid the DNA and RNA contents of haploid and diploid conidia were determined as described in 2.10. The results per 10^8 nuclei were: 11.4 μg DNA and 112 μg RNA for the diploid strain No. 3 and 5.6 μg DNA and 88 μg RNA for the haploid wild type (strain No. 1, Table 24).

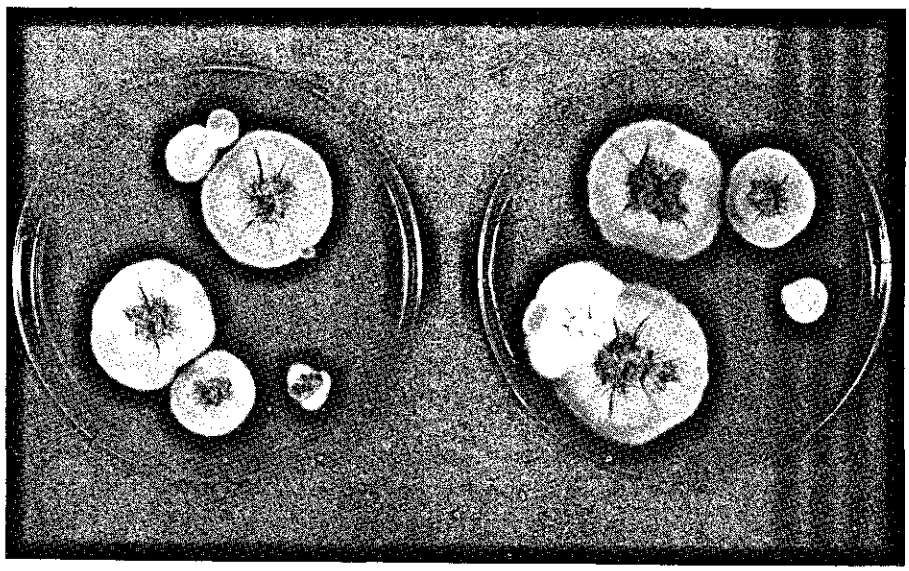


FIG. 51. A diploid strain of *Cladosporium cucumerinum* grown for two weeks on CM containing benomyl, showing segregation.

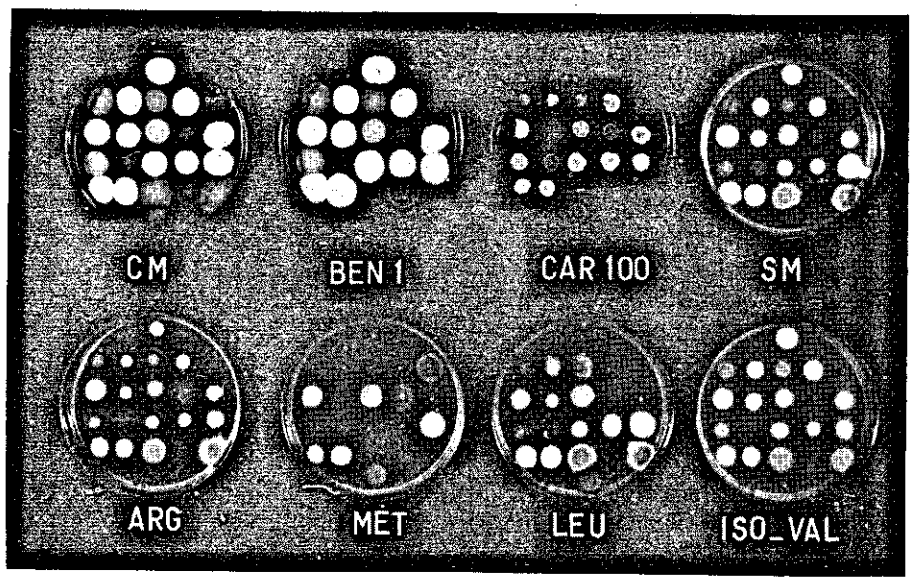


FIG. 52. Diploid analysis in *Cladosporium cucumerinum*. Using the strains *(ben2; pal2; met1; leu1)* and *car1, iva1; arg4; dar1)*, inoculated at 'top' and 'bottom' of the CM plate, a diploid strain was isolated (Fig. 51). The haploids originated from mitotic haploidization of this diploid were tested for the different markers of the parental strains. The various test media were: CM, CM + 1 μg/ml benomyl (BEN 1), CM + 100 μg/ml carboxin (CAR 100); MM + arginine, methionine, leucine, isoleucine and valine (SM); SM – arginine (ARG), SM – methionine (MET); SM – leucine (LEU) and SM – isoleucine and valine (ISO-VAL).

TABLE 25. Haploidization analysis in *Cladosporium cucumerinum* of diploid No. 3 constituted from *(ben2; pal2; met1; leu1)* and *(car1; iva1; arg4; dar1)*. Haploids are classified for pairs of markers, into parental and recombinant genotypes.

Mutant and wild-type alleles	*ben*	+	*pal*	+	*met*	+	*leu*	+	*car*	+	*iva*	+	*arg*	+	*dar*	+
ben																
+																
pal	29	2														
+	14	0														
met	29	0	23	6												
+	14	2	8	8												
leu	14	1	8	7	9	6										
+	29	1	23	7	21	9										
car	27	0	19	8	22	5	11	16								
+	16	2	12	6	8	10	4	14								
iva	16	1	6	11	7	10	10	7	11	6						
+	27	1	25	3	23	5	5	23	16	12						
arg	16	1	5	12	7	10	10	7	11	6	17	0				
+	27	1	26	2	23	5	5	23	16	12	0	28				
dar	29	1	18	12	17	13	13	17	19	11	17	13	17	13		
+	14	1	13	2	13	2	2	13	8	7	0	15	0	15		

7.4.3. *Selection and analysis of haploid segregants*

Although the use of p-fluorophenylalanine as a haploidizing agent according to LHOAS (1961, 1968) has generally been applied in parasexual genetics this chemical was not successfully employed with *C. cucumerinum*. Also griseofulvin (KAPPAS and GEORGOPOULOS, 1974) did not give good results; however, with benomyl as a selective and haploidizing agent, a number of haploid segregants were, though with difficulty, recovered from diploids (Fig. 51). Most of the segregants had the wild-type green color, like the diploid itself, and probably many of these were diploid. Therefore, only the auxotrophic green, the pale, the dark green and the grey (pale and dark green) strains were used for genetic analysis. Table 25 shows the test results of 45 haploids classified for pairwise combinations of eight markers. It appeared that of the eight markers involved in the analysis *arg4* and *iva1* segregated together, indicating that they were located to the same linkage group. Fig. 52 depicts a plate with haploids tested for the different markers.

TABLE 26. Percentage pathogenicity of 15 recombinant haploid strains of *Cladosporium cucumerinum* derived from a diploid heterozygous for deficiencies for arginine *(arg)*, isoleucine + valine *(iva)*, leucine *(leu)* and methionine *(met)*, compared with this diploid and the two parent strains.

Deficiency(-ies) present in the strain	Number of strains	% Pathogenicity
iva, arg (parent strain)		0
met, leu (parent strain)		0
– (diploid No. 3)		100
–	6	52, 65, 82, 85, 92, 104
leu	1	67
met	3	39, 47, 67
met, leu	1	15
arg, iva, leu	1	6
arg, iva, met	3	6, 11, 12

7.4.4. *Pathogenicity of diploids and haploid segregants*

Although five of the six parental strains from which the three diploid strains were derived (7.4.2., Table 24), had lost their pathogenicity, the diploids themselves were as pathogenic as the original wild type. Similar results have been reported for *P. italicum* (BERAHA et al., 1964) and for *P. expansum* (BERAHA and GARBER, 1965) where diploids were as pathogenic as the prototrophic haploid wild type.

Pathogenicity tests of haploid recombinants derived from diploid No. 3, confirmed the supposition that additional mutations but not the new mutation to auxotrophy (or resistance, 4.2.) itself affect pathogenicity. Strain No. 5 (Table 24) showing a pathogenicity of 70%, lost its pathogenicity upon induction of the *leu1* mutation. A recombinant haploid, carrying this *leu1* mutation, showed a pathogenicity of 67%. Table 26 shows the pathogenicity percentages of 15 recombinant haploids, for which only the deficiencies are indicated. On the basis of the data of Table 26, however, it cannot be excluded that an accumulation of requirements in one strain will decrease pathogenicity. This phenomenon can be explained by assuming an insufficient amount of amino acid at the site of inoculation although the ability to attack the plant is not affected (BERAHA and GARBER, 1965; COPLIN et al., 1974).

7.5. DISCUSSION

Results with *Ustilago maydis* and *Aspergillus niger* were in accordance with findings with *Aspergillus nidulans* (6.3.), where a high level of resistance to benomyl and/or thiabendazole and the phenomenon of negative cross-resistance to benomyl proved to be based on mutations at one locus. In *U. maydis* this was demonstrated by sexual crosses, while in *A. niger* the parasexual cycle was used. Both BORCK and BRAYMER (1974) and BEN-YEPHET et al. (1974) also

identified in *Neurospora crassa* and *Ustilago hordei*, respectively, only one gene being involved in benomyl resistance. Therefore, it may be concluded that in various fungal species benomyl resistance including the phenomenon of negative cross-resistance (5.2.1.) is based on mutations in a single gene.

The parasexual cycle in *A. niger*, which was already known to exist (PONTECORVO et al., 1953a), was demonstrated and especially employed for examining the genetics of resistance. In agreement with LHOAS (1967) a number of mutations conferring conidial color, auxotrophy and resistance was assigned to six linkage groups. Only for a few mutations the location differed from Lhoas' results. This difference could be explained by assuming a reciprocal translocation which once occurred in nature in one of the wild-type strains. All 42 mutations analysed in heterozygous diploids segregated in a 1:1 ratio, indicating single nuclear gene inheritance.

Cladosporium cucumerinum is a fungus for which a parasexual cycle was not known to exist. Though with some difficulty, its existence could be demonstrated here. It was exceptional, however, in that no heterokaryons were observed. FINCHAM and DAY (1971) reported that in *Cladosporium fulvum* also attempts to produce balanced heterokaryons were unsuccessful. Another recently published case in which heterozygous diploid strains were isolated without apparent heterokaryon formation has been reported by DE BERTOLDI and CATEN (1975) in a *Humicola* species.

As far as known no parasexual cycle exists in perfect fungi such as *Neurospora crassa* and *Venturia inaequalis* while in the imperfect fungus *Colletotrichum lagenarium*, where well-growing heterokaryons were obtained by DUTTA and GARBER (1960), no diploids could be isolated. On the other hand, the parasexual cycle has been demonstrated in many *Aspergillus* spp. which produce extensively growing heterokaryons, and further in *Penicillium chrysogenum* (PONTECORVO and SERMONTI, 1953, 1954) and *Cephalosporium acremonium* (NÜESCH et al., 1973) where the heterokaryons grew poorly and from which diploids arose directly as vigorously growing sectors. Finally, it exists in fungi like *C. cucumerinum* and a *Humicola* species where diploids were selected without detecting any heterokaryon formation.

Diploid strains of *C. cucumerinum* appeared to be as pathogenic as the haploid wild type, although the parental strains from which the diploids were derived, had lost their pathogenicity. These results are comparable to those of INGRAM (1968), who showed by demonstrating the existance of the parasexual cycle that *Verticillium dahliae var. longisporum* was a stable diploid of *V. dahliae*, and that both haploid and diploid stages occurred in nature.

The parasexual cycle in *C. cucumerinum* provided genetic information on the loss of pathogenicity of certain auxotrophic mutants. The pathogenicity of one of the parental strains of a diploid was lost after the induction of the *leu1* mutation. A recombinant haploid, carrying *leu1*, however, was highly pathogenic. This proved that not the newly induced mutation *leu1* but additional mutations had affected pathogenicity. The pathogenicity of diploids indicated that these additional mutations, which caused the loss of patho-

genicity, were recessive. Similar results were reported by BERAHA et al. (1964) with *Penicillium italicum* on oranges; they also obtained pathogenic diploids derived from two non-pathogenic parental haploid strains.

8. GENERAL DISCUSSION

Since the introduction of systemic fungicides, development of fungicide resistance in plant-pathogenic fungi has been reported frequently although resistance to conventional fungicides is hardly known. Most systemic fungicides are 'specific-site' inhibitors, whereas most conventional fungicides are 'multi-site' inhibitors or general plasma toxicants. Within the group of specific-site inhibitors differences with respect to acquired resistance may exist between fungicides, according to their mechanism of action. DEKKER (1974) argued that the possibility of development of resistance in a fungus depends on various factors, viz. type of fungicide, type of fungus and type of disease. In order to prove this, experiments were carried out with ten fungi and seven different systemic fungicides, viz. benomyl, carboxin, chloroneb, cycloheximide, imazalil, pimaricin and thiabendazole (chapter 3). On the basis of the results obtained these fungicides could be divided into two groups. With one group, consisting of the benzimidazole fungicides, carboxin, chloroneb and cycloheximide high levels of resistance were obtained, whereas with the other group consisting of imazalil and pimaricin only low levels of resistance were found (Table 6). Although the mutation frequencies as observed after mutagenic treatment differed considerably between these fungicides it remains doubtful whether these differences play a significant role with respect to emergence of resistance in the field. In all fungi tested mutations to resistance could be induced to all fungicides; even in the case of the lowest mutation frequency, as observed for the benzimidazole fungicides, development of resistance under field conditions appeared to be a fairly common phenomenon.

In addition to the potential of a fungus to acquire fungicide resistance, the characteristics of the resistant strains are of importance. Whether a resistant population will built up under field conditions depends for a great deal on the pathogenicity of the resistant mutants. This aspect was studied in greenhouse experiments with five pathogens and several fungicides (chapter 4). No direct relation was found between a more or less decreased pathogenicity and an increased resistance to benzimidazole fungicides, carboxin, chloroneb and imazalil. Loss of pathogenicity in these cases has to be considered as the result of mutagenic treatment as proved by genetic analysis in the case of an auxotrophic mutant of *Cladosporium cucumerinum* (chapter 7) and in dodine resistance in *Nectria haematococca* (KAPPAS and GEORGOPOULOS, 1971). Further, it was shown in fungitoxicity tests that the level of resistance in vitro was comparable with that in vivo.

Fungicide resistance cannot be adequately studied without knowledge of fungal genetics. Because of its suitability the non-pathogenic *Aspergillus nidulans* was employed as the main experimental organism in genetic studies (chapter 6). Genetic analysis in this fungus by means of the sexual and parasexual cycles showed that in all cases resistance was heritable and controlled

by nuclear genes. By recombination analysis the number of genes involved in fungicide resistance was determined. In general, it was possible to distinguish either one main locus as established for benomyl (*benA*), chloroneb (*chlA*) and oligomycin (*oliC*) or more than one locus as found for pimaricin (*pimA*, *pimB*), carboxin (*carA*, *carB*, *carC*), cycloheximide and imazalil, the last two cases of resistance being multigenic. For 16 genes conferring fungicide resistance meiotic mapping on six of the eight linkage groups was accomplished.

The identification of more than one locus of resistance in some cases created the possibility of examining additive effects of these genes when recombined in one strain. In the case of imazalil resistance a strain with three genes each conferring a low level of resistance, gave rise to a high level of resistance. Such a phenomenon may happen in the field through the occurrence of a number of successive mutations although probably more slowly than in the case of a single mutation determining a high level of resistance.

The dominance behaviour of mutations to resistance was studied in heterozygous diploids of *A. nidulans*. A range of degrees of dominance could be distinguished: from virtually recessive in the case of mutations to chloroneb resistance to almost completely dominant in the case of one of those to carboxin resistance and of some to cycloheximide resistance.

In the study of mutations affecting amino acid transport in *A. nidulans* KINGHORN and PATEMAN (1975) gave a plausible explanation for dominant and semi-dominant behaviour of mutations conferring resistance to amino acid analogs. The authors state that the mutated loci may determine synthesis of proteins essential for normal amino acid transport function. The heterozygous diploid contains normal and mutant alleles which code for normal and abnormal transport proteins. In this model a 'transport site' is proposed as a complex body containing many interacting copies of each type of transport protein. If all transport sites contain sufficient abnormal components to reduce transport, the mutation would appear to be dominant. In terms of resistance this should mean that a toxicant causes no growth inhibition of a heterozygous diploid owing to a change in many sites involved in uptake or transport. In view of the fact that dominant mutations have as yet mainly been observed in studies on transport systems, in which resistance to amino acid or purine analogs was included, (DARLINGTON and SCAZZOCCHIO, 1967; SINHA, 1969; KINGHORN and PATEMAN, 1975) the question may arise in how far dominant and semi-dominant mutations to fungicide resistance are associated with transport systems. Therefore the known mechanisms of action of the fungicides will be considered, to which dominant or semi-dominant mutations to resistance were found, viz. carboxin, imazalil and cycloheximide. In the case of carboxin mitochondrial respiration is inhibited at or close to the site of succinate oxidation (MATHRE, 1971; WHITE, 1971; GEORGOPOULOS et al., 1972; ULRICH and MATHRE, 1972; GUNATILLEKE et al., 1975a; WHITE and THORN, 1975). Also in this study evidence was presented that reactions in the tricarboxylic acid cycle were involved in the mechanism of action of carboxin.

Thus, since carboxin acts on succinate dehydrogenase this is not in agreement with the explanation put forward for the (semi-) dominance of the mutations to carboxin resistance. The effect of cycloheximide on incorporation of amino acids into proteins (SIEGEL and SISLER, 1964; VOMVOYANNI, 1974) can be ascribed to changed ribosomal properties. An interpretation of dominance could be here, that with sufficient changed ribosomes, cycloheximide should not cause growth inhibition of a heterozygous diploid. The results of the characterization of imazalil-resistant mutants, in which a number of pleiotropic effects were found (chapter 5), indicate that imazalil may interfere with permeability of the cell membrane. Although there is not yet strong evidence for this, interference with sites involved in uptake or transport might be affected, explaining the semi-dominance of the mutations to imazalil resistance.

The importance of characterizing the resistant mutants (chapter 5) was already shown in the considerations about dominance, where evidence was presented for the mode of action of carboxin and imazalil. In the case of resistance to benzimidazole fungicides in *A. nidulans*, as well as in *Aspergillus niger* and *Ustilago maydis*, a number of allelic mutations, determining different levels of benomyl and thiabendazole resistance, were identified; among these were mutations to thiabendazole resistance also conferring increased sensitivity to benomyl, a phenomenon indicated as negative cross-resistance. This allelism gave evidence that one mechanism of action was responsible for resistance to both these fungicides, as was further elucidated with some of these alleles in *A. nidulans* by DAVIDSE (1974, 1975, 1976).

Since the demonstration in *A. nidulans* of heterokaryosis, formation of heterozygous diploids and recovery of diploid and haploid segregants proved the existance of parasexuality, the latter phenomenon has been investigated in a number of imperfect plant-pathogenic fungi. In order to examine whether results obtained with *A. nidulans* were applicable to plant pathogens parasexual genetics were used in *A. niger* and *C. cucumerinum* (chapter 7). With this method it was demonstrated that benomyl and thiabendazole resistance included negative cross-resistance in *A. niger*, like in *A. nidulans*, was determined by one gene. Thirty-nine nuclear gene mutations of *A. niger* were assigned to six linkage groups. These results were similar to those of LHOAS (1967) as far as a comparison can be made.

In the study of the parasexual cycle in *C. cucumerinum* diploid strains were isolated from mixed cultures of auxotrophic mutants, however unexpectedly, without a detectable heterokaryotic state. Recently, this was also found in a *Humicola* spp. (DE BERTOLDI and CATEN, 1975). The diploid strains of *C. cucumerinum*, which occurred in a rather low frequency of about 10^{-9}, were very stable. Although the haploid parental strains of two of the three isolated diploid strains were non-pathogenic, the diploids themselves were as pathogenic as the prototrophic wild-type strain. Pathogenicity tests of haploid segregants recovered from one of these diploids showed segregants with both high and low pathogenicity. This proved that loss of pathogenicity was due to additional mutations, which must have been induced by the same mutagenic

treatment as the mutation on the basis of which the strain was selected (color, auxotrophy, resistance). It means, that *C. cucumerinum*, for various reasons an outstanding experimental organism in phytopathology, is also an invaluable tool in an integrated genetic and biochemical study on fungicide resistance and pathogenicity.

SUMMARY

Since the introduction of the systemic fungicides, fungicide resistance has become a serious problem in plant disease control. This study was carried out in order to contribute to the knowledge about the genetics of fungal resistance to fungicides both from a practical and a fundamental point of view.

The potential of a fungus to acquire fungicide resistance, measured as mutation frequency and degree of resistance, was investigated in various fungi. The fungicides employed were: the benzimidazole fungicides benomyl and thiabendazole; carboxin, an oxathiin compound; chloroneb, an aromatic hydrocarbon; imazalil, an imidazole derivative; and the two antibiotics cycloheximide and pimaricin, a glutarimide and a polyene antibiotic, respectively (chapter 3). Induced resistance occurred in all cases, in which it was searched for. The mutation frequencies varied from 10^{-7} for pimaricin and benomyl to 2.10^{-4} for chloroneb resistance (Table 6). Both mutation frequency and degree of resistance were independent on the fungal species. However, considerable differences between the fungicides were found in the highest level of resistance observed among the resistant strains. From the results it was concluded that imazalil and pimaricin, for which the level of resistance in relation to the wild type increased at most by a factor 10 and 4, respectively, might be used in agriculture without a considerable chance of interference with control. On the other hand the development of resistance to the other fungicides of 50–5000 times, makes their practical use rather questionable.

Pathogenicity and in vivo fungitoxicity tests were carried out with benomyl-resistant strains of five plant pathogens, viz. *Cladosporium cucumerinum*, *Cercosporella herpotrichoides*, *Fusarium nivale*, *Penicillium expansum* and *Phialophora cinerescens* (chapter 4). Generally, a close correlation between resistance in vitro and in vivo could be established. With respect to pathogenicity some strains were as pathogenic as the wild type, others were less pathogenic and a few had lost their pathogenicity. However, the results did not allow the conclusion that loss of pathogenicity was due to the mutation to resistance.

Characterization of the resistant mutants in vitro was performed especially in *Aspergillus nidulans* (chapter 5). Dosage response relationships of fungicide resistance in agar growth tests were presented. With the four fungi examined, viz. *A. nidulans*, *Aspergillus niger*, *P. expansum* and *Ustilago maydis*, cross-resistance of the chloroneb-resistant strains was observed to pentachloronitrobenzene (PCNB), a related aromatic hydrocarbon fungicide, and to 3-phenylindole. Usually, resistance to either benomyl or thiabendazole involved resistance to the other compound but in rare cases resistance to benomyl only or more frequently to thiabendazole only occurred, the latter involving increased sensitivity to benomyl. This phenomenon, known as negative cross-resistance was found in all five fungi examined, viz. *A. nidulans*, *A. niger*, *P. expansum*, *Rhodotorula rubra* and *U. maydis*. A number of pleiotropic effects in the imazalil-resistant mutants of *A. nidulans* and *A. niger* was found, viz.

resistance and hypersensitivity to various inhibitors. This suggested that the action of imazalil might be sought in changes in cell membrane permeability.

It is known that for *Saccharomyces cerevisiae* and *U. maydis* carboxin is ten times more toxic on a medium with acetate as sole carbon source than with glucose. This effect was verified in the wild-type strain of *A. nidulans*. However, in the case of the carboxin-resistant strains this difference was the smaller the more resistant the strains were. This strengthened the observations that reactions in the tricarboxylic acid cycle are involved in the mechanism of action of carboxin, since carboxin has been shown to inhibit mitochondrial succinate oxidation.

Using genetically well-defined strains of *A. nidulans*, it was possible to establish the genetic basis of characters such as patterns of cross-resistance. Genetic analysis with the non-pathogenic *A. nidulans* was carried out by means of the sexual and parasexual cycles (chapter 6). A single nuclear gene relationship of all mutations to resistance was found. The number of loci involved in the resistance to a fungicide was determined by recombination analysis of different mutants. It appeared that with some fungicides such as chloroneb and oligomycin only one locus was responsible for all the mutations to resistance; on the other hand in the case of resistance to pimaricin and to carboxin two and three genes, respectively, were identified, whereas in the case of cycloheximide and imazalil resistance a multigenic system was involved. High-level resistance to benomyl and/or thiabendazole appeared to be based on mutations at one locus. Twenty-three genes conferring resistance were assigned to seven different linkage groups (chromosomes), sixteen of which were mapped. Using heterozygous diploids the dominance behaviour of the mutations to resistance was studied. A range of degrees of dominance was found from a practically recessive condition of mutations to benomyl, chloroneb and pimaricin resistance to an almost complete dominance of some genes conferring carboxin and cycloheximide resistance. In the case of imazalil resistance a positive interaction of genes resulting in a high degree of resistance in recombinant strains was observed.

In order to verify the results obtained with *A. nidulans* in plant-pathogenic fungi, genetic analysis was also performed with *U. maydis*, using the sexual cycle, and with *A. niger* and *C. cucumerinum*, by means of the parasexual cycle (chapter 7). As a result of this approach it was demonstrated that in *A. niger* and *U. maydis* benomyl and thiabendazole resistance including negative cross-resistance was based, as in *A. nidulans*, on one single gene in which all resistant strains carry a different mutation. Analysis through the parasexual cycle, which involves heterokaryosis, diploid formation and recovery of haploid and diploid segregants, gave similar results in *A. niger* as known from the literature. In *C. cucumerinum*, for which a parasexual cycle was not known, diploid strains were isolated, from which recombinant haploids were also recovered, although, surprisingly, without observing a heterokaryotic state. The parasexual cycle in *C. cucumerinum* provided information on the genetic basis of pathogenicity, because loss of pathogenicity of auxotrophic mutants was

fully complemented in the heterozygous diploid strains, which appeared to be as pathogenic as the haploid prototrophic wild-type strain.

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SAMENVATTING

Sedert de introductie van de systemische fungiciden is het optreden van resistentie hiertegen een ernstig probleem geworden bij de bestrijding van fytopathogene schimmels. Deze studie heeft ten doel een bijdrage te leveren aan de kennis van de genetica van resistentie tegen fungiciden bij schimmels, zowel vanuit praktisch als fundamenteel oogpunt.

De potentie van een schimmel om tegen een fungicide resistentie te ontwikkelen waarvoor de mutatiefrequentie en de mate van resistentie als maatstaf golden, werd onderzocht in een aantal schimmels. De onderzochte fungiciden waren: de benzimidazool fungiciden benomyl en thiabendazol; carboxin, een oxathiine verbinding; chloroneb, een aromatische koolwaterstof; imazalil, een imidazool derivaat en de twee antibiotica cycloheximide en pimaricine, respectievelijk een glutarimide en een polyeen antibioticum (hoofdstuk 3). In alle onderzochte gevallen bleek resistentie te kunnen ontstaan. De mutatiefrequentie waarmee resistentie optrad, varieerde van 10^{-7} bij benomyl en pimaricine tot 2.10^{-4} bij chloroneb (Tabel 6). Zowel mutatiefrequentie als mate van resistentie bleken nagenoeg onafhankelijk van het type schimmel. Belangrijke verschillen tussen de fungiciden bestonden echter ten aanzien van de hoogste graad van resistentie die verkregen kon worden bij de resistente stammen. Op grond hiervan werd geconcludeerd dat imazalil en pimaricine, waarvan de resistentie ten opzichte van het wild type maximaal een factor 10 respectievelijk 4 was, weinig kans zouden kunnen geven op voor de praktijk belangrijke resistentie. In tegenstelling hiermee is de kans op resistentie ontwikkeling tegen de andere fungiciden waarvoor de resistente stammen 50–5000 keer minder gevoelig waren dan het wild type, zodanig dat dit tot problemen aanleiding zou kunnen geven.

Pathogeniteits- en fungitoxiciteitsproeven in vivo werden uitgevoerd met benomyl-resistente stammen van vijf plantpathogene schimmels: *Cladosporium cucumerinum*, *Cercosporella herpotrichoides*, *Fusarium nivale*, *Penicillium expansum* en *Phialophora cinerescens* (hoofdstuk 4). In het algemeen werd tussen de mate van resistentie in vitro en in vivo een positieve correlatie waargenomen. Wat betreft de pathogeniteit van de resistente stammen kon onderscheid gemaakt worden tussen stammen die even pathogeen waren als het wild type, andere die minder pathogeen waren en enkele die hun pathogeniteit geheel verloren hadden. De resultaten gaven echter geen uitsluitsel omtrent het feit of verlies van pathogeniteit te wijten was aan de mutatie voor resistentie.

Karakterisering van de resistente mutanten in vitro werd in het bijzonder uitgevoerd met *Aspergillus nidulans* (hoofdstuk 5). Het effect van fungiciden op de groei van resistente stammen op agar-media werd weergegeven in dosis-respons curven. De chloroneb-resistente stammen van de vier onderzochte schimmels *A. nidulans*, *Aspergillus niger*, *P. expansum* en *Ustilago maydis* bleken kruisresistentie te vertonen ten aanzien van pentachloornitrobenzeen

(PCNB), een verwante aromatische koolwaterstof, en 3-phenylindool. Als regel ging resistentie tegen benomyl of thiabendazol gepaard met resistentie tegen beide verbindingen. In een uitzonderlijk geval trad resistentie op tegen benomyl alleen of frequenter tegen thiabendazol alleen maar dan gekoppeld aan een verhoogde gevoeligheid voor benomyl. Het laatstgenoemde verschijnsel werd aangeduid als negatieve kruisresistentie en bleek voor te komen in alle vijf onderzochte schimmels *A. nidulans*, *A. niger*, *P. expansum*, *Rhodotorula rubra* en *U. maydis*. Er werd een aantal pleiotrope effecten van de imazalil-resistente mutanten van *A. nidulans* en *A. niger* gevonden zoals resistentie tegen en/of hogere gevoeligheid voor verschillende toxische stoffen. Deze effecten hebben tot de conclusie geleid dat de werking van imazalil wel eens gezocht zou kunnen worden in relatie tot de permeabiliteit van de celmembraan.

Het is bekend dat carboxin voor *Saccharomyces cerevisiae* en *U. maydis* tien maal zo toxisch is in een medium met acetaat als enige koolstofbron als in een medium met glucose. Dit effect werd ook gevonden voor het wild type van *A. nidulans*. In het geval van de carboxin-resistente stammen was dit verschil echter kleiner naarmate de resistentie groter was. Dit bevestigde de waarnemingen dat reacties in de citroenzuurcyclus betrokken zijn bij het werkingsmechanisme van carboxin, daar van carboxin is aangetoond dat het de mitochondriale succinaatoxidatie remt.

Dankzij het gebruik van genetisch goed gedefinieerde stammen van *A. nidulans* was het mogelijk om met zekerheid de genetische basis van eigenschappen, zoals de verschillende kruisresistenties, vast te stellen. Genetische analyses werden met de niet-pathogene *A. nidulans* uitgevoerd met behulp van de sexuele en parasexuele cycli (hoofdstuk 6). Alle mutaties voor resistentie bleken monogeen chromosomaal te vererven. Het aantal loci dat bij de resistentie tegen een fungicide betrokken was, werd bepaald door recombinatie analyse van verschillende mutanten. Het bleek dat in sommige gevallen zoals bij resistentie tegen chloroneb en oligomycine slechts één locus verantwoordelijk was voor de resistentie, anderzijds werden in de resistentie tegen pimaricine twee en tegen carboxin drie genen geïdentificeerd terwijl resistentie tegen cycloheximide en imazalil op een multigene basis berustte. Een hoge graad van benomyl en/of thiabendazole resistentie bleek gebaseerd te zijn op mutaties in één gen. Drieentwintig resistentiegenen bleken op zeven verschillende koppelingsgroepen (chromosomen) gelegen te zijn, van welke er zestien exact gelokaliseerd werden. Met behulp van heterozygote diploiden werd de mate van dominantie van mutaties voor resistentie bestudeerd. Dit leverde een reeks van graden van dominantie op, variërend van de bijna volkomen recessiviteit van de mutaties voor benomyl, chloroneb en pimaricine resistentie tot bijna volledige dominantie van enkele carboxin en cycloheximide resistentiegenen. In het geval van imazalil resistentie werd een positieve interactie van geneh waargenomen welke een hoge graad van resistentie opleverde in stammen die na recombinatie van verschillende resistentiegenen waren ontstaan.

Om de met *A. nidulans* verkregen resultaten te kunnen vergelijken met die van plantpathogene schimmels, werd ook genetisch onderzoek verricht met

U. maydis, door gebruik te maken van de sexuele cyclus en met *A. niger* en *C. cucumerinum*, met behulp van de parasexuele cyclus (hoofdstuk 7). Dankzij deze benadering kon worden aangetoond dat overeenkomstig de resultaten met *A. nidulans*, ook in *A. niger* en *U. maydis* de benomyl en thiabendazol resistentie met inbegrip van de negatieve kruisresistentie was gebaseerd op veranderingen in slechts één gen waarin alle resistente stammen een verschillende mutatie hadden ondergaan. Onderzoek door middel van de parasexuele cyclus, bestaande uit heterokaryose, diploidvorming en het daaruit verkrijgen van haploide en diploide recombinanten, leverde bij *A. niger* met de literatuur overeenstemmende resultaten op. Bij *C. cucumerinum*, waarvoor nog geen parasexuele cyclus bekend was, lukte het om diploide stammen te isoleren, waaruit ook weer haploide recombinanten werden verkregen. Opvallend hierbij was dat geen heterokaryonvorming werd waargenomen. De parasexuele cyclus in *C. cucumerinum* verschafte informatie aangaande de genetische basis van de pathogeniteit, want een verlies van de pathogeniteit van auxotrofe mutanten werd volledig gecomplementeerd in de heterozygote diploide stammen, die even pathogeen bleken te zijn als het haploide prototrofe wild type.

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CURRICULUM VITAE

Jacob Marius van Tuyl, geboren op 14 januari 1950 te Herwijnen, behaalde het einddiploma H.B.S.-B. in 1968 aan de Prins Bernhard H.B.S. te Velsen. Van 1968 tot 1974 studeerde hij aan de Landbouwhogeschool te Wageningen in de richting planteziektenkunde, waarvan het doctoraal examen met als hoofdvak entomologie en de bijvakken fytopathologie (verzwaard) en genetica op 11 juni 1974 met lof werd behaald. Vanaf februari 1974 was hij als promotie-assistent werkzaam op het Laboratorium voor Fytopathologie te Wageningen, alwaar dit proefschrift werd bewerkt.