MECHANISM OF RESISTANCE IN *PENICILLIUM ITALICUM* TO FUNGICIDES WHICH INHIBIT STEROL 14α-DEMETHYLATION

Promotor : dr. ir. J. Dekker,
Emeritus-hoogleraar in de fytopathologie.

Co-promotor : dr. ir. M. A. de Waard,
Universitair hoofddocent.

J. Guan

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Proefschrift
ter verkrijgen van de graad van doctor
in de Landbouw- and Milieuwetenschappen
op gezag van de rector magnificus,
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van de Landbouwuniversiteit te Wageningen.

Theorems

1. The mechanism of resistance in *Penicillium italicum* to fungicides which inhibit sterol 14α-demethylation is related to factors which prevent the fungicides from reaching their target site.

   This thesis.

2. The cell-free bioassay of ergosterol biosynthesis in cell-free extracts of *Penicillium italicum* is suitable to evaluate fungicides for their potency to inhibit sterol 14α-demethylation and other enzymes involved in ergosterol biosynthesis.

   This thesis.

3. Application of fungicides is part of a complex crop protection system that involves more than merely spraying a chemical on a crop.

   H. Cole jr. 1986. Fungicide Chemistry (M. B. Green & D. A. Spilker (Eds.), p. 128-134.

4. Pesticide use is justified on grounds that the benefits outweigh the risks. However, those benefits can change over time with weather, crop prices, shifts in pest populations, changes in crops and cropping practices, and resistance.

   M. Dover & B. Croft, 1984. Getting Tough - Public policy and the management of pesticide resistance. World resources Institute. p. 38.

5. Because the possibility always exists that disease-causing fungi will break through non-chemical crop protection measures, the use of fungicides will remain essential.

   N. N. Ragsdale. 1991. Health and environmental factors associated with agricultural use of fungicides. National Agricultural Pesticide Impact Assessment Program. p. xi.

6. Banning the use of a fungicide causing a very small risk may expose the public to fungal food contamination presenting much greater risks due to mycotoxins and fungal induced phytoalexins.

N. N. Ragsdale. 1991. Health and environmental factors associated with agricultural use of fungicides. National Agricultural Pesticide Impact Assessment Program. p. 18.

7. In the popular media crop protection chemicals are often called "agricultural poisons". As this term wrongly frightens the consumer that his food has been treated with poisons, it is important to provide the public with realistic information about the risks of crop protection chemicals.

J. Dekker. 1990. British Crop Protection Council Reviews, p. 1.

8. Development of resistance to pesticides is the consequence of mutation and selection. In fact, it is a speeding-up of evolution, and therefore it can be considered as a natural process.

9. The contribution from ordinary food to occurrence of cancer is about as great as that from smoking.

P. H. Abelson. 1992. Science 255: 141.

10. If Christopher Columbus returned today, and looked at the social and environmental damage which the "age of discovery" has led to, he might well decide that he should have stayed at home.

D. Dickson. 1992. New Scientist 1808: 2.

Theorems with the thesis "Mechanism of Resistance in *Penicillium italicum* to Fungicides which Inhibit Sterol 14α-demethylation".

Wageningen, 25 March 1992. J. Guan

To my parents

Foreword

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CONTENTS

CHAPTER 1

General introduction

Fungicides

Plant diseases have probably influenced human welfare before recorded history. Several plant diseases such as blights, blasts and mildews were already mentioned in the Bible. Serious human sufferings from plant diseases have also occurred in more recent history. In the 1840s, potato late blight caused by *Phytophthora infestans* was directly responsible for the Irish potato famine (Large, 1940). About a century later another plant disease, brown spot of rice caused by *Helminthosporium oryzae* severely reduced rice yield in Bengal and in consequence, about two million people died of starvation (Padmanabhan, 1973).

Discovery of Bordeaux mixture in 1885 opened an era of chemical control of plant diseases. Since then, chemical control has experienced three generations of fungicides. In the first generation, sulphur, copper and mercury were used as disease controlling agents. Introduction and use of organic fungicides, like dithiocarbamates (ferbam and ziram), ethylenebisdithiocarbamates (zineb, maneb and mancozeb), quinones (dichlone and chlorothalonil) and phthalimides (captan, captafol and folpet) represents the second generation. The third generation of fungicides is highlighted by the discovery of systemic fungicides. The first and second generation of fungicides are in general non-systemics and react with various cellular sites or components of the pathogen, resulting in inhibition of many metabolic functions. These fungicides have a broad spectrum of activity and are used for control of diseases as protectants. The third generation of fungicides represents the modern fungicides. Most of these fungicides have systemic activity and generally react specifically at one site resulting in inhibition of a single metabolic function (Lyr, 1977). Among systemic fungicides, a future group of compounds is developing, which may represent the fourth generation of fungicides. These compounds are nonfungitoxic. They provide disease control by interference with the infection process of the pathogen or stimulate host defence mechanisms. Therefore, they have several advantages over conventional systemic fungicides: less environmental hazard, active at extremely low level and less likely to encounter fungal resistance (Sisler and Ragsdale, 1987).

Mode of action of systemic fungicides

The mode of action of systemic fungicides is ascribed to their primary site of inhibition in fungal metabolism. Any secondary effects occurring are either due to additional activity of the compounds at a concentration higher than necessary to inhibit fungal growth or due to changes in metabolism of the fungus as a consequence of the primary effect of the fungicide (Kaars Sijpesteijn, 1977). Based on their mode of action, the commercial systemic fungicides can be divided into four major groups: inhibitors of a) respiration, b) membrane permeability, c) mitosis and cell division and d) biosynthetic processes.

Inhibitors of respiration. Any interference with fungal respiration results in inhibition of many metabolic processes and finally leads to death when the energy supply is under the level for maintenance of essential cell performance. The mode of action of carboxamide fungicides such as carboxin, oxycarboxin, benodanil and mebenil is inhibition of succinate dehydrogenase activity in the citric acid cycle (Schewe and Lyr, 1987). These compounds act selectively against rusts and smuts.

Inhibitors which influence membrane permeability. Many cationic agents have fungicidal activity although only few of them have been developed as commercial fungicides. Dodine is a well known example although it has only a limited systemic activity. It accumulates in membranes and causes disruption of membrane permeability. This results in leakage of vital cell components and cell death (Corbett *et al.*, 1984).

Inhibitors of mitosis. Representatives of this group of fungicides are benzimidazoles (benomyl) and benzimidazole-generating compounds (thiophanate-methyl). These fungicides belong to the most important groups of systemic fungicides. They have a broad spectrum of action against Ascomycetes and Fungi Imperfecti. These fungicides bind to ß-tubulin, a sub-unit of the microtubules and therefore, interfere with microtubule assembly. As a consequence, mitosis, cell division and other microtube-dependent processes are inhibited (Davidse, 1987a).

Inhibitors of biosynthetic processes. Many biosynthetic processes in fungi can be inhibited by systemic fungicides. Well known targets are RNA polymerase, adenosine deaminase and sterol biosynthesis enzymes. Phenylamide fungicides, like furalaxyl and metalaxyl, inhibit RNA polymerase I, which is responsible for

the synthesis of ribosomal RNA (Davidse *et al.*, 1983). These compounds are used for control of fungi belonging to Peronosporales (*e. g.* potato late blight and downy mildews). The mode of action of the aminopyrimidine fungicides ethirimol and dimethirimol is inhibition of the enzyme adenosine deaminase which catalyses deamination of adenosine to inosine (Hollomon and Chamberlain, 1981). These fungicides are mainly used for control of powdery mildews. Fungicides which inhibit sterol biosynthesis (SBIs) have been introduced since the early 1970's. This group of fungicides includes a large number of structurally unrelated compounds. They have a wide antifungal spectrum including Ascomycetes, Basidiomycetes and Fungi Imperfecti and are at present among the most important groups of fungicides and antimycotics (Scheinpflug and Kuck, 1987; Vanden Bossche, 1985).

Ergosterol biosynthesis in fungi and its inhibition

Fungal sterols. Most species of Ascomycetes, Basidiomycetes and Fungi Imperfecti contain ergosterol as a major sterol (Nes, 1984; Weete, 1989). However, in rust fungi (Uredinales) (Weete, 1989) and powdery mildews (Loeffler *et al.*, 1984) ergosterol is not the major sterol. These fungi contain other related C14-desmethyl sterols. Oomycetes are unable to synthesize any sterols and are therefore insensitive to fungicides which inhibit sterol biosynthesis (Nes, 1987).

Sterols are known to play at least two roles in cells: a) The bulk membrane function for which large quantities of sterols are needed, but structural requirements are in this case less specific and b) the regulatory or sparking function for which the amount necessary is much less, but structural requirements are highly specific (Nes, 1987; Burden *et al.*, 1989). Ergosterol appears to satisfy both functions.

Ergosterol biosynthesis. Ergosterol biosynthesis in fungi was reviewed by Weete (1989) and Mercer (1984). The biosynthetic pathways found in fungi is presented in Fig. 1. All sterols are synthesised from acetic acid via mevalonic acid and squalene as key intermediates. Lanosterol is the first cyclization product. The conversion of lanosterol to ergosterol occurs through two alternative pathways in yeasts and filamentous fungi. In filamentous fungi, a methyl group is first introduced at the C-24 position of lanosterol to produce 24-methylenedihydrolanosterol (eburicol) (A). The next step is to remove a methyl group at the C-14 position (B). This reaction is cytochrome P450-dependent.

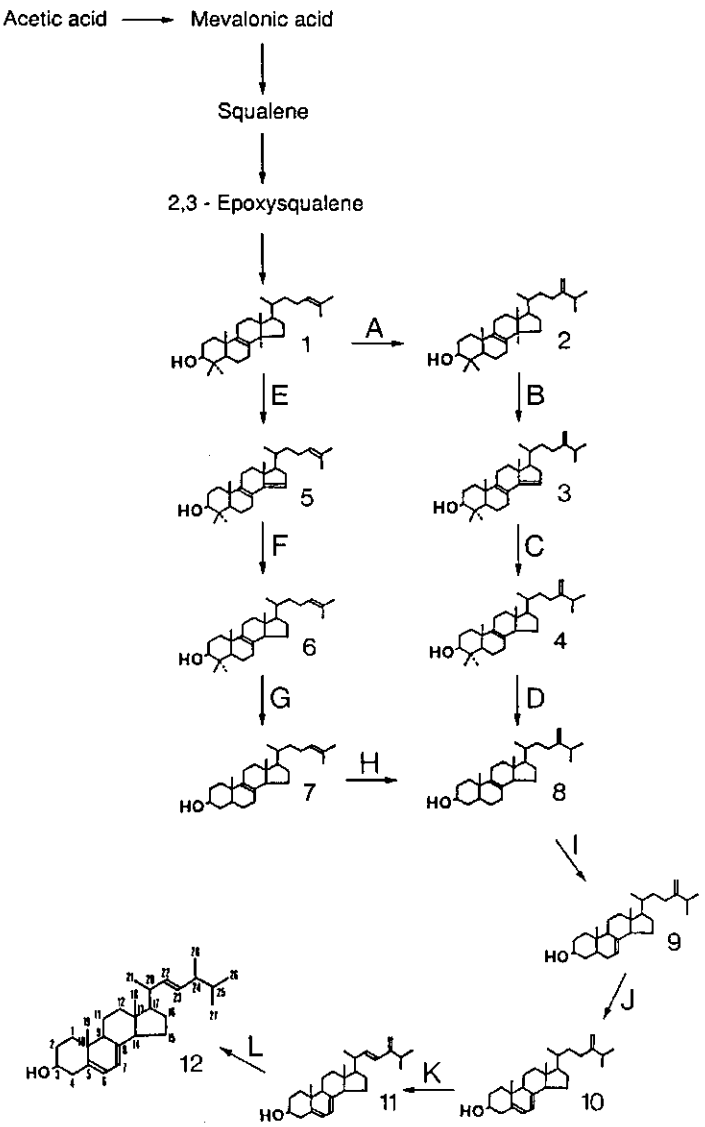

Fig. 1. Ergosterol biosynthesis pathway in fungi and its inhibition steps by various compounds. 1, Lanosterol; 2, 24-Methylenedihydrolanosterol; 3, 4,4-Dimethyl-ergosta-8,14,24(28)-trienol; 4, 4,4-Dimethyl-ergosta-8,24(28)-dienol; 5, 4,4-Dimethyl-cholesta-8,14,24-trienol; 6, 4,4-Dimethyl-cholesta-8,24-dienol; 7, Cholesta-8,24-dienol (zymosterol); 8, Ergosta-8,24(28)-dienol (fecosterol); 9, Episterol; 10, Ergosta-5,7,24(28)-trienol; 11, Ergosta-5,7,22,24(28)-tetraenol; 12, Ergosterol (Kato, 1986).

The enzyme involved is cytochrome P450-dependent sterol 14α-demethylase (cytochrome $P450_{14DM}$) (Aoyama *et al.*, 1984). The demethylation product is 4,4-dimethyl-ergosta-8,14,24(28)-trienol. Then, the 14,15-double bond is reduced (C) and two methyl groups at the C-4 position are removed (D). This results in formation of ergosta-8,24(28)-dienol (fecosterol). In yeast, introduction of the methyl group at the C-24 position (H) appears only after removal of the methyl group at the C-14 position (E), reduction of the 14,15-double bound (F) and removal of two methyl groups at the C-4 position (G). The 8,9-double bond of fecosterol is isomerized to a 7,8-double bond (I). Ergosterol is finally synthesised by further introduction of a 5,6-double bond (J), a 22,23-double bond (K) and reduction of the 24,28-double bond (L).

Inhibition of ergosterol biosynthesis. Ergosterol biosynthesis may be inhibited at different steps (Scheinpflug and Kuck, 1987; Burden *et al.*, 1989; Kelley *et al.*, 1990). Representative inhibitors are summarized as follows:

a) Squalene epoxidase inhibitors: allylamines, terbinafine and naftifine.
b) 2,3-oxidosqualene cyclase inhibitors: 2-aza-2,3-dihydrosqualene and 2,3-iminosqualene.
c) C-24 sterol methyltransferase inhibitors (Step A and H): 25-azacholesterol and 24-epiiminolanosterol.
d) 14α-demethylase inhibitors (Step B and E): imidazole and triazole derivatives.
e) Δ^{14} reductase inhibitors (Step C and F): morpholine derivatives.
f) Δ^{8}-Δ^{7} isomerase inhibitors (Step I): morpholine derivatives.
h) Δ^{22} desaturase inhibitor (Step K): triarimol.
i) $\Delta^{24(28)}$ reductase inhibitor (Step L): 23-azacholesterol.

Among the various steps in ergosterol biosynthesis, sterol 14α-demethylation has proved to be the most fruitful target, for which many currently important sterol 14α-demethylase inhibitors (DMIs) are being developed. Most DMIs are imidazole and triazole derivatives, such as the agricultural fungicides imazalil, fenarimol, triadimenol, penconazole, propiconazole and the antimycotics clotrimazole, itraconazole, ketoconazole and miconazole. A limited number of DMIs are derivatives of piperazine, pyridine and pyrimidine (Scheinpflug and Kuck, 1987). A common feature of DMIs is the heterocyclic ring that contains at least one nitrogen with a free electron pair. The inhibitory effect of DMIs on sterol 14α-demethylase (cytochrome $P450_{14DM}$) activity is based on interaction

between the free electron pair of this nitrogen atom and the heme iron of the cytochrome $P450_{14DM}$, and on interaction between the *N*1-substituent of the fungicide and the apoprotein of the enzyme (Vanden Bossche, 1987; Vanden Bossche *et al.*, 1986; Yoshida and Aoyama, 1987; Yoshida, 1988).

Inhibition of sterol biosynthesis by DMIs results in direct depletion of ergosterol and accumulation of abnormal sterol intermediates (Sisler and Ragsdale, 1984). Lack of ergosterol alters membrane fluidity which results in interference with membrane-bound enzymes, such as chitinase (Vanden Bossche, 1990). Abnormal sterols which accumulate upon inhibition of sterol biosynthesis by DMIs may be incorporated into the plasma membrane and cause membrane disturbance (Weete *et al.*, 1985). Therefore, it is generally believed that the mode of fungitoxic action of DMIs is mainly based on the primary inhibition of ergosterol biosynthesis resulting in depletion of intracellular ergosterol and accumulation of abnormal sterols (Vanden Bossche, 1985; 1990; Köller and Scheinpflug, 1987). DMIs are also found to interfere with processes other than sterol biosynthesis (Vanden Bossche, 1985). Examples are: a) interference with fatty acid metabolism resulting in accumulation of free fatty acids and increased fatty acid desaturation, b) inhibition of activity of microsomal ATPases (Mg^{2+}, Na^{+}, K^{+}), mitochondrial cytochrome c oxidase and cytochrome c peroxidase, and c) direct membrane damage of *Saccharomyces cerevisiae* and *Botrytis cinerea*. However, these effects were only observed at concentrations in excess of those required for inhibition of growth.

Development of resistance to DMIs

Development of resistance in fungi to DMIs has been reported in both laboratory and field after their introduction since the 1970's (Hollomon *et al.*, 1990; Köller and Scheinpflug, 1987). Laboratory isolates with resistance to DMIs often have a decreased fitness with respect to spore germination, mycelial growth and pathogenicity. These fitness parameters are negatively correlated with the degree of resistance (Fuchs and De Waard, 1982; De Waard and Fuchs, 1982). In the field, development of resistance to DMIs was reported first for cereal and cucurbit powdery mildews (Brent and Hollomon, 1988). Inadequate disease control has been observed (Hollomon *et al.*, 1990). Resistant isolates of cucumber powdery mildew (*Sphaerotheca fuliginea*) were found to exhibit a normal fitness compared with that of the wild-type isolate (Schepers, 1985). Development of field resistance to DMIs was also reported for the plant pathogens *Venturia inaequalis* (Hildebrand *et al.*, 1988) and *Penicillium digitatum*

(Eckert, 1987).

Various studies indicated that although DMIs are site specific fungicides, the risk of development of resistance may be low (Dekker, 1982). This may relate to the fact that resistance is often polygenic (Van Tuyl, 1977; Hollomon *et al.*, 1984; Butters *et al.*, 1986; Kalamarakis *et al.*, 1991). A single mutation results in only a low level of resistance (Stanis and Jones, 1985). A high level of resistance can be obtained by interaction of polygenes (De Waard and Van Nistelrooy, 1990; Kalamarakis *et al.*, 1991). These characteristics are in clear contrast with benzimidazole and phenylamide fungicides which encountered often a rapid development of resistance through a single gene mutation (Davidse, 1987a, 1987b).

A common mechanism of resistance to site specific fungicides in fungi appears to be based on a change in their target sites (Dekker, 1985). Such a mechanism of resistance to DMIs seems to be restricted to yeast-like fungi. For instance, the mechanism of resistance in mutants of *Ustilago maydis* (Walsh and Sisler, 1982), *Candida albicans* (Bard *et al.*, 1987) and *S. cerevisiae* (Aoyama *et al.*, 1983) is based on a lack of the target enzyme or the presence of a nonfunctional one while in some mutants of *C. albicans* a decreased affinity of the target enzyme is considered to be responsible for resistance (Vanden Bossche *et al.*, 1990). Such changes in the target site have not yet been reported for filamentous fungi. In this type of fungi, a well documented mechanism of resistance is an increased energy-dependent efflux of the compounds. This mechanism has been observed with *Aspergillus nidulans* (De Waard and Van Nistelrooy, 1979), *Penicillium italicum* (De Waard and Van Nistelrooy, 1984; 1988), *Monilinia fructicola* (Ney, 1988) and *Nectria haematococca* var. *cucurbitae* (Kalamarakis *et al.*, 1991). Increased energy-dependent efflux of DMIs results in a lower accumulation of the toxicant in mycelium, by which the target enzyme in sterol biosynthesis becomes less readily inhibited. Other potential mechanisms of resistance, such as tolerance to toxic sterols, detoxification of toxic sterols, failure to activate fungicides, deposition of fungicides in lipid droplets, changes in pH leading to protonation of fungicides (Hollomon *et al.*, 1990) and an induced resistant response related to a transient accumulation of sterol precursors (Smith & Köller, 1990) only seem to have limited relevance.

Aims and outlines of the present study

Studies on mechanisms of resistance to fungicides in fungi are valuable to

design anti-resistance strategies and will also contribute to a better fundamental understanding of fungal physiology and biochemistry. Such knowledge may in turn provide useful information to developing new fungicides. In most fungal pathogens with resistance to DMIs the mechanism of resistance is still unknown, except for the few cases mentioned above. The present study was aimed at elucidating mechanisms of resistance to DMIs in the fungus *P. italicum*. Special attention was paid to the DMI fungicide imazalil. *P. italicum* was used as the target fungus since it is an important plant pathogen and easy to culture *in vitro* and *in vivo*. In addition, laboratory-isolates of this fungus with various degrees of resistance to DMIs were available (De Waard and Van Nistelrooy, 1990). Studies with this fungus may function as a model to investigate the mechanism of resistance in filamentous plant pathogens which are more difficult to handle.

Various aspects which may relate to potential mechanisms of resistance have been studied. In the first place, sterol composition of wild-type and DMI-resistant isolates was studied in order to find out whether a mutation of the target site would be involved which would affect sterol synthesis (Chapter 2). A major part of the study was used to develop methods to obtain cell-free preparations of *P. italicum* in which activity of the target enzyme of DMIs, sterol 14α-demethylase, could be demonstrated (Chapter 3). This technique was never described for a filamentous plant pathogen. The success of these experiments made it possible to compare the inhibitory effect of DMIs on activity of sterol 14α-demethylase of different isolates. In this way, the hypothesis that decreased affinity of the target enzyme to DMIs would be involved as a mechanism of resistance was tested (Chapter 4). In addition, another approach to study the sensitivity of the target enzyme to DMIs was developed by demonstrating cytochrome P450 isozymes in fungal microsomes (Chapter 5). In consequence, the affinity of P450 isozymes in microsomes of different isolates to various DMIs was compared (Chapter 6). In order to study whether metabolism of imazalil would be involved as a mechanism of resistance, the capability of the fungal isolates to metabolize imazalil was compared (Chapter 7). Finally, accumulation of imazalil and fenarimol in mycelia of these isolates was studied in order to establish whether differential accumulation of these fungicides between DMI-resistant isolates with various degrees of resistance plays a role (Chapter 8). Chapter 9 gives a final discussion of the results obtained.

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CHAPTER 2

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Effects of imazalil on sterol composition of sensitive and DMI-resistant isolates of Penicillium italicum

J. GUAN[1], A. KERKENAAR[2] and M.A. DE WAARD[1]

[1] Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands
[2] Netherlands Organization of Applied Scientific Research, TNO Institute of Applied Chemistry, P.O. Box 108, 3700 AC Zeist, the Netherlands

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Abstract

Imazalil differentially inhibited dry weight increase of 10-hour-old germlings of wild-type and DMI-resistant isolates of *Penicillium italicum* in liquid malt cultures. EC_{50} values ranged from 0.005 to 0.27 $\mu g\ ml^{-1}$. In all isolates ergosterol constituted the major sterol (over 95% of total sterols) in the absence of the fungicide. Therefore, DMI-resistance cannot be associated to a deficiency of the C-14 demethylation enzyme in the ergosterol biosynthetic pathway. Imazalil treatment at concentrations around EC_{50} values for inhibition of mycelial growth resulted in a decrease in ergosterol content and a simultaneous increase in 24-methylene-24,25-dihydrolanosterol content in all isolates. A correlation existed between the imazalil concentration necessary to induce such changes in sterol composition and the EC_{50} values for inhibition of mycelial growth of the different isolates. The reason for the differential effects of imazalil on sterol composition in the various *P. italicum* isolates may be due to decreased accumulation of the fungicide in the mycelium and to other yet non-identified mechanisms of resistance.

Additional keywords: imazalil, *Penicillium italicum*, fungicide resistance, sterols.

Introduction

Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1H-imidazole) is a systemic fungicide with a broad antifungal spectrum. Its fungitoxic property against fruit decay caused by *Penicillium* spp. was first reported by Laville (1973). Later on, control of various other plant pathogens has been reported as well. The fungicide interferes with ergosterol biosynthesis by inhibition of C-14 demethylation of lanosterol or 24-methylene-24,25-dihydrolanosterol, resulting in accumulation of C-14 methyl sterol precursors (Buchenauer, 1977; Siegel and Ragsdale, 1978; Vanden Bossche et al., 1984; Kerkenaar et al., 1984 and 1986). The fungitoxicity of demethylation inhibitors (DMIs) has been attributed to depletion of ergosterol and to accumulation of sterol precursors in fungal membranes. This abnormal sterol content causes membrane hyperfluidity, leading to changes in membrane permeability and activity of membrane-bound enzymes (Kato, 1986). The latter effect is, for instance, responsible for abnormal chitin deposition in DMI-treated mycelial tips and explains the abnormal swelling and branching of the tips (Kerkenaar and Barug, 1984; Kerkenaar et al., 1984). Studies with imazalil indicate that its toxicity is pH-dependent. It exhibits a

higher toxicity at pH values above the pKa value (6.53) of the fungicide, due to a higher uptake of the fungicide in its undissociated form than in its protonated form at lower pH values (Siegel et al., 1977).

Development of resistance to fungicide with a site-specific action is a potential threat for chemical disease control. With DMIs, so far, it has only been detected in a limited number of pathogens after prolonged periods of selection pressure. Examples of DMI-resistance in practice have been noticed with *Erysiphe graminis* (Fletcher and Wolfe, 1981), *Sphaerotheca fuliginea* (Schepers, 1985), *Penicillium digitatum* (Eckert, 1987), *Venturia inaequalis* (Stanis and Jones, 1985) and *Pseudocercosporella herpotrichoides* (Leroux et al., 1988). Under laboratory conditions, however, development of resistance occurs more readily as has been demonstrated for various other fungi (cf. De Waard and Fuchs, 1982). Laboratory isolates are often used to study the mechanism of resistance to DMIs, which can be of various nature. A well-described mechanism has been observed in mutants of budding fungi with resistance to polyene antibiotics and cross-resistance to DMIs (cf. Kato, 1986). Such mutants are defective in C-14 demethylation since the cytochrome $P\text{-}450_{14DM}$ of the enzyme lost its catalytic activity (Aoyama et al., 1987). These mutants may utilize sterol intermediates instead of ergosterol, so that they can survive in the presence of the toxicants (Walsh and Sisler, 1982). The altered sterol composition usually reduces growth and other fitness parameters of the mutants as compared to the wild-type. A second mechanism of resistance to DMIs detected in laboratory isolates of *Aspergillus nidulans* and *Penicillium italicum* is based on an increased energy-dependent efflux of the fungicides from mycelium into medium. It is assumed that in these cases the target enzyme in sterol biosynthesis probably becomes less readily saturated (De Waard and Van Nistelrooy, 1979 and 1984).

In the present work the following aspects have been studied. a) The level of resistance under various culture conditions; b) A possible deficiency of the target enzyme for imazalil in sterol biosynthesis and c) The effect of imazalil on sterol composition in the various isolates.

Materials and methods

Fungal isolates. The following isolates of *P. italicum* have been used. Isolate W_5 with wild-type sensitivity to DMIs (De Waard et al., 1982), isolate $E_{300\text{-}3}$ with low resistance to imazalil (De Waard et al., 1982), isolate H_{17}, I_{33} and J_4, all obtained via selection of conidia of isolate $E_{300\text{-}3}$ on imazalil amended PDA (De Waard, 1988). Isolate J_4 was used in only part of the experiments. The isolates were maintained on PDA.

Media. (1) Synthetic agar medium (Bartz and Eckert, 1972) was buffered to pH 5.53, 6.53 and 7.53 with potassium phosphate (KH_2PO_4/K_2HPO_4 0.05 M). (2) Malt medium, containing 2% malt extract (Difco) and 1% mycological peptone (Oxoid), was buffered with 2-morpholino-ethanesulphonic acid (MES; 0.05 M) and dipotassium hydrogen phosphate (0.05 M). Malt media were adjusted to pH 7.0 with NaOH. In case of solid media 1.2% agar was included.

Chemicals. Imazalil sulphate was a gift from Janssen Pharmaceutica (Beerse,

Belgium). Stock solutions were made in methanol. The final methanol concentration in amended medium never exceeded 0.1%. Sodium [^{14}C] acetate (sp. act. 57 mCi mmole^{-1}, Amersham) was diluted to an activity of 50 μCi ml^{-1} with 50% methanol. Sodium acetate (Merck) was dissolved in 50% methanol (25 mM).

Fungitoxicity tests. Spore germination. The effect of imazalil on spore germination was tested in synthetic agar medium buffered at pH 5.53, 6.53 and 7.53 and in malt agar buffered at pH 7.0. Spores were collected from 7 to 10-day-old cultures. Three drops of a spore suspension (10^6 spores ml^{-1}) were spread over the agar surface of a Petri dish amended with imazalil at different concentrations. The Petri dishes were incubated at 24 °C. Spore germination on the synthetic agar medium at pH 5.53, 6.53 and 7.53 was counted after 14, 22 and 36 h of incubation, respectively, and on malt agar pH 7.0 after 13 h. In each treatment 100 spores were counted. The experiment was carried out in triplicate.

Mycelial growth on agar. The effect of imazalil on mycelial growth was tested in the same media. The inoculum was prepared by spreading 3 drops of the spore suspension (10^6 spores ml^{-1}) over the agar surface of a Petri dish. The Petri dishes were incubated at 24 °C overnight. Agar discs (5 mm) with a thin mycelial mat were transferred upside down to test agar plates amended with imazalil. After five days of incubation at 24 °C the size of colonies was determined.

Mycelial growth in liquid media. Liquid cultures were prepared by inoculation of liquid malt medium (100 ml) in flasks (300 ml) with 1 ml spore suspension (10^9 spores ml^{-1}). The flasks were incubated on a rotary shaker at 24 °C and at 200 rpm for 10 h. These cultures, homogeneous suspensions of germlings, were diluted 6 times with the same fresh medium. This suspension of germlings is described as the standard mycelial suspension and was used to determine the effect of imazalil on dry weight increase. Portions (10 ml) of this suspension were transferred into flasks (50 ml). Imazalil was added from stock solution to final concentrations ranging from 0.005 to 2.5 μg ml^{-1}. Incubation of these flasks was continued on the rotary shaker and mycelial dry weights were determined after 0, 4, 8 and 12 h of incubation.

Lipid extraction. Standard mycelial suspension (100 ml) in flasks (300 ml) were incubated with imazalil at final concentrations of 0.001, 0.01 and 0.1 μg ml^{-1} for 8 h under the same conditions as described for the toxicity experiments. Mycelium was harvested on a Büchner funnel and freeze-dried. Lipids were extracted by homogenization of 0.1 g dry mycelium in a Potter-type homogenizer with 20 ml chloroform/methanol (2:1, v/v) for about 5 min. The homogeneous mycelial suspension was shaken on a rotary shaker for 1 h at room temperature. The chloroform/methanol extract was collected by filtration on a Büchner funnel. Water was added to the extract to a final concentration of 20% for phase separation (Folch et al., 1957). The upper phase was removed. The lower chloroform phase was taken to dryness by evaporation under reduced pressure at 40 °C. The residue was resuspended in about 5 ml of chloroform. Water content in the chloroform was absorbed by adding 0.5 g Na_2SO_4. The chloroform was filtered and dried under a stream of nitrogen. Dry weights of the residues, constituting total lipids, were determined. Lipids were kept under nitrogen in the dark at −20 °C until further analysis.

Ultraviolet absorption. Total lipids were dissolved in 1 ml of chloroform and were analysed for the presence of ergosterol by UV-spectroscopy with a Beckman spectrophotometer (Model 25). The ergosterol content was calculated on basis of its molar extinction coefficient at 282 nm (E_m = 11,900).

GLC and MS analysis. Total lipids (from 0.1 g dry wt mycelium) were saponified with 10% KOH in 80% methanol under nitrogen in closed vials at 90 °C for 2 h. Free sterols were extracted three times with equal volumes of hexane. The combined hexane extracts were washed with 2 N KOH and water, respectively, and dried under a stream of nitrogen and kept at −20°C in the dark. The free sterol fractions were subjected to gas liquid chromatography (GLC) and analysed by mass spectrometry (GC-MS) (Kerkenaar et al., 1984).

Incorporation of [^{14}C] acetate into lipids. Standard mycelial suspensions (100 ml) in flasks (300 ml) were incubated with 2.5 μCi of sodium [^{14}C] acetate (act. 50 μCi ml^{-1}) and 25 μmole sodium acetate in the presence or absence of imazalil (0.1 μg ml^{-1}) at 24 °C on a rotary shaker for 8 h. Mycelium was harvested and total lipids were extracted as described above. The total lipid fractions were separated by thin layer chromatography (TLC) using precoated silicagel plates (F254, 0.25 mm thick, Merck). From each chloroform extract (1 ml), 200 μl was spotted on the TLC plates. Authentic ergosterol, lanosterol, cholesterol acetate and palmitic acid were used as references in a comparable TLC experiment with non-labeled lipids. The TLC plates were developed in a solvent system of heptane/isopropylether/acetic acid (60:40:4, v/v/v). Lipids on the plate were visualized by spraying with H_2SO_4/methanol (1:1, v/v) and heating at 150 °C for about 5 min. The radioactive areas in the plate were detected with a thin layer scanner (Berthold). Radioactive areas were scraped off and measured quantitatively for radioactivity with a liquid scintillation system (Beckman LS 5800).

Results

Growth and fungitoxicity. Growth of *P. italicum* isolates in various media and fungitoxicity of imazalil were tested to find a medium which would support fast fungal growth and in which imazalil would display a high fungitoxicity and a differential toxicity to the various isolates. Such a medium is necessary to study the effect of imazalil on sterol biosynthesis.

Growth on a liquid minimal glucose mineral salt medium (Kerkenaar et al., 1984) was too slow (results not shown). Growth of all isolates on synthetic agar according to Bartz and Eckert (1972) was optimal at pH 5.53. In that case, spore germination of all isolates (W_5, E_{300-3}, H_{17} and I_{33}) after 14 h of incubation was about 98%. Spore germination of the isolates at pH 6.53 after 22 h of incubation and at pH 7.53 after 36 h of incubation varied between 52-57% and 26-44%, respectively. Germination on malt agar at pH 7.0 after 13 h of incubation was 82-88%. In all cases no obvious differences between the isolates were observed. On the basis of these results toxicity of imazalil to spore germination and radial growth of all isolates was tested using synthetic agar pH 5.53, 6.53 and 7.53 and malt agar pH 7.0. Results (Table 1) show that imazalil is inhibitory to both spore germination and mycelial radial growth in both types of media. Toxicity of imazalil to mycelial growth was highest in malt agar

Table 1. Effect of imazalil on spore germination and radial growth of *Penicillium italicum* isolates.

Isolate	EC_{50} of imazalil ($\mu g\ ml^{-1}$)							
	spore germination[1]		radial growth[2]					
	SA pH 5.33	Malt pH 7.0	SA pH 5.33		SA pH 6.53		SA pH 7.53		Malt pH 7.0	
W_5	0.028	0.011	0.06		0.006		0.005		0.005	
E_{300-3}	0.14 (5)[3]	0.019 (2)	0.21	(3.5)	0.046	(8)	0.023	(4.6)	0.03	(6)
H_{17}	0.95 (34)	0.12 (11)	3.4	(57)	0.42	(70)	0.23	(46)	0.25	(50)
I_{33}	1.09 (39)	0.16 (15)	6.5	(108)	0.70	(117)	0.55	(110)	0.35	(70)
J_4	–	–	–		–		–		0.38	(76)

[1] Spore germination on synthetic agar (SA) and malt agar determined after 14 and 13 hours of incubation, respectively. EC_{50} values of imazalil on spore germination on synthetic agar pH 6.53 and 7.53 were not calculated because of extremely low germination percentages in controls (see text).

[2] Radial growth on synthetic agar pH 5.33, 6.53 and 7.53 and malt agar pH 7.0 was determined after 5 days of incubation; diameter of colonies in control treatments were 17, 13, 11 and 19 mm (W_5); 19, 15, 13 and 19 mm (E_{300-3}); 20, 15, 10 and 17 mm (H_{17}); 21, 16, 10 and 20 mm (I_{33}), respectively. Radial growth of isolate J_4 in control treatment on malt agar pH 7.0 was 19 mm after 5 days of incubation.

[3] Data between brackets: degree of resistance.

pH 7.0 and synthetic agar pH 7.53. Since malt agar pH 7.0 supported radial growth much better than synthetic agar pH 7.53, the malt medium was chosen in future studies.

In liquid malt medium (pH 7.0) imazalil showed a concentration-dependent inhibition of dry weight increase in time (Fig. 1). EC_{50} values of imazalil for inhibition of growth measured after 12 h of incubation for isolates W_5, E_{300-3}, H_{17} and I_{33} are 0.005, 0.016 (3.2), 0.10 (20) and 0.27 (54) $\mu g\ ml^{-1}$, respectively. Between brackets, degrees of resistance (EC_{50} resistant isolate: EC_{50} wild-type) are given. The results show that the increased degree of resistance in isolates E_{300-3}, H_{17} and I_{33} is also expressed during growth in a rich liquid malt medium. The EC_{50} values found are even slightly lower than the corresponding values established for radial growth on malt agar. On the basis of these results 10-hour-old germlings in liquid malt medium were used to determine the effect of imazalil on sterol biosynthesis.

Total lipids. In control treatments, the total lipid content of wild-type isolate W_5 and DMI-resistant isolates E_{300-3}, H_{17}, I_{33} and J_4 was 67.7 ± 15, 75.7 ± 24, 63.3 ± 3, 63.0 ± 1 and 61.1 ± 12 mg g^{-1} dry mycelium, respectively. The figures were not significantly different. Treatment with imazalil (0.001, 0.01 and 0.1 $\mu g\ ml^{-1}$) did not effect the total lipid content.

UV spectroscopy. Ultraviolet absorption spectra of total lipid extracts from all isolates in all treatments were identical to the one of a reference ergosterol as given by

Table 2. Effect of imazalil on ergosterol content of *Penicillium italicum* isolates during incubation for 8 h in a liquid malt medium pH 7.0.

Imazalil (μg ml^{-1})	Ergosterol (mg g^{-1} dry mycelium)[1]				
	W_8	E_{300-3}	H_{17}	I_{33}	J_4
–[2]	1.39 ± 0.01	1.37 ± 0.03	1.37 ± 0.02	1.28 ± 0.01	1.25 ± 0.04
0[3]	1.99 ± 0.01 (100)[4]	1.98 ± 0.02 (100)	1.94 ± 0.08 (100)	1.97 ± 0.02 (100)	1.87 ± 0.01 (100)
0.001	1.98 ± 0.03 (98)	1.98 ± 0 (100)	1.83 ± 0.05 (81)	1.98 ± 0.02 (>100)	1.93 ± 0.08 (>100)
0.01	1.45 ± 0.30 (10)	1.82 ± 0.20 (74)	1.74 ± 0.08 (65)	1.98 ± 0.20 (>100)	1.87 ± 0.20 (100)
0.1	1.20 ± 0.01 (<0)	1.26 ± 0.01 (<0)	1.61 ± 0.20 (42)	1.81 ± 0.20 (76)	1.82 ± 0.20 (91)

[1] Content calculated from UV absorption of total lipid extract at 282 nm.
[2] Ergosterol content of 10-hour-old standard mycelium before incubation with imazalil.
[3] Ergosterol content of control treatment after incubation of standard mycelium for 8 h.
[4] Standard deviation. Data between brackets: increase of ergosterol level as percentage of increase in controls.

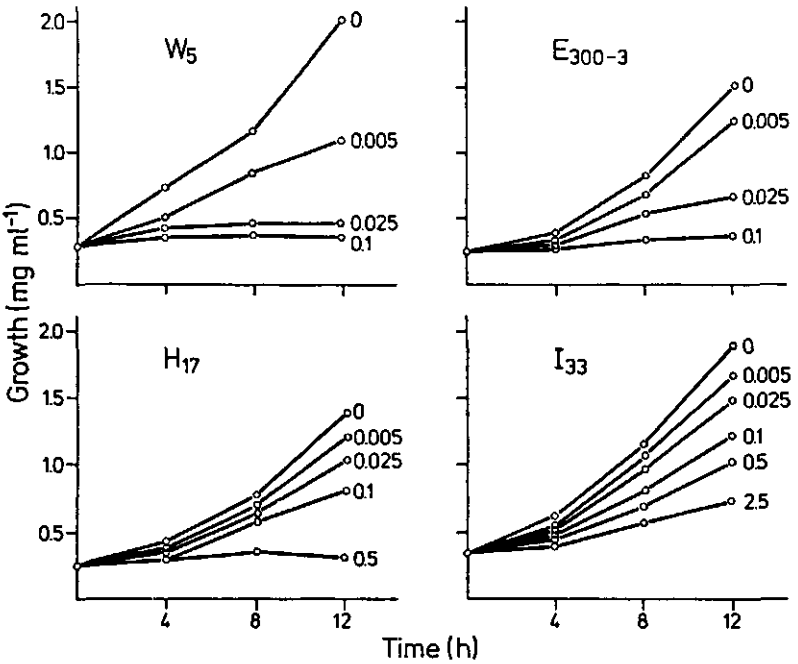

Fig. 1. Toxicity of imazalil to growth of 10-hour-old germlings of *Penicillium italicum* isolates in liquid malt medium pH 7.0. The figures represent imazalil concentrations (μg ml^{-1}) in the medium.

Kerkenaar et al. (1981). The maximum absorption at 282 nm was used to calculate the ergosterol content in mycelium (Table 2). The ergosterol content of standard 10-hour-old mycelium was almost similar in all isolates (1.25-1.39 mg g^{-1} dry mycelium). This content increased to 1.87-1.99 mg g^{-1} dry mycelium during an additional 8-hour-growing period. Treatment with imazalil reduced ergosterol levels in both wild-type and resistant isolates. The levels of ergosterol decreased with increasing concentrations of imazalil. The decrease was inversely proportional to the degree of resistance of the various isolates. Concentrations of imazalil which inhibited the increase of ergosterol content during the 8-hour-growing period in W_5, E_{300-3}, H_{17}, I_{33} and J_4 for 50% are 0.004, 0.019, 0.05, 0.18 and 0.42 μg ml^{-1}. respectively.

GLC and GC-MS. Analysis of free sterols extracted from saponified total lipids of control-treated mycelia of all isolates revealed one major peak with a relative retention time (RR_t) of 1.17 compared with that of cholesterol. The peak was identified by its mass spectrum and fragmentation pattern (Kerkenaar et al., 1984) as ergosterol (data not shown). One minor peak had a RR_t of 1.70. This one was identified by its mass spectrum and fragmentation pattern (Kerkenaar et al., 1984) as 24-methylene-24,25-dihydrolanosterol (data not shown) and it constituted in all isolates less than 5% of the total quantity of sterols. In all isolates no differences in composition of sterols between 10-hour-old and 18-hour-old mycelia were observed (Table 3). Imazalil treatment

Table 3. Effect of imazalil on sterol composition of *Penicillium italicum* isolates during 8 h of incubation in liquid malt medium pH 7.0.

Imazalil ($\mu g\ ml^{-1}$)	Exp.	Sterol composition (% of total sterols)									
		W_5		E_{300-3}		H_{17}		I_{33}		J_4	
		Erg.[3]	Mlan.	Erg.	Mlan.	Erg.	Mlan.	Erg.	Mlan.	Erg.	Mlan.
–[1]	I	97.2	2.8	98.3	1.7	97.5	2.5	98.0	2.0	95.6	4.4
	II	98.2	1.8	96.3	3.7	96.4	3.6	95.6	4.4	–	–
0[2]	I	96.5	3.4	97.4	2.6	96.7	3.3	93.7	2.3	96.3	3.7
	II	98.9	1.1	98.9	1.1	97.3	2.7	98.1	1.9	95.4	4.6
0.001	I	91.2	8.8	95.5	4.5	87.3	12.7	94.7	5.3	91.7	8.3
	II	96.5	3.5	95.9	4.1	–	–	–	–	98.0	2.0
0.01	I	38.0	62.0	76.5	23.5	80.8	19.2	86.8	13.2	86.4	13.6
	II	41.0	59.0	74.8	25.2	72.3	27.7	89.6	10.4	90.8	9.2
0.1	I	22.7	67.3	29.2	70.8	50.5	49.5	65.8	34.2	69.1	30.9
	II	25.8	74.2	28.1	71.9	42.7	57.3	80.0	20.0	80.3	19.7

[1] Sterol composition of 10-hour-old standard mycelium before incubation with imazalil.
[2] Sterol composition of control treatment after incubation of standard mycelium for 8 h.
[3] Erg. = ergosterol. Mlan. = 24-methylene-24,25-dihyrolanosterol.

of mycelium resulted in reduction of the proportion of ergosterol and accumulation of 24-methylene-24,25-dihydrolanosterol. This shift in sterol composition occurred in both wild-type and DMI-resistant isolates. In the resistant isolates relatively high concentrations of imazalil were required to produce the same shift, indicating that the effect of imazalil on sterol composition is correlated to the degree of resistance in these isolates. Other minor sterols, which accumulate after inhibition of C-14 demethylation in *P. italicum* (Kerkenaar et al., 1984 and 1986) were not detected in two independent experiments.

[^{14}C] acetate incorporation. Total lipids were separated into various fractions by thin layer chromatography. The various fractions were identified in separate experiments by comparing their R_f values with those of references after charring and with literature data (Buchenauer, 1977; Vanden Bossche et al., 1978). Scans of the radioactivity in the TLC plates indicated that imazalil treatment (0.1 $\mu g\ ml^{-1}$) of isolate W_5 and E_{300-3} resulted in a decrease in incorporation of [^{14}C]-label into C-4 desmethyl sterols and an increase in incorporation of [^{14}C]-label into C-4,4 dimethyl sterols. Such a shift was much less with highly resistant isolates I_{33} and J_4. With isolate H_{17} an intermediate effect was observed (Fig. 2). Quantitative data were obtained by counting [^{14}C]-labeled lipids separated by TLC in a liquid scintillation system. The results (Table 4) support the above observations. The data also show that the strong inhibition of incorporation of [^{14}C]-label into C-4 desmethyl sterols in wild-type isolate W_5 and low resistant isolate E_{300-3} was coupled with an increase in incorporation of [^{14}C]-label into C-4 methyl sterols. Besides sterols, imazalil treatment also resulted in an increase in incorporation of [^{14}C]-label into free fatty acids, triglycerides and

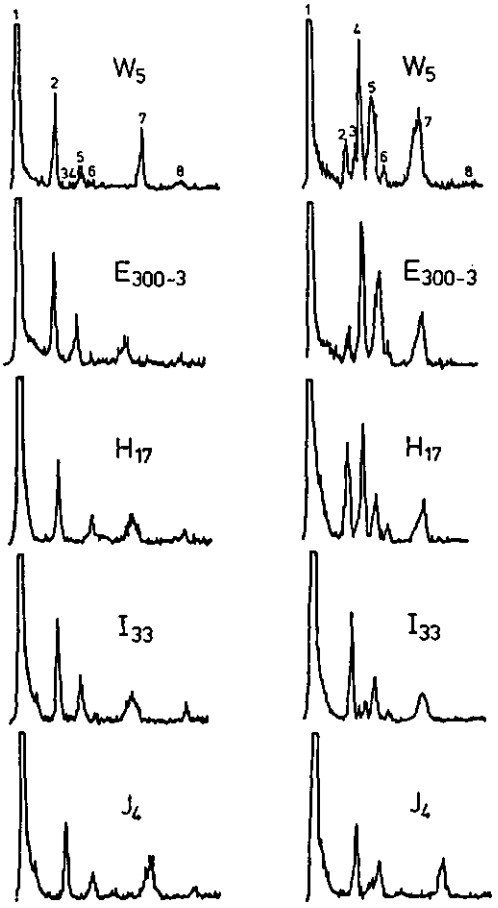

Fig. 2. Thin layer scans of [^{14}C]-labeled lipid fractions of *Penicillium italicum* isolates incorporated with [^{14}C] acetate in presence and absence of imazalil (0.1 μg ml^{-1}). 1. Polar lipids. 2. C-4 desmethyl sterols. 3. C-4 methyl sterols 4. C-4,4 dimethyl sterols. 5. Unknown compounds. 6. Free fatty acids. 7. Triglycerides. 8. Steryl esters. Left: controls. Right: imazalil treatments.

unknown compounds and a decrease in incorporation of [^{14}C]-label into polar lipids and sterol esters. These effects, again, were more pronounced in wild-type isolate W_5 and low resistant isolate $E_{300\text{-}3}$ and were almost absent in highly resistant isolates I_{33} and J_4. With isolate H_{17} these effects were intermediate.

Discussion

The liquid malt medium (pH 7.0) supported fungal growth well and toxicity of imazalil in this medium was as high as in synthetic medium. Therefore this medium

Table 4. Effect of imazalil on incorporation of [^{14}C] acetate into lipid fractions of *Penicillium italicum* isolates during incubation for 8 h in a liquid malt medium pH 7.0.

Isolate	Imazalil (μg ml^{-1})	Percentage of radioactivity in lipid fractions[1]							
		1	2	3	4	5	6	7	8
W5	0	77.6 ± 6.1[2]	10.4 ± 2.2	0.2 ± 0.2	0.4 ± 0.1	3.2 ± 0.8	0.7 ± 0.1	6.6 ± 2.0	0.8 ± 0.1
W5	0.1	32.9	3.9	3.5	19.6	17.0	4.4	18.4	0.3
E_{300-3}	0	65.2	14.9	1.1	0.9	8.7	1.7	6.3	1.2
E_{300-3}	0.1	34.8	5.1	3.1	22.9	15.7	5.8	12.3	0.3
H17	0	78.5 ± 3.4	8.9 ± 0.4	0.3 ± 0.1	0.5 ± 0.1	3.3 ± 0.6	1.1 ± 0.4	6.4 ± 2.1	0.8 ± 0.1
H17	0.1	72.6 ± 4.0	6.6 ± 1.3	1.1 ± 0.2	8.9 ± 0.2	3.9 ± 1.6	1.2 ± 0.2	5.6 ± 0.9	0.2 ± 0.1
I33	0	70.9	11.1	0.2	0.3	7.6	0.7	8.1	1.1
I33	0.1	71.6	9.0	1.1	2.2	7.2	1.0	7.6	0.5
J4	0	71.6	9.7	0.1	0.2	4.9	0.6	11.4	1.5
J4	0.1	69.7	8.2	0.5	2.8	7.4	0.9	9.7	0.8

[1] 1. Polar lipids; 2. C-4 desmethyl sterols; 3. C-4 methyl sterols; 4. C-4,4 dimethyl sterols; 5. Unknown compounds; 6. Free fatty acids; 7. Triglycerides; 8. Sterol esters.
[2] Standard deviation.

proved to be most suitable to study effects of imazalil on growth and sterol composition of *P. italicum*.

The age of the standard mycelial suspension (10-hour-old) appeared to be critical for high toxity of imazalil. Sensitivity of 16-hour-old mycelium to imazalil is relatively low (De Waard, unpublished results). This discrepancy is probably caused by the high proportion of growing biomass (hyphal tips) in the total biomass in 10-hour-old cultures of germlings, compared to 16-hour-old cultures when mycelial pellets are formed. In the latter case non-growing mycelial cells are abundantly present and it is likely that imazalil may accumulate into lipid fractions of these cells, and as a consequence of which the actual concentration is lowered. Another explanation might be the relatively low content of ergosterol in 10-hour-old germlings (Table 2), leading to a fast depletion of this sterol in the presence of imazalil.

Wild-type as well as DMI-resistant isolates of *P. italicum* contained ergosterol as the major sterol (Table 3). This rules out the possibility that resistance in *P. italicum* to imazalil is associated to C-14 demethylation deficiency in sterol biosynthesis. Such a mechanism of resistance has been reported for polyene-resistant budding fungi (Walsh and Sisler, 1982; Aoyama et al., 1983), which are cross-resistant to DMIs. These mutants may possess an ability to utilize sterol intermediates instead of ergosterol so that they can survive in the presence of the fungicides. In these mutants the C-14 demethylation deficiency is often coupled with a highly reduced fitness (Walsh and Sisler, 1982). The normal spore germination and mycelial growth rate of the DMI-resistant isolates of *P. italicum* under the various cultural conditions tested (Table 1) indicates no obvious loss in saprophytic fitness. This is in agreement with the assertion mentioned above that the resistance mechanism in *P. italicum* isolates is not based on C-14 demethylation deficiency. DMI-resistant isolates with a normal sterol composition and fitness were also described for *Candida albicans* (Hitchcock et al., 1986), *Ustilago avenae* (Hippe and Köller, 1986) and *U. maydis* (Leroux and Gredt, 1984).

Imazalil had a profound effect on ergosterol biosynthesis of the wild-type isolate W_5. Imazalil at about the EC_{50} value for inhibition of mycelial dry weight increase (0.005 $\mu g\ ml^{-1}$) caused a significant reduction of the ergosterol content and a simultaneous accumulation of 24-methylene-24,25-dihydrolanosterol (Tables 2 and 3). These results once more indicate that inhibition of C-14 demethylation is the primary site of action of imazalil. Similar effects on major sterol composition of the other isolates were observed, but only at relatively higher concentrations. In fact, a close correlation was present between the imazalil concentration necessary to induce similar changes in sterol composition and the EC_{50} values of the fungicide for inhibition of mycelial growth of the various isolates. These results indicate that the target enzyme for DMIs, cytochrome $P\text{-}450_{14DM}$, is present in all isolates but it is only inhibited at different extracellular concentrations of imazalil.

The [^{14}C] acetate incorporation experiments (Table 5) support the above conclusions. Inhibition of the incorporation of [^{14}C]-label into C-4 desmethyl sterols and accumulation of [^{14}C]-label in C-4,4 dimethyl sterols by imazalil (0.1 $\mu g\ ml^{-1}$) was most obvious in the wild-type isolate W_5. This inhibitory effect was lower in resistant isolates in the order of $E_{300\text{-}3}$, H_{17}, I_{33} and J_4. Both groups of [^{14}C]-labeled sterols were not further analysed. It is suggested that [^{14}C]-labeled C-4 desmethyl sterols and [^{14}C]-labeled dimethyl sterols consist mainly of ergosterol and 24-methylene-24,25-dihydrolanosterol, respectively (Table 4). Imazalil treatment of the wild-type isolate

W_5 and low-resistant isolate $E_{300\text{-}3}$ also resulted in incorporation of [^{14}C]-label into C-4 methyl sterols. This is probably obtusifoliol. However, obtusifoliol was not detected in the GC-MS experiments. This discrepancy is ascribed to the fact that in the latter tests total sterols were analysed, of which C-4 methyl sterols may constitute an undetectable amount. In [^{14}C] acetate incorporation experiments *de novo* synthesized sterols were analysed.

At pH values above the pKa value of imazalil treatment of *P. italicum* with imazalil resulted in formation of C-14 methyl sterols with C-3 keto groups (Kerkenaar et al., 1984 and 1986). These steroids were not detected in the present GC-MS experiments. This difference may be related with the use of a complex medium instead of a synthetic one and use of germlings instead of two-day-old mycelium.

Imazalil did not strongly affect total lipid content in all isolates. This is also found in a comparable study by Hitchcock et al. (1986). However, imazalil did affect incorporation of [^{14}C]-label not only into sterols as described above but also into various other lipids, like polar lipids, free fatty acids, triglycerides and unknown compounds. Again these effects were most obvious with wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$ and were almost absent with isolate I_{33} and J_4. The effect on isolate H_{17} was again intermediate. It is supposed that the relatively weak effect of imazalil on incorporation of [^{14}C]-label into the various lipid fractions of highly-resistant isolates is a consequence of reduced inhibition of C-14 demethylation of 24-methylene-24,25-dihydrolanosterol in these isolates.

The mechanism responsible for reduced inhibition of C-14 demethylation in the low-resistant isolate $E_{300\text{-}3}$ may be based on an increased energy-dependent efflux of imazalil from mycelium as has been demonstrated for various other DMIs (De Waard and Van Nistelrooy, 1984 and 1988). In view of the supposed polygenic nature of resistance to DMIs, in general (Van Tuyl, 1977), and, in particular, in *P. italicum* (De Waard, 1988), additional mechanisms of resistance may operate in isolates with a higher degree of resistance. The nature of these mechanisms is not known, but may be related to additional permeability barriers for the fungicide, increased detoxification, decreased affinity of cytochrome $P450_{14DM}$, or changes in total lipid composition different from sterols. The validity of these potential mechanisms of resistance will be the subject of further studies.

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Samenvatting

Effecten van imazalil op de sterolsamenstelling van gevoelige en DMI-resistente isolaten van Penicillium italicum

Imazalil remt differentieel de toename in drooggewicht van 10-uur-oude gekiemde sporen van wild-type en DMI-resistente isolaten van *Penicillium italicum* in vloeistofcultures van moutextract. De EC_{50} waarden voor groei van de verschillende isolaten lopen uiteen van 0,005 tot 0,27 μg ml^{-1}. In afwezigheid van het fungicide is in alle isolaten ergosterol het belangrijkste sterol (meer dan 95% van het totaal). DMI-

resistentie kan daarom niet in verband staan met deficiëntie van het C-14 demethyleringsenzym in de ergosterol biosynthese. Imazalilbehandeling van mycelium bij concentraties rond de EC_{50} waarde voor groeiremming, resulteerde bij alle isolaten in een afname van het ergosterolgehalte en een gelijktijdige toename van het gehalte aan 24-methyleen-24,25-dihydrolanosterol. Er bestaat dus een nauwe correlatie tussen de imazalilconcentratie die noodzakelijk is om vergelijkbare veranderingen in sterolsamenstelling te induceren en de EC_{50} waarde voor remming van myceliumgroei van de verschillende isolaten. De differentiële effecten van imazalil op de sterolsamenstelling van de verschillende *P. italicum* isolaten kunnen worden veroorzaakt door verminderde accumulatie van het fungicide in het mycelium en door andere, nog niet geïdentificeerde resistentiemechanismen.

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CHAPTER 3

Ergosterol biosynthesis in a cell-free preparation of *Penicillium italicum* and its sensitivity to DMI fungicides

J. Guan[1], C. Stehmann[1], S. W. Ellis[1], A. Kerkenaar[2,3] and M. A. de Waard[1]

[1]Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

[2]Netherlands Organization of Applied Scientific Research, TNO Institute of Applied Chemistry, P.O. Box 108, 3700 AC Zeist, The Netherlands.

[3]Present address: Denka International B.V., Hanzeweg 1, 3771 NG Barneveld, The Netherlands.

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Abstract

A method has been developed to study ergosterol biosynthesis in cell-free extracts of the filamentous plant pathogen *Penicillium italicum*. The method is based on a mild mechanical disruption of conidial germlings in a Bead-Beater apparatus. The cell-free extract was effective in synthesizing C4-desmethyl sterols from [2-^{14}C]mevalonate. Ergosterol was the only C4-desmethyl sterol synthesized and amounted to 25.6% of total non-saponifiable lipids. Other sterols identified in the non-saponifiable lipid fraction were lanosterol and a trace amount of 24-methylenedihydrolanosterol. Inhibition of ergosterol synthesis by fungicides which inhibit sterol 14α-demethylation (DMIs) led to accumulation of 24-methylenedihydrolanosterol indicating inhibition of cytochrome P450-dependent sterol 14α-demethylase activity. IC_{50} values (concentrations which inhibit incorporation of [2-^{14}C]mevalonate into ergosterol for 50%) of the highly toxic DMI fungicides imazalil, itraconazole, ketoconazole, penconazole and propiconazole ranged from $6.5 \pm 0.5 \times 10^{-9}$ to $1.7 \pm 0.7 \times 10^{-8}$ M. This indicates that DMI fungicides are very potent

inhibitors of sterol 14α-demethylase activity in cell-free extracts of the fungus. Less-toxic DMI-analogues R14821 and R42243 had much higher IC_{50} values, suggesting that these compounds have a significantly lower potency to inhibit sterol 14α-demethylase activity.

Introduction

Sterol demethylation inhibitors (DMIs) are the largest group of sterol biosynthesis inhibiting fungicides. DMIs affect ergosterol biosynthesis by inhibition of sterol 14α-demethylation of lanosterol or 24-methylenedihydrolanosterol (1, 2). Although DMIs have a broad antifungal spectrum, remarkable differences exist in the sensitivity of various fungi to DMIs (1). Insensitivity of fungi to DMIs may relate to a low affinity of the target enzyme (sterol 14α-demethylase). Low affinity of this target enzyme may also be a mechanism of acquired resistance in fungi (3, 4). In order to test these hypotheses, an enzyme assay in which the intrinsic inhibitory activity of DMIs to sterol 14α-demethylation can be tested, is essential. The assay can also be helpful in optimising toxicity of candidate DMIs to specific fungal pathogens (5). Studies of sterol 14α-demethylase assays have been largely confined to *Saccharomyces cerevisiae* (5-7) and the yeast form of *Candida albicans* (2, 8-10). The development of a sterol 14α-demethylase assay for filamentous fungi has only recently been reported for *Aspergillus fumigatus* (11). Lack of such enzyme assays may be due to instability of the membrane-bound enzyme in filamentous fungi (12) or to a relatively low enzyme concentration. A complication is also that sterol 14α-demethylation in filamentous fungi occurs after methylation at C24 of lanosterol (13). The side chain alkylation of lanosterol takes place in mitochondria (14). This implies that synthesis of C4-desmethyl sterols in cell-free extracts of filamentous fungi may require conditions different from those described for *S. cerevisiae* and the yeast form of *C. albicans*.

In this paper a method to obtain cell-free extracts of the filamentous plant pathogen *Penicillium italicum* effective in synthesis of ergosterol is described. To evaluate the assay, the inhibitory effects of various DMI-fungicides and two less-toxic DMI-analogues on ergosterol biosynthesis were tested.

Materials and methods

Chemicals. Imazalil sulphate (imazalil), imazalil analogues 1-[2(2,4-dichlorophenyl)-2-(hydroxy)ethyl-1*N*-imidazole (R14821), 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*N*-imidazole (R42243), ketoconazole

and itraconazole were gifts from Janssen Pharmaceutica (Beerse, Belgium); penconazole and propiconazole from Ciba Geigy A. G. (Basle, Switzerland). Imazalil and the other compounds were used in 1000× concentrated solutions in water and dimethyl sulfoxide (DMSO), respectively. Mevalonic acid DBED salt (RS-[mevalonic-2-^{14}C] in ethanol, sp. act. 1.9 GBq mmol^{-1}) was purchased from Amersham (UK). NAD^+, $NADP^+$, NADPH, ATP, glucose 6-phosphate and reduced glutathione were purchased from Sigma (St. Louis, Mo.). The preparation of cofactor solution was according to Ballard *et al.* (11) in 100 mM potassium phosphate buffer pH 7.5 (1 ml) and contained 20 μmol NAD^+, 20 μmol $NADP^+$, 20 μmol NADPH, 100 μmol ATP, 60 μmol glucose 6-phosphate and 60 μmol reduced glutathione. The pH of the cofactor solution was adjusted to 7.3 with KOH (10 M). Divalent cation solutions of $MgCl_2$ (0.5 M) and $MnCl_2$ (0.4 M) were prepared in distilled water and adjusted with K_2HPO_4 $3H_2O$(5 M) to pH 7.0 and 6.7, respectively.

Fungus and culture conditions. Wild-type isolate W_5 of *P. italicum* was maintained on malt extract agar medium. Preparation of fungal cultures (11 hours old) and mycelial suspensions were carried out according to methods described previously (15).

Preparation of cell-free extracts. Mycelium was collected by filtration on a Büchner funnel and washed twice with 100 mM ice cold potassium phosphate buffer pH 7.5 (250 ml). Subsequent steps were carried out at 0-4°C. Mycelium was resuspended in buffer in a ratio of 80 mg wet wt ml^{-1} (30 mg dry wt ml^{-1}). A vessel (32 ml) of a Bead-Beater (Biospec Products, Bartlesville, Ok.) containing glass beads (15 g, diameter 1 mm) was completely filled with mycelial suspension. Remaining air in the vessel was removed by evacuation for 5 min at -1 bar. The vessel was again fully filled with mycelial suspension. The mycelium was disrupted 4 times for 30 sec with 30 sec intervals while the outer jacket around the vessel was filled with ice-water. The homogenate was filtered over two layers of cheese cloth (pre-soaked in the buffer). Glycerol (88% purity) was gently mixed with the filtrate to a final concentration of 20% (v/v). The mixture was centrifuged twice at 3,000*g* for 10 min. The top part of the resulting supernatant (cell-free extract) was immediately used in the sterol 14α-demethylation assay. In order to check whether intact cells were present in this cell-free extract, a few drops were added to malt extract agar medium in a Petri dish, incubated overnight at 25°C and assessed for fungal growth. The protein content of cell-free extracts was determined with Bio-Rad Protein Assay (Bio-Rad

Laboratories, Veenendaal, the Netherlands) using bovine γ-globulin as a standard.

Sterol biosynthesis assay. Sterol biosynthesis assays were carried out according to the method of Ballard *et al.*(11). The reaction mixture (10 ml) consisting of cell-free extract (9.3 ml), cofactor solution (0.5 ml) and divalent cation solutions of $MgCl_2$ (0.1 ml) and $MnCl_2$ (0.05 ml) was adjusted to pH 7.3 with K_2HPO_4 $3H_2O$ (5 M). The volume of K_2HPO_4 solution added never exceeded 1% of the total volume. The mixture was divided into 0.995 ml portions in 10 ml screw-capped test tubes. Test compounds (1 μl) were added to the mixture. In controls, the corresponding amounts of water or DMSO were added. The reaction was started by adding [2-^{14}C]mevalonate (5 μl). The tubes were incubated in a reciprocal water bath shaker (80 strokes per min) at 25°C for 3 h in the dark. The caps of tubes were opened every hour. The pH of the incubation mixtures was measured again after incubation. Effects of pH on sterol biosynthesis was studied in a similar manner after adjusting the pH of incubation mixtures from 7.0 to 7.5 with KH_2PO_4 (3 M) or K_2HPO_4 $3H_2O$ (5 M). Experiments were carried out at least in 3-fold with different cell-free extracts.

Saponification and sterol extraction. Saponification of total lipids was carried out by adding 1 ml of freshly prepared 20% KOH (w/v) in ethanol (90%) to incubation mixtures. The tubes were tightly closed and heated in a water bath at 80°C for 1 hour. Non-saponifiable lipids were extracted from cooled saponification mixtures by Vortex mixing three times with 7 ml petroleum ether (b. p. 40-60°C). The combined organic extracts were evaporated to dryness on a rotary evaporator at 40°C under vacuum. The residues were redissolved in 2 - 4 ml of petroleum ether. Residual water in the organic solvent was removed by adding anhydrous sodium sulphate (0.5 g). The solvent was reduced to dryness under nitrogen. Residues containing non-saponifiable lipids were stored under nitrogen at -20°C.

Thin-layer chromatography (TLC). Non-saponifiable lipid extracts from incubation mixtures (1 ml) were dissolved in petroleum ether (300 μl) and applied to TLC plates (Silica gel plate F_{254}, Merck, Darmstadt, Germany). The plates were developed in cyclohexane : ethylacetate / 4 : 1 (v/v) in the dark. Authentic ergosterol and lanosterol were used as standards. Radioactive areas on the plates were located by autoradiography by exposing the TLC plates to a Kodak diagnostic film (X-Omat™, Eastman Kodak Company, Rochester, NY.) for three days.

Identification of non-saponifiable lipid fractions was carried out by comparing the R_f values of different fractions with those of the authentic compounds as observed under UV light and with literature data. Sterol and other non-saponifiable lipid fractions separated on TLC plates were scraped off and counted for radioactivity in a liquid scintillation counter (Beckman LS 5800). The radioactivity recovered from TLC plates was considered as radioactivity incorporated into non-saponifiable lipids. The amount of radioactivity incorporated into different sterol fractions was expressed as a percentage of radioactivity in total non-saponifiable lipids.

Radio-HPLC of sterols. Non-saponifiable lipids extracted from incubation mixtures (1 ml) were dissolved in 95% methanol (300 μl) containing ergosterol and lanosterol (0.3 mg ml^{-1}) as internal standards. Analyses of non-saponifiable lipids were carried out with a Radio-HPLC equipment consisting of a Waters 510 HPLC pump, two UV detectors (Waters 484 and 481, Millipore, Milford, Ma.) set at 210 and 280 nm, respectively and an on-line radioactivity monitor (Canberra Radiomatic, A-200, Radiomatic Instruments and Chemical Co., Inc., Tampa, Fl.) using Pico-Aqua™ (Packard Instrument Company, Inc., Downers Grove, Ill.) as scintillant at a flow rate of 3 ml min^{-1}. Samples (100 μl) were eluted with 95% methanol (HPLC grade, J. T. Baker B.V., Deventer, the Netherlands) on a Zorbax C_8 column (4.6×250 mm, Chrompack, Middelburg, the Netherlands) at a flow rate of 1 ml min^{-1} at 30°C. Identification of sterol fractions was carried out by comparing retention times of peaks with those of internal standards and literature data. Sterol compositions of individual bands from TLC plates were also analyzed. Radioactivity on TLC plates was located after only 6 h of exposure of the film in order to avoid oxidation of sterols. Bands with radioactivity were scraped off and radioactive compounds were eluted with chloroform. The elutes were taken to dryness under nitrogen. Residues were dissolved in 95% methanol (100 μl) and analyzed with the Radio-HPLC as described above.

Results

TLC analysis. Under optimal condition (pH 7.3) incorporation of radioactivity into non-saponifiable lipids was on average (n = 13) 23 ± 2.4% of total radioactivity added. Separation of the non-saponifiable lipids by TLC revealed several distinct bands (Fig. 1, control treatment). Band 2, 3 and 4 were tentatively identified as C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols by comparing their R_f values with authentic ergosterol and lanosterol. The

identity of band 6 was probably squalene (11). The identities of bands 1 and 5 (including several zones) were unknown. Incorporation of radioactivity into C4-desmethyl sterols was found to be linear with time up to 3 h (results not shown). Percentages of radioactivity incorporated into C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols after 3 h of incubation were 25.6 $\pm$ 3.5, 11.5 $\pm$ 4.2 and 31.7 $\pm$ 7.3% of non-saponifiable lipids, respectively (n = 13). Water and DMSO added in controls did not have any obvious effect on synthesis of sterols.

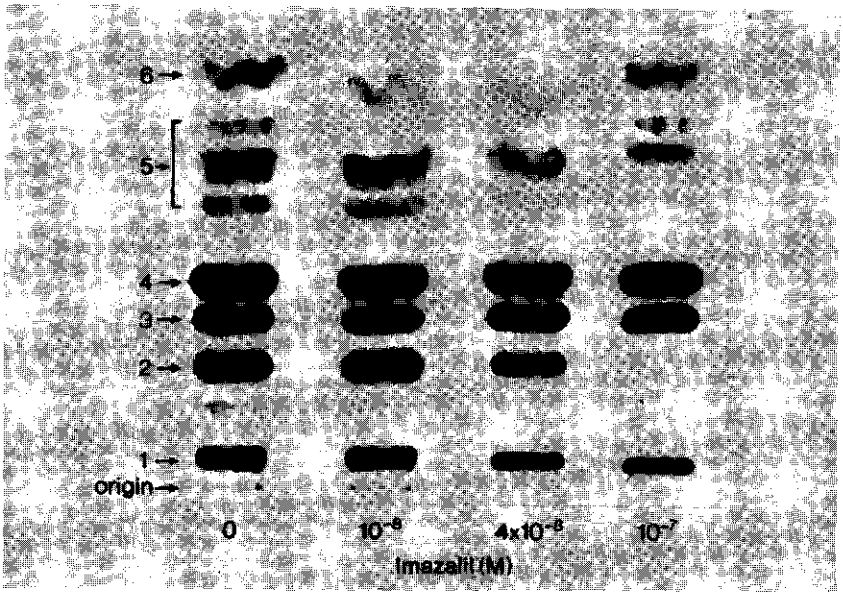

Fig. 1. Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C]mevalonate in cell-free bioassays of *Penicillium italicum* W_5 in the absence and the presence of imazalil at pH 7.3. Band 2, 3 and 4 contained C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols, respectively. Band 6 contained squalene. The identities of bands 1 and 5 are unknown.

Malt agar plates inoculated with a few drops of cell-free extract did not show any mycelial growth confirming that formation of radiolabelled sterols was due to cell-free synthesis and not to synthesis in contaminating mycelial fragments.

The average protein content of the cell-free extracts was 1.1 $\pm$ 0.25 mg ml^{-1} (n = 13). The average protein content of cell-free extracts in assays with individual compounds (n $\geq$ 3) did not differ from the average value mentioned above.

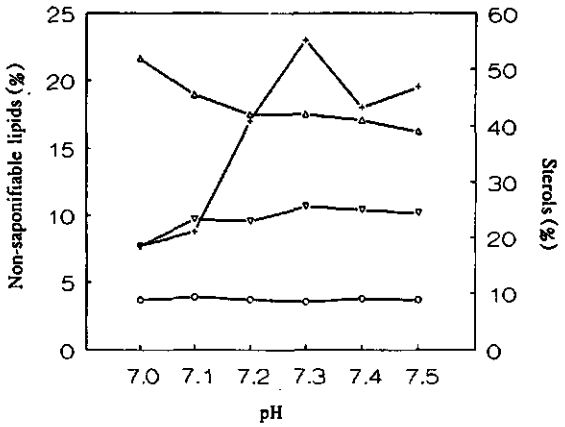

Fig. 2. Effect of pH on incorporation of [2-^{14}C]mevalonate into non-saponifiable lipids (+), C4,4-dimethyl sterols (▵), C4-monomethyl sterols (○) and C4-desmethyl sterols (▽) in a cell-free extract of *Penicillium italicum* W_5. Left ordinate: radioactivity in non-saponifiable lipids as percentage of total radioactivity added. Right ordinate: radioactivity in sterols as percentage of radioactivity in non-saponifiable lipids.

Essential factors for C4-desmethyl sterol synthesis.. A gentle disruption of the mycelium was critical. Microscopical examination showed that the majority of the mycelia remained intact after disruption. More severe disruption of mycelia obtained by increasing the speed of the disruptor or by using smaller glass beads (diameter 0.5 mm) yielded cell-free extracts with a relatively higher protein content. However, although incorporation of radioactivity into C4,4-dimethyl sterols did occur, synthesis of C4-desmethyl sterols in these extracts was poor (results not shown). Similar results were observed when the air in the disruption vessel was not removed. An essential condition was also the addition of glycerol to cell-free extracts immediately after filtration of disrupted mycelial homogenates. The activity of the cell-free extract was also affected by the pH. In the pH range from 7.0 to 7.5 the highest amount of radioactivity incorporated into total non-saponifiable lipids was found at pH 7.3 (Fig. 2). After 3 h of incubation the pH of the incubation mixtures dropped to pH 7.1. Radioactivity incorporated into C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols, expressed as a percentage of non-saponifiable lipids, varied only slightly in this pH range (Fig. 2). At pH values lower than 7.0 and higher than

7.5, incorporation of radioactivity into C4-desmethyl sterols decreased sharply (results not shown). Therefore, studies on the inhibitory effect of the test compounds on sterol 14α-demethylation were carried out at pH 7.3. In order to avoid experimental variation caused by differences in quality of cell-free extracts, data were only collected from assays in which incorporation of radioactivity into C4-desmethyl sterols was 25.6 $\pm$ 3.5% of total non-saponifiable lipids synthesized (control treatments). This was the case for 90% of all assays carried out.

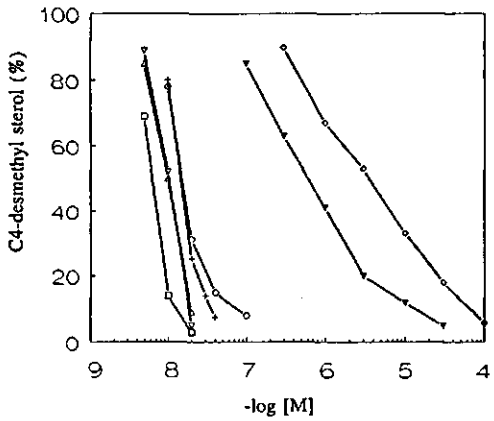

Fig. 3. Inhibition of incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free bioassays of *Penicillium italicum* W_6 at pH 7.3 by imazalil (+), itraconazole (▽), ketoconazole (□), penconazole (○), propiconazole (△) and imazalil analogues R14821 (▼) and R42243 (◇). Abscis: concentration of test compounds. Ordinate: radioactivity in C4-desmethyl sterols as percentages of control treatment.

Inhibition studies. Fig. 1 shows an autoradiogram of a TLC plate on which total non-saponifiable lipids extracted from control and imazalil-treated samples were separated. Imazalil inhibited the incorporation of radioactivity into C4-desmethyl sterols (band 2). Effects of imazalil on incorporation of radioactivity into C4-monomethyl (band 3) and C4,4-dimethyl sterols (band 4) in autoradiograms could not be observed. Resolution of unknown lipids in band 5 was not always clear. This was probably caused by traces of water present in such samples. However, results indicate that incorporation of radioactivity into lipids

of this band was only slightly affected by imazalil (Fig. 1). Other DMI fungicides itraconazole, ketoconazole, penconazole, propiconazole and two less-toxic DMI-analogues R14821 and R42243 showed a similar effect. Dosage response curves for inhibition of incorporation of radioactivity into C4-desmethyl sterols by the test compounds are presented in Fig. 3. IC_{50} values (concentrations which inhibited incorporation of radioactivity into C4-desmethyl sterols for 50%) of the DMI-fungicides ranged from 6.5×10^{-9} (ketoconazole) to 1.7×10^{-8} M (penconazole) indicating a strong inhibitory effect. The less-toxic DMI-analogues R14821 and R42243 also showed an inhibitory effect but only at much higher concentrations (Fig. 3, Table 1).

Table 1. IC_{50} values of DMIs and imazalil analogues on incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free extracts of *Penicillium italicum* W_5 at pH 7.3.

Compound	$IC_{50} \pm$ SEM (M)[1]	n[2]
Imazalil	$1.6 \pm 0.4\times10^{-8}$	10
Itraconazole	$1.0 \pm 0.3\times10^{-8}$	3
Ketoconazole	$6.5 \pm 0.5\times10^{-9}$	3
Penconazole	$1.7 \pm 0.7\times10^{-8}$	4
Propiconazole	$1.0 \pm 0.2\times10^{-8}$	3
R14821	$6.0 \pm 0.7\times10^{-6}$	3
R42243	$4.0 \pm 0.3\times10^{-5}$	4

[1] Concentration which inhibits incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols for 50%.

[2] Number of replications with different cell-free extracts.

Identification of radiolabelled sterols. Non-saponifiable lipids were analyzed with radio-HPLC. In control samples, 5 major peaks were observed with retention times of 4.9 (peak 1), 9.6 (peak 2), 16.2 (peak 3), 22.4 (peak 4) and 24.8 min (peak 5), respectively (Fig. 4A). Peak 3 and 4 had retention times identical to those of authentic ergosterol and lanosterol, respectively (results not shown). In imazalil treated samples (10^{-7} M), only 4 major peaks were observed. Compared with the control samples, peak 3 disappeared and peak 5 increased significantly (Fig. 4B). Peaks 1 and 2 did not change after imazalil treatment. Radiolabelled lipids recovered from individual bands on TLC plates of control

and imazalil-treated (10^{-7} M) samples were also analyzed with radio-HPLC. A comparison of the results obtained from TLC and radio-HPLC (Table 2) suggests that the identities of peak 3, 4 and 5 are mostly likely ergosterol, lanosterol and 24-methylenedihydrolanosterol, respectively. The identity of other lipids was not studied any further.

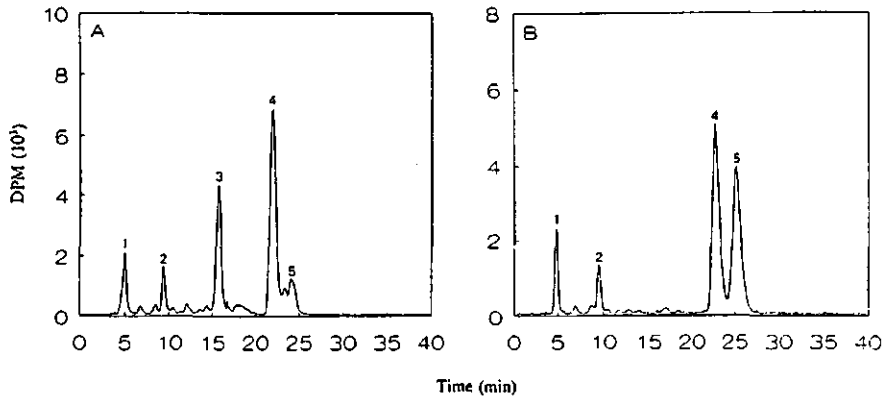

Fig. 4. Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from control (A) and imazalil-treated (10^{-7} M) (B) cell-free bioassays of *Penicillium italicum* W_5 at pH 7.3. The identities of peak 3, 4 and 5 are ergosterol, lanosterol and 24-methylenedihydrolanosterol, respectively.

Table 2. Comparison of analyses by TLC and radio-HPLC of non-saponifiable lipids formed during incorporation of [2-^{14}C]mevalonate in cell-free extracts of *Penicillium italicum* W_5.

TLC[1]		Radio-HPLC[2]	
Band	Identity	Peak	Identity
1	Unknown	1	Unknown
2	C4-desmethyl sterols	3	Ergosterol
3	C4-monomethyl sterols	2	Unknown
4	C4,4-dimethyl sterols	4	Lanosterol
		5	24-methylenedihydrolanosterol
5	Unknown	Not recovered	
6	Squalene	Not recovered	

[1] See Fig. 1.

[2] See Fig. 4A.

Discussion

Cell-free extracts of *P. italicum* W_5 were active in incorporation of [2-^{14}C]-mevalonate into various non-saponifiable lipids. Radioactivity incorporated into C4-desmethyl sterols was on average 25.6% of that incorporated into total non-saponifiable lipids (Fig. 2). Radio-HPLC analysis of this C4-desmethyl sterol fraction revealed only one peak with a retention time identical to that of standard ergosterol, and its presence disappeared upon incubation with imazalil and other DMIs (Table 2; Fig. 4). These data suggest that ergosterol is the only C4-desmethyl sterol synthesized in the cell-free extracts. Other sterols identified in the non-saponifiable lipids were lanosterol and 24-methylenedihydrolanosterol (Table 2; Fig. 4). Identification of lanosterol was based on its recovery from the C4,4-dimethyl sterol band on TLC plates and an identical retention time as standard lanosterol on radio-HPLC (Table 2 and Fig. 4). The tentative identification of 24-methylenedihydrolanosterol was based on the following arguments. a) It is present in the C4,4-dimethyl sterol band of TLC plates. b) The retention time of the compound in radio-HPLC analysis was similar to that of 24-methylenedihydrolanosterol (17). c) Mass spectrometry of the compounds in peak 5 demonstrated the presence of 24-methylenedihydrolanosterol (Vanden Bossche, personal communication). Intact mycelium of *P. italicum* contains ergosterol and only a trace amount of 24-methylenedihydrolanosterol (16). Therefore, synthesis of a large amount of lanosterol and a trace amount of 24-methylenedihydrolanosterol in cell-free extracts suggests a rate limiting step in ergosterol biosynthesis. This is probably the C24 side chain alkylation step of lanosterol and may result from hampered transport of lanosterol to mitochondria (14). Incubation of cell-free extracts with imazalil led to accumulation of 24-methylenedihydrolanosterol (Fig. 4B). This suggests that inhibition of sterol 14α-demethylase activity is responsible for inhibition of ergosterol biosynthesis in cell-free extracts and that synthesis proceeded according to the same pathway as in intact mycelium (16).

Incorporation of [2-^{14}C]mevalonate into ergosterol, expressed as a percentage of radioactivity in total non-saponifiable lipids was almost the same between pH 7.0 and 7.5 (Fig. 2). This result is different from that reported for the cell-free assay of *A. fumigatus*, which had an optimal pH sharply confined between 7.2 and 7.4 (11). This may reflect slight differences in sterol 14α-demethylation in different fungi. In cell-free assays of *P. italicum* at pH 7.3, the mean amount of the radioactivity incorporated into ergosterol was 25.6% of the total radioactivity incorporated into non-saponifiable lipids. This is higher than reported for

the cell-free assay of *A. fumigatus* (11). Another difference is that bioassays with cell-free extracts of *P. italicum* led to synthesis of lower amounts of 24-methylenedihydrolanosterol than with cell-free extracts of *A. fumigatus* (11). This is probably not due to a significant difference in the physiological age of the germlings used. The disruption methods used to make cell-free extracts of both fungi are also comparable. In both cases disruption resulted in hardly any damage of the germlings. Still, disruption of *P. italicum* may have been more gentle than that of *A. fumigatus* in view of the relatively lower protein content of cell-free extracts of *P. italicum*. The main reason for the difference in ergosterol biosynthesis activity may be the addition of glycerol to cell-free extract of *P. italicum* to stabilize activity of microsomal enzymes involved in sterol biosynthesis.

All DMI-fungicides tested gave extremely low IC_{50} values for inhibition of sterol 14α-demethylation (6.5×10^{-9} M to 1.7×10^{-8} M). Values for the less-toxic DMI-analogues (R14821 and R42243) were much higher (6×10^{-6} M to 4×10^{-5} M) (Table 1). These results are in general in agreement with the results obtained in CO displacement studies in which the DMI fungicides showed a relatively higher binding affinity to cytochrome P450 isozymes than the less-toxic DMI-analogues (15). This suggests a relation between fungitoxicity and inhibition of sterol 14α-demethylase activity. However, the IC_{50} values of the DMI fungicides tested did not correlate with their fungitoxicity. For instance, imazalil which was the most toxic DMI fungicide tested (15) had a significantly higher IC_{50} value than ketoconazole (Table 1). It is not possible that deviations in IC_{50} values are due to variations in quality of cell-free extracts with respect to protein content and incorporation efficiency of mevalonate into ergosterol. Apparently, other factors such as accumulation in mycelium also play a role in toxicity of DMIs.

In conclusion, the present assay system for the synthesis of ergosterol in cell-free extracts of *P. italicum* is suitable to evaluate DMI fungicides for their potency to inhibit sterol 14α-demethylation. This implies that the bioassay can be used to optimize the activity of DMI fungicides and is useful to compare the sensitivity of sterol 14α-demethylase in cell-free extracts of DMI-sensitive and resistant isolates. The bioassay is probably also useful to study other target sites in ergosterol biosynthesis.

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CHAPTER 4

Inhibition of sterol 14α-demethylase activity in *Penicillium italicum* does not correlate with resistance to the DMI fungicide imazalil

J. Guan and M. A. de Waard

Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

(Submitted to Pesticide Biochemistry and Physiology)

Abstract

Sensitivity of sterol 14α-demethylase activity in cell-free extracts of *Penicillium italicum* isolates $E_{300\text{-}3}$, H_{17} and I_{33} with increasing degrees of resistance to imazalil was studied and compared with that of the wild-type isolate W_6. Incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free extracts of medium- and high-resistant isolates H_{17} and I_{33} was about 33% of non-saponifiable lipids. This was slightly higher than in those of wild-type isolate W_6 (26%) and low-resistant isolate $E_{300\text{-}3}$ (24%). The difference may be due to a relatively higher protein content of cell-free extracts of isolates H_{17} and I_{33}. Ergosterol appeared to be the only C4-desmethyl sterol synthesized in cell-free extracts of all isolates. Imazalil inhibited incorporation of [2-^{14}C]mevalonate into ergosterol in cell-free extracts of all isolates tested, resulting in accumulation of radioactivity in 24-methylenedihydrolanosterol. These results indicate that the sterol biosynthetic pathway and the target site for imazalil in cell-free extracts of both wild-type and DMI-resistant isolates are the same. IC_{50} values of imazalil (concentrations which inhibit incorporation of [2-^{14}C]mevalonate into ergosterol by 50%) for isolates $E_{300\text{-}3}$, H_{17} and I_{33} ranged from $1.6 \pm 0.4 \times 10^{-8}$ to $2.1 \pm 0.4 \times 10^{-8}$ M. These values were not significantly different from that of the wild-type isolate ($1.6 \pm 0.4 \times 10^{-8}$ M). It is concluded that the affinity of sterol 14α-demethylase to imazalil in both

wild-type and DMI-resistant isolates is similar and that decreased affinity of the target enzyme to imazalil does not play a major role as a mechanism of resistance in *P. italicum*.

Introduction

The mechanism of resistance in *Penicillium italicum* to demethylation inhibitors (DMIs) has previously been studied (1-3). An increased energy-dependent efflux of the fungicides seems to be responsible for a low level of resistance to these fungicides. The mechanism of resistance in medium- and high-resistant isolates H_{17} and I_{33} remained unclear. Because of the specific mode of action of DMIs on sterol 14α-demethylation in ergosterol biosynthesis (4, 5), resistance might be based on decreased affinity of the target enzyme: sterol 14α-demethylase. So far, this mechanism has only been proposed for certain mutants of the yeast-like form of the fungus *Candida albicans* (6, 7). Comparable studies with filamentous fungi have been hampered due to difficulties in demonstrating enzyme activity *in vitro*. Recently, a method to study sterol 14α-demethylation in cell-free extracts of the filamentous plant pathogen *P. italicum* has been developed (8). This method paves the way to study the sensitivity of sterol 14α-demethylase activity to DMIs in DMI-resistant isolates of the fungus. This paper describes the intrinsic sensitivity of the enzyme in the various isolates to the DMI fungicide imazalil.

Materials and methods

Fungus and culture conditions. Wild-type isolate W_5 and DMI-resistant isolate $E_{300\text{-}3}$ (low degree of resistance), H_{17} (medium degree of resistance) and I_{33} (high degree of resistance) of *Penicillium italicum* (9, 10) were maintained on malt extract agar medium. Mycelium suspensions (11 h old) were obtained according to methods described previously (11).

Chemicals. Imazalil sulphate (imazalil) was a gift from Janssen Pharmaceutica (Beerse, Belgium). Imazalil was used in 1000 × concentrated solutions in water. Mevalonic acid DBED salt (RS-[mevalonic-2-^{14}C] in ethanol, sp. act. 1.9 GBq mmol^{-1}) was purchased from Amersham, (UK). A cofactor solution (NAD^+, $NADP^+$, NADPH, ATP, glucose 6-phosphate and reduced glutathione) and divalent cation solutions ($MgCl_2$ and $MnCl_2$) were prepared as described previously (8).

Sterol biosynthesis assay. Preparation of cell-free extracts from DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} was carried out according to a standard method described for the wild-type isolate W_5 (8). The protein content in the cell-free extracts was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Veenendaal, the Netherlands) using bovine γ-globulin as standard. Sterol biosynthesis assay, extraction and analysis of non-saponifiable lipids by thin layer chromatography (TLC) and radio-HPLC were conducted according to methods described previously (8).

Results

TLC. Fig. 1 (control) shows an autoradiogram of a TLC plate of non-saponifiable lipids extracted from cell-free extracts of resistant isolate I_{33} after incubation with [2-^{14}C]mevalonate. By comparing the R_f values of authentic ergosterol and lanosterol, compounds in bands 2, 3 and 4 were tentatively

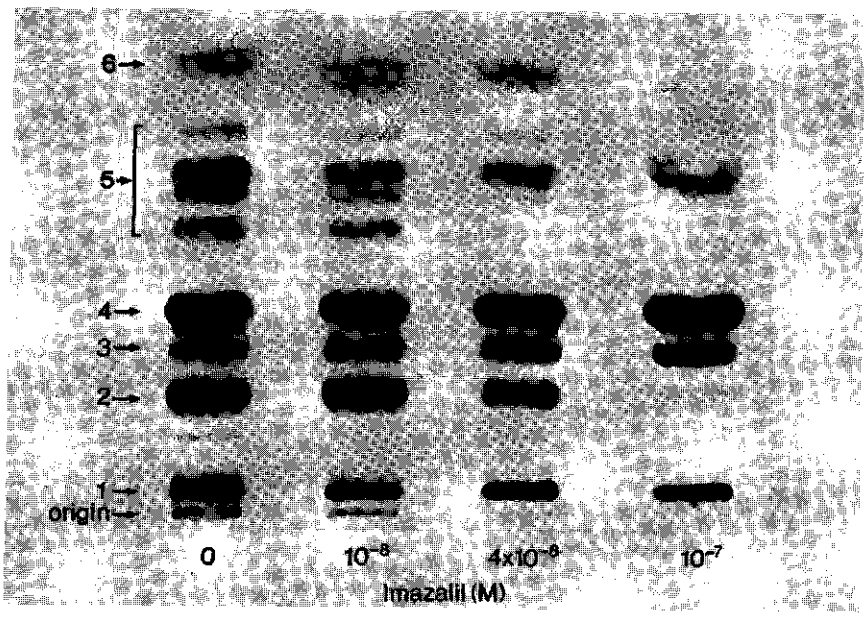

Fig. 1. Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C]mevalonate in cell-free bioassays of a DMI-resistant isolate I_{33} of *Penicillium italicum* in the absence and presence of imazalil (10^{-7} M) at pH 7.3. Band 2, 3 and 4 contained C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols, respectively. Band 6 contained squalene. The identities of bands 1 and 5 are unknown.

assigned as C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols, respectively. Identity of band 6 is probably squalene. Non-saponifiable lipids in bands 1 and 5 (including several zones) were not studied any further. The same results were observed for non-saponifiable lipids extracted from cell-free extracts of isolates $E_{300\text{-}3}$ and H_{17} (results not shown). The data are also similar to those of the wild-type isolate (8). Radioactivity of non-saponifiable lipids in all bands was counted (Table 1). The data indicate that in cell-free extracts of isolate $E_{300\text{-}3}$ a similar amount of radioactivity was incorporated into C4-desmethyl sterols as in that of isolate W_5. Incorporation of radioactivity into C4-desmethyl sterols in cell-free extracts of isolates H_{17} and I_{33} were 29 and 26% higher than in that of isolate W_5, respectively. Incorporation of radioactivity into C4-monomethyl sterols in cell-free extracts of isolates H_{17} and I_{33} was relatively low. The protein content of cell-free extracts of isolates H_{17} and I_{33} was higher than those of isolates W_5 and $E_{300\text{-}3}$ (Table 1).

Imazalil inhibited incorporation of radioactivity into C4-desmethyl sterols in cell-free extracts of isolate I_{33} (Fig. 1). A similar inhibitory effect was observed with resistant isolates $E_{300\text{-}3}$ and H_{17} (Fig. 2) and with wild-type isolate W_5 (8). Mean IC_{50} values of imazalil (concentration which inhibits incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%) for wild-type and DMI-resistant isolates are shown in Table 2. Statistical analysis demonstrated that these figures do not differ significantly from each other. Imazalil (10^{-7} M) did not affect synthesis of C4-monomethyl sterols, but significantly increased accumulation of C4,4-dimethyl sterols in all isolates (results not shown). The effect on incorporation of radioactivity into other non-saponifiable lipid was not clear, since in various samples separation of lipids in bands 5 and 6 was hampered. This may be due to trace amounts of water in the samples. These lipid fractions were not studied any further.

Radio-HPLC. Total non-saponifiable lipids were analyzed with a radio-HPLC. Radio-HPLC chromatograms of non-saponifiable lipids extracted from the control treatment of cell-free extracts of isolate I_{33} revealed 5 major peaks with retention times of 4.9 (peak 1), 9.6 (peak 2), 16.2 (peak 3), 22.4 (peak) and 24.8 (peak 5) (Fig. 3A). Peaks 3 and 4 had retention times identical to that of ergosterol and lanosterol (results not shown). Radio-HPLC analysis of radiolabelled lipids recovered from individual bands in the TLC plates showed that C4-desmethyl sterol bands gave a single peak with a retention time identical to that of peak 3; C4-monomethyl sterol bands also gave a single peak with a retention time identical to that of peak 2; C4,4-dimethyl sterol bands gave a major and a minor peak with retention times identical to those of peak 4 and 5, respectively.

Table 1. Sterols formed during incorporation of [2-^{14}C]mevalonate in cell-free extracts of the wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} of *Penicillium italicum*.

Isolate	Protein content (mg ml^{-1})[1]	Sterol composition[2,3]		
		I	II	III
W_5[4]	1.1 ± 0.3	31.7 ± 7.3	11.5 ± 4.2	25.6 ± 3.1
$E_{300\text{-}3}$	1.6 ± 0.1	39.8 ± 2.8	10.7 ± 0.6	23.9 ± 1.9
H_{17}	2.4 ± 0.2[5]	36.3 ± 5.2	6.2 ± 0.9[5]	33.0 ± 2.1[5]
I_{33}	2.1 ± 0.3[5]	33.4 ± 8.6	8.0 ± 2.9	32.2 ± 6.2[5]

[1] Cell-free extracts.
[2] Radioactivity incorporated into sterol fractions as a percentage of radioactivity incorporated into non-saponifiable lipids. I, C4,4-dimethyl sterols; II, C4-monomethyl sterols; III, C4-desmethyl sterols.
[3] Mean and standard deviation. W_5 (n = 13), $E_{300\text{-}3}$ (n = 3), H_{17} (n = 5) and I_{33} (n = 7).
[4] Data from Guan *et al.* (8).
[5] Significantly different from W_5 (P = 0.05).

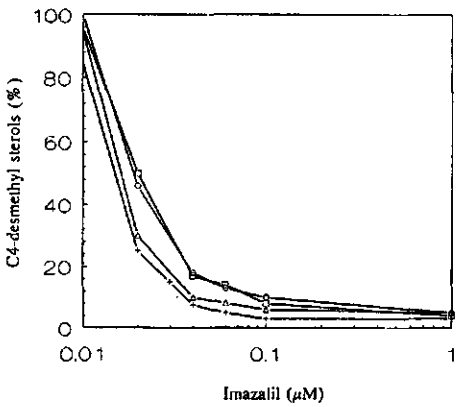

Fig. 2. Inhibition of incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free bioassays of wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○) and I_{33} (□) of *Penicillium italicum* by imazalil at pH 7.3. Abscis: concentration of imazalil. Ordinate: radioactivity in C4-desmethyl sterols. Figures are mean values of 13 (W_5) and 4 ($E_{300\text{-}3}$, H_{17} and I_{33}) experiments.

Table 2. IC_{50} values of imazalil on incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free extracts of wild-type W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and J_4 of *Penicillium italicum*.

Isolate	$IC_{50} \pm$ SEM (M)[1,2]
W_5[3]	$1.6 \pm 0.4 \times 10^{-8}$
$E_{300\text{-}3}$	$1.7 \pm 0.5 \times 10^{-8}$
H_{17}	$2.0 \pm 0.5 \times 10^{-8}$
I_{33}	$2.1 \pm 0.4 \times 10^{-8}$

[1] Concentrations of imazalil which inhibit incorporation of radioactivity into C4-desmethyl sterols by 50%.

[2] Mean and standard deviation. W_5 (n = 13); $E_{300\text{-}3}$, H_{17} and I_{33} (n = 4).

[3] Data from Guan *et al.* (8).

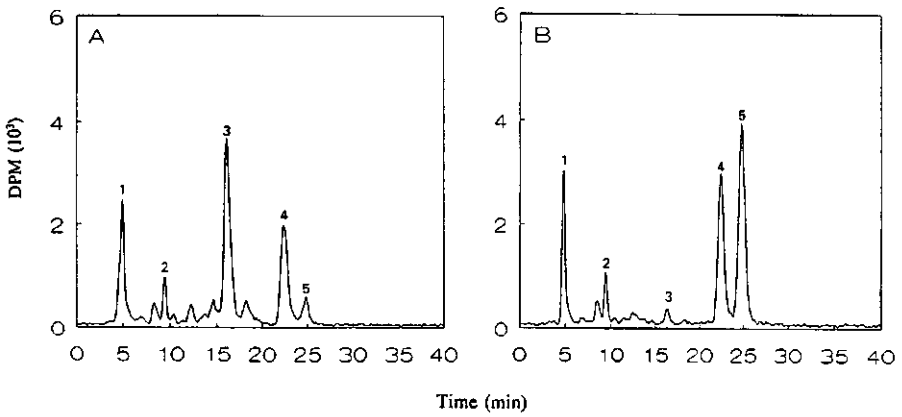

Fig. 3. Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from control (A) and imazalil-treated (10^{-7} M) (B) cell-free bioassays of DMI-resistant isolate I_{33} of *Penicillium italicum* at pH 7.3. The identities of peak 3, 4, and 5 are ergosterol, lanosterol, and 24-methylenedihydrolanosterol, respectively.

Radio-HPLC analysis of non-saponifiable lipids extracted from imazalil-treated (10^{-7} M) samples of isolate I_{33} revealed only 4 major peaks. Compared with the

control treatment, peak 3 disappeared and peak 5 increased in sized (Fig. 3B). Similar sterol patterns and inhibitory effects of imazalil on C4-desmethyl sterol biosynthesis were observed in non-saponifiable lipids extracted after incubation with [2-^{14}C]mevalonate from cell-free extracts of isolates $E_{300\text{-}3}$ and H_{17} (results not shown). All these results were qualitatively identical to those obtained for the wild-type isolate W_5 (8).

Discussion

Cell-free extracts of DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} of *P. italicum* were active in synthesizing C4-desmethyl sterols from [2-^{14}C]mevalonate (Fig. 1). The radio-HPLC chromatograms of non-saponifiable lipids of the DMI-resistant isolate I_{33} (Fig. 3A) were similar as compared to those of the wild-type isolate (8). The identities of peaks 3, 4 and 5 of non-saponifiable lipids of this isolate were established as ergosterol, lanosterol and 24-methylenedihydro-lanosterol, respectively (8). On basis of the similarity in retention times it is assumed that the identities of the lipids in all resistant isolates of *P. italicum* are the same. This is in agreement with the experiments carried out with intact mycelium which showed that ergosterol was the major sterol in these isolates (12). Cell-free extracts of medium- and high-resistant isolates H_{17} and I_{33} synthesized not only relatively higher amount of ergosterol than those of isolates W_5 and $E_{300\text{-}3}$ but also less amounts of C4-monomethyl sterols (Table 1). These results may, at least in part, be due to the relatively high protein content of the cell-free extracts of isolates H_{17} and I_{33} (Table 1), suggesting that these extracts also contain a higher concentration of sterol 14α-demethylase. The relatively higher sterol biosynthesis activity in cell-free extracts of resistant isolates H_{17} and I_{33} may also be a consequence of a better quality of the enzyme preparations due to slight variation in physiological age of the mycelium at the time of harvest and a better disruption of the mycelium. The specific content of sterol 14α-demethylase in isolates H_{17} and I_{33} is probably not higher than in the wild-type isolate W_5, since previous studies suggest that isolates H_{17} and I_{33} have a relatively low cytochrome P450 isozyme content (13). The relatively high sterol synthesis activity of cell-free extracts from resistant isolates can also be due to an increased specific activity of sterol 14α-demethylase. The relevance of this hypothesis seems to be low but can, as yet, not be judged.

Ergosterol biosynthesis in cell-free extracts of resistant isolates could be inhibited by imazalil. Despite of the fact that cell-free extracts of different isolates slightly differed in quality, IC_{50} values of imazalil for inhibition of incorporation

of [2-^{14}C]mevalonate into ergosterol for low-, medium- and high-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} were similar and did not significantly different from that of the wild-type isolates W_5 (Table 2). On basis of these results one may conclude that the sensitivity of sterol 14α-demethylase in cell-free extracts of sensitive and DMI-resistant isolates is similar. Therefore, a mechanism of resistance based on decreased affinity of the target enzyme to DMIs is not probable. This conclusion is not in line with results obtained in carbon monoxide (CO) displacement tests in which imazalil, itraconazole and ketoconazole were more readily displaced by CO from cytochrome P450 isozymes of isolates H_{17} and I_{33} than from those of the wild-type isolate (13). These results suggested that the affinity of P450 isozymes in isolates H_{17} and I_{33} to DMIs was relatively low. However, the reliability of the conclusion from these spectrophotometric analyses is challenged by a number of factors which hamper a proper interpretation of the results. These factors include the necessary modification of the standard isolation procedure of P450 isozymes from resistant isolates, the possible difference in quality of the isozymes in wild-type and resistant isolates and a possible underestimation of the concentration of the isozymes in microsomal fractions of the resistant isolates. Furthermore, data obtained in spectrophotometric tests are only of a semi-quantitative nature. Hence, results derived from the spectrophotometric assays are not sufficiently reliable to compare affinity of cytochrome $P450_{14DM}$ to DMIs in different isolates and therefore, do not provide sound evidence to reject the conclusion that affinity of sterol 14α-demethylase from resistant isolates to imazalil is similar to that of the wild-type.

In isolate $E_{300\text{-}3}$ of *P. italicum*, the low level of resistance to imazalil is related to a mechanism of increased energy-dependent efflux of the fungicide (3). This is in agreement with the present results (Table 1). The possibility that the mechanism of resistance in medium- and high-resistant isolates H_{17} and I_{33} is based on metabolism of the fungicide has been excluded (14). The mechanism of resistance in isolates H_{17} and I_{33} must, therefore, relate to factors which prevent the fungicide to reach its target site. A possibility is that increased energy-dependent efflux in these isolates may still be relevant but can not be noticed because of a relatively high background adsorption of the fungicides to mycelium. A second argument is that resistance to DMIs in isolates H_{17} and I_{33} is probably polygenic (15). It is known that various polygenes in *Aspergillus nidulans* (16) and *Nectria haematococca* var. *cucurbitae* (17) can be responsible for increased energy-dependent efflux and that these genes may have an additive effect in this respect (17). Therefore, it can not be excluded that further decreased accumulation of imazalil in isolates H_{17} and I_{33} may still be responsible for their

relatively high degree of resistance to DMIs.

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CHAPTER 5

Interaction of microsomal cytochrome P450 isozymes isolated from *Penicillium italicum* with DMI fungicides

J. Guan[1], H. M. J. Braks[1], A. Kerkenaar[2,3] and M.A. de Waard[1]

[1]Department of Phytopathology, Wageningen Agricultural University
P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

[2]Netherlands Organization of Applied Scientific Research,
TNO Institute of Applied Chemistry, P.O. Box 108,
3700 AC Zeist, The Netherlands.

[3]Present address: Denka International B.V.,
Hanzeweg 1, 3771 NG Barneveld, The Netherlands.

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Abstract

A procedure has been developed for the isolation of microsomal cytochrome P450 isozymes from the filamentous fungus *Penicillium italicum*. The content of cytochrome P450 isozymes in the microsomal fraction was 95 ± 18 pmol per mg protein. Maximum absorbance of the reduced carbon monoxide (CO) difference spectrum was at 449 nm. Absorbance at 420 nm was absent. The fungitoxic sterol 14α-demethylation inhibitors (DMIs) imazalil, itraconazole, ketoconazole, penconazole and propiconazole, and two less-toxic imazalil analogues R14821 and R42243 interacted with oxidized cytochrome P450 isozymes resulting in type II binding difference spectra. Equimolar concentration (10^{-7} M) of cytochrome P450 isozymes and DMIs induced about 80% of the maximum absorbance difference, indicating a close stoichiometric interaction. The concentration of DMIs which induced 50% of the maximum absorbance difference in the type II spectra (IC_{50}) ranged from 3.7×10^{-8} to 5.0×10^{-8} M and did not correlate with fungitoxicity of the DMIs. Imazalil

analogues did not interact stoichiometrically with cytochrome P450 isozymes and gave relatively higher IC_{50} values. The binding of the DMIs and imazalil analogues to cytochrome P450 isozymes was studied by CO displacement tests. The ease of displacement of the test compounds decreased in the order of R42243, R14821, penconazole, propiconazole and imazalil. This correlated with the fungitoxicity of these compounds. However, DMIs with a relatively large *N*-1 substituent, like itraconazole and ketoconazole, did not show such a correlation, indicating that factors in addition to binding affinity to the heme iron and apoprotein of cytochrome P450 isozymes also influence toxicity.

Introduction

Sterol demethylation inhibitors (DMIs) are a large and structurally diverse group of fungicides used to control fungal diseases in plants and mammals (1, 2). The most important groups of DMIs are derivatives of imidazoles and triazoles. Their target site is the cytochrome P450-dependent sterol 14α-demethylase (cytochrome $P450_{14DM}$) which catalyses the removal of the C14 methyl group of lanosterol or 24-methylenedihydrolanosterol in ergosterol biosynthesis (2, 3).

Studies with cytochrome P450 isozymes from *Candida albicans* (2, 4, 5) and purified cytochrome $P450_{14DM}$ from *Saccharomyces cerevisiae* (6-9) indicate that DMIs bind to at least two sites in the enzyme. Firstly, the free electron pair of the *N*-3 atom in the imidazole ring or of the *N*-4 atom of the triazole ring interacts with the 6th coordination place of the heme iron; and secondly, the *N*-1 substituent of the azole ring binds to the apoprotein of cytochrome $P450_{14DM}$. The second site of binding is considered to be more important for enzyme inhibition, since not all derivatives of azoles are potent DMIs although they bind to the 6th coordination place of the iron moiety. Studies by Vanden Bossche *et al.* (10) have provided direct evidence for this conclusion. They found that substitution of the triazole ring of itraconazole with an imidazole ring did not change the binding affinity of the compound to cytochrome P450 isozymes from *C. albicans*. The same was true for ketoconazole when its imidazole ring was substituted with a triazole ring. Therefore, studies on the interaction between DMIs and the apoprotein of cytochrome $P450_{14DM}$ will probably provide the most relevant information on the intrinsic fungitoxicity of candidate DMI fungicides (6). However, such studies have been largely confined to *C. albicans* and *S. cerevisiae* (2, 8, 11). The lack of reports of comparable studies with filamentous fungi probably reflects the unstable nature of the membrane-bound enzyme in these organisms rather than a lack of interest and effort (12). Recent reports of the isolation of cytochrome P450 isozymes from *Aspergillus fumigatus*, a human

pathogen (13, 14), has opened up the possibility of conducting such studies with other filamentous fungi.

The present study describes a novel method for isolating a microsomal fraction from the filamentous phytopathogenic fungus *Penicillium italicum* which contains cytochrome P450 isozymes. The study also compares the toxicity of a number of DMIs and two less-toxic imazalil analogues to *P. italicum* and the binding of these compounds to microsomal cytochrome P450 isozymes (Type II binding) and their ease of displacement from the isozymes upon CO treatment.

Materials and methods

Chemicals. Imazalil sulphate (imazalil), imazalil analogues R14821 and R42243, ketoconazole and itraconazole were gifts from Janssen Pharmaceutica (Beerse, Belgium) and penconazole and propiconazole from Ciba Geigy (Basel, Switzerland). NovoZym™ 234 was purchased from Novo Bio Labs (Novo Allé, Bagsvaerd, Denmark); ethylenediaminetetraacetic acid (EDTA), mercaptoethanol (MCE), sodium dithionite and dithiothreitol (DTT) from Sigma (Deisenhofen, Germany); toluenesulfonyl fluoride (TSF) from Janssen Chimica (Beerse, Belgium). TSF was used as a solution in acetone (2 M).

Buffers. Composition of buffers is based on data of Marichal *et al.* (13). Buffer A was a 100 mM potassium phosphate buffer pH 5.8 containing 0.5 M ammonium sulphate; buffer B a 10 mM potassium phosphate buffer pH 7.4 containing 2 M sorbitol; buffer C a 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M sorbitol, 0.1 mM DTT and 0.1 mM EDTA; buffer D a 100 mM potassium phosphate buffer pH 7.4, containing 0.1 mM DTT, 10 mM EDTA and 20% glycerol (v/v); and buffer E a 100 mM phosphate buffer pH 7.4, containing 17% glycerol (v/v).

Fungus, growth conditions and toxicity tests. Wild-type isolate W_5 of *P.italicum* was maintained on malt extract agar plates. Spores were collected from 7 to 10 days old agar plates. Liquid cultures of mycelium were prepared by inoculation of liquid malt medium (100 ml) in flasks (300 ml) with 1 ml spore suspension (10^9 spores ml^{-1}). The flasks were incubated at 25°C on a rotary shaker (200 rpm) for 11 h. The effect of the test compounds on radial growth of the fungus was tested on malt agar pH 7 according to the method described previously (15).

Isolation of microsomal fractions. Mycelium was harvested on a sieve (0.21 mm) and collected on a second sieve (0.038 mm) by washing intensively with tap water. The mycelium was washed with buffer A and the suspension centrifuged at 5,000*g* for 10 minutes. The pellet was resuspended in buffer A containing 0.1% MCE. After centrifugation at 5,000*g* for 10 min, the pellet (10 g wet wt) was resuspended in buffer A (250 ml) in a ratio of 40 mg wet wt ml^{-1} (15 mg dry wt ml^{-1}). NovoZym and TSF were added to the suspension in a flask (300 ml) to final concentrations of 1 mg ml^{-1} and 1 mM, respectively. The suspension was incubated in a reciprocal water bath shaker at 40 strokes per min at 25°C for 30 min. All subsequent steps were carried out at 0 - 4°C. The NovoZym-treated mycelium was centrifuged at 5,000*g* for 5 min and the pellet washed once with buffer A (100 ml). After centrifugation at 5,000*g* for 5 min, the pellet was carefully resuspended in buffer B (30 ml) using a loose fitting teflon pestle to minimize serious damage to the mycelium. Further buffer B was added to a volume of 150 ml. This suspension was allowed to stand for 10 min to permit osmotic equilibration of mycelium and then centrifuged at 5,000*g* for 10 min. The resulting pellet was transferred to a Potter-type homogenizer (B.Braun, Melsungen AG, Germany), which was filled with buffer C to a volume of 70 ml and MCE (90 μl) and TSF (45 μl) added. Disruption of mycelium was carried out by slowly moving the teflon pestle up and down while the pestle was rotating at 100 rpm thereby avoiding any generation of vacuum. After 10 strokes of the pestle, 7 ml of glycerol (88% purity) was added. The pestle was moved up and down for another 10 strokes and further 13 ml of glycerol (88% purity) was added. Hence this final suspension (90 ml) contained 20% glycerol (v/v), 0.1% MCE and 1mM TSF. The disruption of mycelium was completed with a further 10 strokes. When the homogeneous suspension was examined microscopically it appeared that most of the hyphae were disrupted. The homogeneous suspension was successively centrifuged at 7,000*g* for 10 min and at 25,000*g* for 20 min. The resultant milky-like supernatant was filtered through glass wool to remove a floating lipid layer. The filtrate was ultracentrifuged at 125,000*g* for 90 min. After ultracentrifugation two layers of pellets could be observed, a microsomal solid pellet and a microsomal loose pellet "floating" just above the solid pellet. The supernatant was removed by aspiration and the loose pellet collected with a Pasteur pipette without disturbing the solid pellet. The loose pellet was diluted with buffer D containing 0.1% MCE to a volume of 15 ml and ultracentrifuged again at 125,000*g* for 60 minutes. The loose pellet formed after this step was diluted with buffer E (about 5-10 ml), divided into 1 ml samples in Eppendorf centrifuge tubes (1.5 ml), frozen in liquid nitrogen and

stored at -75°C until use. The solid pellet was discarded. The protein content of microsomal samples was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) with bovine γ-globuline as standard.

Estimation of cytochrome P450 content. The cytochrome P450 isozyme content of the isolated microsomal fraction was determined according to the method of Omura and Sato (16). Frozen microsomal fractions were allowed to thaw on ice. Fractions (1 ml) were reduced with sodium dithionite (10 mg) for 30 sec. The sample was centrifuged at 10,000*g* for 1 min and divided between sample and reference cuvettes which were immediately covered with tight rubber plugs and sealed with parafilm. A stable baseline was adjusted and recorded on an Aminco spectrophotometer (DW TM/2a, UV/VIS). The sample was saturated with about 100 bubbles of CO over a 30 sec period. Reduced CO difference spectra were recorded at 20°C at time intervals from 2 min up to 30 min after CO bubbling. The content of cytochrome P450 isozymes was estimated from the absorbance difference between 449 and 490 nm after 5 min of CO bubbling using a molar extinction coefficient of E_M=91,000. The suspension was diluted to 100 pmol per ml with buffer E and used immediately.

Stability of cytochrome P450 at different pH values. Stability of the isolated isozymes with time was tested at pH 7.1, 6.7 and 6.2. The different pH values were obtained by reducing cytochrome P450 samples with different amounts of sodium dithionite, either added in a solid form or dissolved in buffer E (adjusted to pH 7.3 with 10 M KOH). Samples at pH 7.1 were obtained by reduction of 0.9 ml of a microsomal suspension containing 100 pmol cytochrome P450 with 0.1 ml 10% sodium dithionite in buffer E. Samples at pH 6.7 and 6.2 were obtained by reduction of 1.0 ml of microsomal suspension containing 100 pmol cytochrome P450 with 10 mg and 30 mg solid sodium dithionite, respectively. CO difference spectra were recorded as described above.

Type II binding spectra. Microsomal samples (1 ml), containing 100 pmol cytochrome P450 were centrifuged at 10,000*g* for 1 min and divided between sample and reference cuvettes. A baseline was adjusted and recorded. The content of sample cuvette was titrated with 1000 × concentrated solutions of the test compounds (imazalil in water and the other compounds in dimethyl sulfoxide) and the resultant type II difference spectra recorded. Since water and dimethyl sulfoxide did not change the baseline, no corresponding amounts of solvents

were added to the reference cuvette. The final volume of titrated compounds never exceeded 1% of the total volume.

CO displacement. Standard tests were carried out at pH 7.1 and 6.2. Microsomal samples (1 ml or 0.9 ml) containing 100 pmol cytochrome P450 were incubated with test compounds (10^{-6} M) at 20°C for 1 min. Thereafter, the 0.9 ml samples were reduced for 30 sec with 0.1 ml of 10% sodium dithionite in buffer E (pH 7.3) or the 1.0 ml samples with 30 mg of solid sodium dithionite. Then, samples were centrifuged at 10,000*g* for 1 min, divided between sample and reference cuvettes and a baseline was recorded as described before. The content of the sample cuvette was saturated with CO (100 bubbles over 30 sec). The pH values of both cuvettes after measurement were 7.1 and 6.2, respectively. The CO difference spectra were recorded as described above.

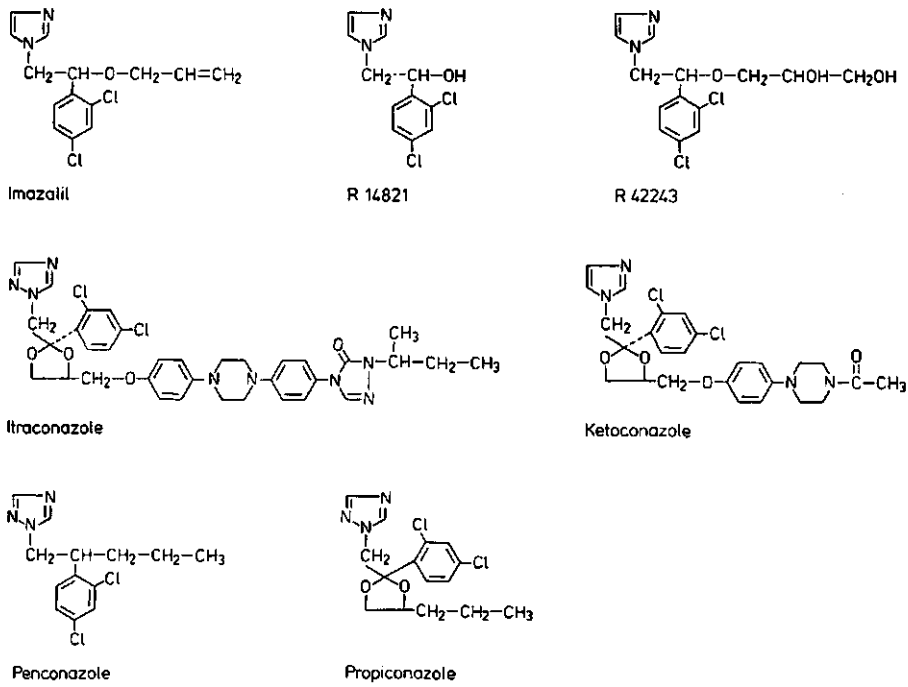

Fig. 1. Chemical structures of DMIs and imazalil analogues R14821 and R42243.

Results

Toxicity studies. The effect of the test compounds (Fig. 1) on radial growth, as determined by EC_{50} values is shown in Table 1. The most toxic azole was imazalil (1.4×10^{-8} M). The imazalil analogues R14821 and R42243 were much less toxic than all other compounds tested.

Table 1. Toxicity of DMIs and imazalil analogues to radial growth of *Penicillium italicum* W_5 on malt extract agar medium pH 7.0.

Compound	$EC_{50} \pm SEM$ (M)[1]
Imazalil	$1.4 \pm 0.2 \times 10^{-8}$
Itraconazole	$6.0 \pm 0.2 \times 10^{-8}$
Ketoconazole	$5.0 \pm 0.4 \times 10^{-8}$
Penconazole	$4.0 \pm 0.2 \times 10^{-8}$
Propiconazole	$2.2 \pm 0.3 \times 10^{-8}$
R14821	$6.6 \pm 0.4 \times 10^{-6}$
R42243	$9.7 \pm 0.4 \times 10^{-5}$

[1] Concentrations which inhibit growth by 50%.

Isolation of cytochrome P450. Microsomes isolated by the standard method showed a reduced CO difference spectrum with a maximum absorbance at 449 nm (pH 7.1). An absorbance peak at 420 nm region was absent (Fig. 2A). The average yield of cytochrome P450 isozymes was 365 ± 33 pmol per g dry weight of mycelium and the maximum yield was 415 pmol. This is equivalent to 95 ± 18 pmol per mg microsomal protein.

Various factors were important for successful isolation. a) A mycelial age of 11 h was optimal. At this age the average length of the germ tubes was about 250 μm. Isolations from younger and older mycelia contained relatively high amounts of denatured form of cytochrome P450. b) The incubation temperature during NovoZym treatment of mycelium was also important. The optimal temperature was 25°C. A higher temperature of 28°C which is optimal for NovoZym cell wall lytic activity enhanced the denaturation of cytochrome P450 isozymes. c) Different batches of NovoZym had different lytic activity. Hence, the optimal

enzyme concentration varied from 1.0 to 1.3 mg of NovoZym per ml standard mycelial suspension. Best cytochrome P450 preparations were obtained when NovoZym treatment resulted in slight lysis of mycelium. At this stage, only few protoplasts were formed. d) A high glycerol content of 20% (v/v) in the disruption buffer was necessary for the formation of the loose pellet which contained the cytochrome P450 isozymes. The detection of cytochrome P450 isozymes in the solid pellet was difficult since they were masked by an abundant amount of cytochrome P420.

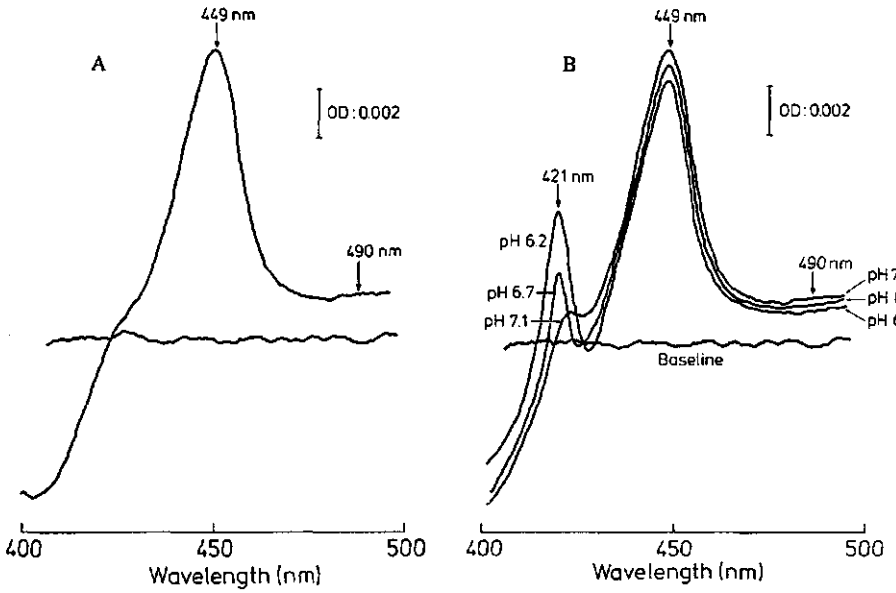

Fig. 2. Effect of pH on the reduced carbon monoxide difference spectra of microsomal cytochrome P450 preparations (10^{-7} M) of *Penicillium italicum* W_5. A. pH 7.1, spectrum recorded 2 min after carbon monoxide treatment. B. pH 7.1, 6.7 and 6.2, spectra recorded 30 min after carbon monoxide treatment.

Stability of cytochrome P450 at different pH. Reduction with sodium dithionite decreased the pH of the microsomal samples and affected the stability of the isozymes. For instance, at pH 6.7 and 6.2 the spectra recorded 2 min after CO saturation of microsomal samples showed a similar maximum absorbance of 449 nm as that observed at pH 7.1 but the absorbance peak at 421 nm was higher (results not shown). Further, after 30 min incubation at 20 °C the sample at

pH 7.1 showed a small increase in absorbance around 421 nm while samples at pH 6.7 and 6.2 showed a much larger increase at this wavelength (Fig. 2B). Increase in absorbance at 421 nm correlated with a slight decrease in absorbance at 449 nm. When the microsomal loose pellet was resuspended in buffer pH 6.4 (100 mM potassium phosphate containing 17% glycerol, v/v) and reduced with 10 mg of sodium dithionite the pH of the samples dropped to 5.9. In this case the reduced CO difference spectrum recorded showed absorbance peaks at 421 and 449 nm of the same heights (result not shown).

Type II spectra. All test compounds, including the two imazalil analogues, interacted with microsomal cytochrome P450 isozymes at pH 7.4 by showing type II difference spectra. Results of a typical titration experiment with imazalil are given in Fig. 3. The maximum absorbance of type II difference spectra of all test compounds was at 430 nm. However, the wavelength for the minimum absorbance varied from one test compound to another (Table 2). Upon titration the absorbance difference between the maximum and minimum of the type II

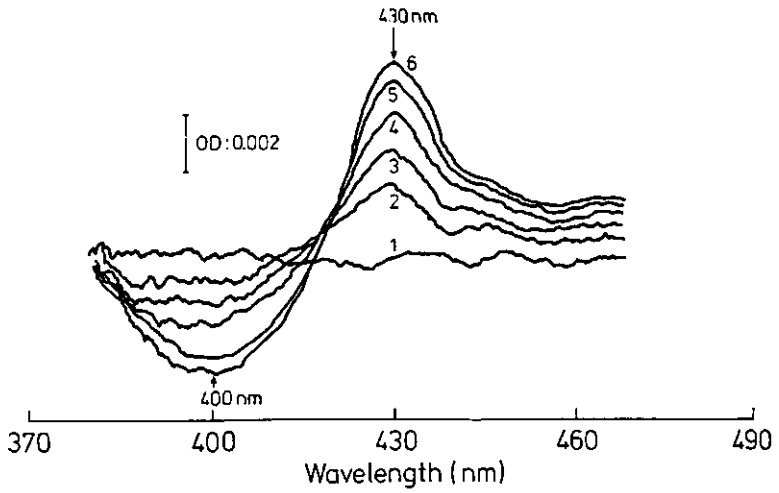

Fig. 3. Type II difference spectra obtained after stepwise addition of imazalil to a microsomal cytochrome P450 isozyme preparation (10^{-7} M) of *Penicillium italicum* W_5 at pH 7.4. 1, baseline; 2, 10^{-8} M; 3, 4×10^{-8} M; 4, 6×10^{-8} M; 5, 10^{-7} M; 6, 2×10^{-6} M.

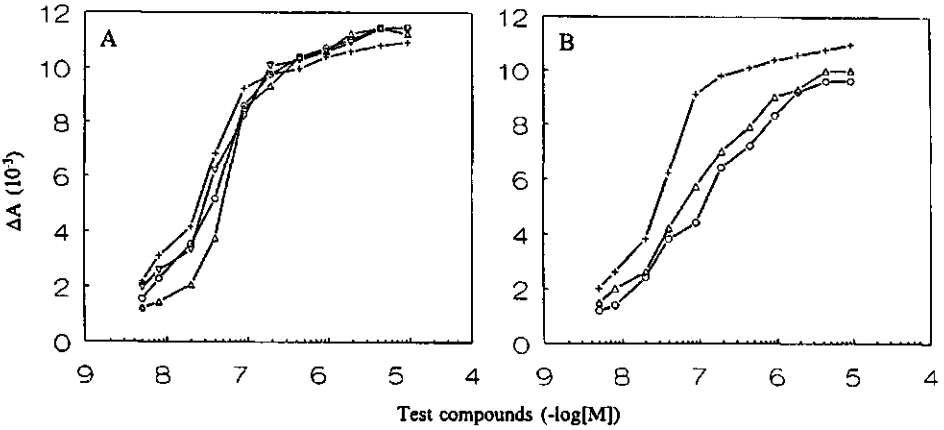

Fig. 4. Effect of concentrations of test compounds on the magnitude of the type II difference spectra of microsomal cytochrome P450 isozymes (10^{-7} M) of *Penicillium italicum* W_5 at pH 7.4. A. Titration curves of itraconazole (+), ketoconazole (△), penconazole (○) and propiconazole (▽); B. Titration curves of imazalil (+), R14821 (△) and R42243 (○). Absorbance difference (ΔA) between maximum and minimum of type II difference spectra plotted against concentration of DMIs and related compounds.

Table 2. Characteristics of type II difference spectra of microsomal cytochrome P450 isozymes (10^{-7} M) of *Penicillium italicum* W_5, titrated with DMIs and related compounds at pH 7.4

Compound	Type II spectra (nm)		$IC_{50} \pm SEM$ (M)[1]
	minimum	maximum	
Imazalil	400	430	$4.0 \pm 1.2 \times 10^{-8}$
Itraconazole	390	430	$3.7 \pm 1.0 \times 10^{-8}$
Ketoconaole	393	430	$5.0 \pm 1.1 \times 10^{-8}$
Penconazole	385	430	$4.2 \pm 1.3 \times 10^{-8}$
Propiconazole	385	430	$4.1 \pm 1.1 \times 10^{-8}$
R14821	390	430	$7.0 \pm 0.8 \times 10^{-8}$
R42243	390	430	$1.2 \pm 0.9 \times 10^{-7}$

[1] Concentrations of the compounds which induced half saturation response in type II difference spectrum.

spectra can be plotted against compound concentration (Fig. 4) and IC_{50} values of the compounds (induced half saturation response in type II spectra) calculated (Table 2). The IC_{50} values for most of the compounds ranged from 3.7×10^{-8} to 7×10^{-8} M, but was relatively lower for R42243 (1.2×10^{-7} M).

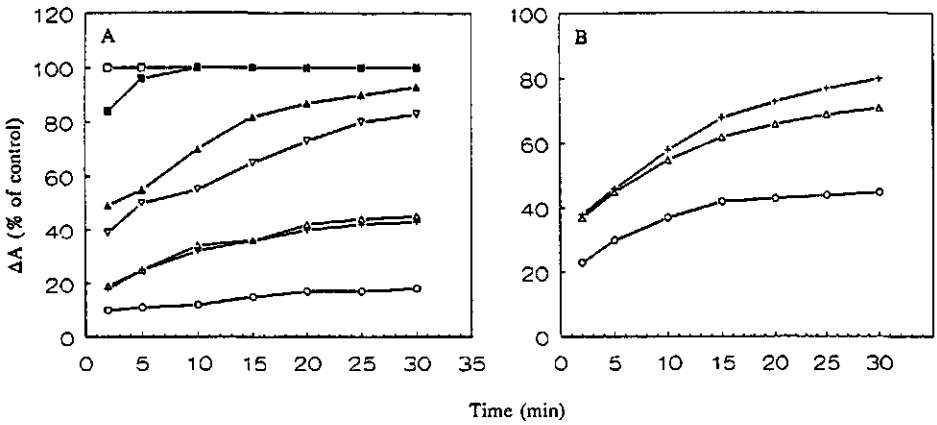

Fig. 5. Displacement of DMIs and related compounds (10^{-6} M) from microsomal cytochrome P450 isozymes (10^{-7} M) of *Penicillium italicum* W_5 at pH 7.1 (A) and pH 6.2 (B) after reduction with sodium dithionite and treatment with carbon monoxide. Absorbance difference (ΔA) between 449 and 490 nm of the reduced carbon monoxide difference spectrum plotted against time after carbon monoxide treatment. Imazalil (+), itraconazole (△), ketoconazole (○), penconazole (▲), propiconazole (▽), R42243 (□) and R14821 (■).

Displacement tests. CO displacement of the DMIs was concentration dependent. At equimolar concentrations of DMIs and cytochrome P450 isozymes (10^{-7} M) most of the DMIs were readily displaced at pH 7.1 (results not shown). Therefore, the experiments were repeated at a concentration of 10^{-6} M (Fig. 5A). Samples pre-incubated with compounds R14821 and R42243 showed rapid displacement by CO. Penconazole and propiconazole were more gradually displaced with time. The same was true for itraconazole and imazalil although to a much slower extent. Ketoconazole was the most difficult DMI to displace. After 30 min of incubation, the average displacement efficiencies (± SEM) for ketoconazole, imazalil, itraconazole, propiconazole, penconazole, R14821 and R42243 were 15 ± 2%, 42 ± 8%, 44 ± 6%, 80 ± 6%, 94 ± 8%, 99 ± 3%

and 101 ± 3%, respectively (n=5). CO displacement of imazalil, itraconazole and ketoconazole was also tested with microsomal samples at pH 6.2 (Fig. 5B). At this pH these DMIs were more readily displaced than at pH 7.1. However, at both pH values ketoconazole remained the most difficult DMI to displace.

Discussion

The cytochrome P450 enzyme involved in sterol 14α-demethylation in microorganisms has been intensively studied only in *C. albicans* and *S. cerevisiae* (2, 5, 8, 10). Recently the isolation of cytochrome P450 isozymes from the filamentous human pathogen, *A. fumigatus*, has also been reported (13, 14). The present work represents the first report of the isolation of cytochrome P450 isozymes from the filamentous plant pathogen *P. italicum*.

Several factors such as mycelial age, incubation temperature during NovoZym treatment and glycerol content of mycelial homogenate were important for the isolation of cytochrome P450 from *P. italicum*. Modification of any of these parameters resulted in either low yield or poor quality of the P450. Symmetry of the reduced CO difference spectrum and absence of absorbance at 420 nm indicate the high quality of the isolated cytochrome P450 isozymes (Fig. 2A). The cytochrome P450 content of the microsomal samples of *P. italicum* was 95 ± 18 pmol mg^{-1} protein. Marichal *et al.*(13) and Ballard *et al.*(14) found in microsomal preparations of *A. fumigatus* a cytochrome P450 content of 40-50 and 19 pmol mg^{-1} protein, respectively. Hence, the content of cytochrome P450 isozymes of *P. italicum* microsomes is relatively high and is, in fact, approaching the cytochrome P450 content in yeast microsomes (17). The high specific content of cytochrome P450 isozymes in *P. italicum* microsomes is probably a consequence of isolating the loose pellet after the ultracentrifugation step. A glycerol content of 20% (v/v) was essential to obtain such a loose pellet. The isolation of the loose pellet can be regarded as a purification step since the solid pellet with a relatively high protein and cytochrome P420 content was discarded.

The stability of cytochrome P450 isozymes decreased at lower pH whereas maximum absorbance of the CO difference spectra remained at the same wavelength (Fig. 2B). This indicates that low pH (6.7 and 6.2) did not affect the heme environment but increased the denaturation of the isozymes. Therefore, spectral analysis was mainly carried out at pH 7.4 (type II binding) and 7.1 (CO displacement tests). These pH values are in the range of pH values essential for

synthesis of sterols in cell-free preparations of *A. fumigatus* (18).

The maximum absorbance of the reduced CO difference spectrum was at 449 nm (Fig. 2). This is different from the maximum absorbance of 451 nm and 448 nm reported for the microsomal cytochrome P450 isozymes of *A. fumigatus* (13, 14) and *C. albicans* (17), respectively. This probably reflects a difference in the heme environment of the P450s from various fungi.

Equimolar concentrations (10^{-7} M) of cytochrome P450 isozymes and DMI fungicides gave almost a saturation response in the type II spectra (Fig. 4A), indicating a close stoichiometric interaction. The less-toxic imazalil analogues R14821 and R42243 did not show such an interaction (Fig. 4B). The IC_{50} values of the DMIs and imazalil analogues did not correlate with their EC_{50} values for fungal radial growth (Table 1 and 2). This suggests that all compounds tested, whether or not toxic, interact with the heme iron atom of cytochrome P450 isozymes. In consequence, the formation of the type II spectrum is not a good criterium for the evaluation of the fungitoxicity of a DMI. The test may only identify much less toxic compounds as was the case for the imazalil analogue R42243 (Table 2, Fig. 4B). These results are in agreement with findings of Yoshida and Aoyama (7) who reported that various azoles interacted stoichiometrically with ferric cytochrome $P450_{14DM}$ purified from *S. cerevisiae* although their inhibitory effect on sterol 14α-demethylation differed from each other. The non-toxic 1-methylimidazole also reacted with cytochrome $P450_{14DM}$ but not stoichiometrically. The most potent isomer of triadimenol also bound almost stoichiometrically, while the less potent isomers did not (9).

Studies with purified cytochrome $P450_{14DM}$ from *S. cerevisiae* (6, 7, 9) and microsomal cytochrome P450 isozymes from *C. albicans* (2, 4, 5, 10, 17, 19) suggest that although the interaction of the azole moiety of DMIs with the heme iron of cytochrome $P450_{14DM}$ is necessary for fungitoxicity, the binding affinity to the enzyme is largely dependent upon the *N*-1 substituent of the compound. This can be investigated by studying the displacement of DMIs from reduced cytochrome P450-DMI complexes by CO. The work of Yoshida and Aoyama (6, 7) indicates that the difference in inhibitory effect of itraconazole, ketoconazole and triadimefon on sterol 14α-demethylation correlated with the ability of CO to displace these DMIs from cytochrome $P450_{14DM}$. Yoshida and Aoyama (9) also reported that it was more difficult for CO to displace the most potent isomer of triadimenol than other less potent isomers. Vanden Bossche *et al.* (4) did the same type of study by using microsomal cytochrome P450 isozymes from *C. albicans* and found that itraconazole, which was more difficult to displace by CO than ketoconazole, had higher inhibitory effect on sterol

14α-demethylation than ketoconazole. Therefore, it is suggested that the ability of CO to displace DMIs from cytochrome $P450_{14DM}$ is a good parameter to evaluate candidate DMI fungicides (2, 5, 6, 7).

Results of CO displacement tests indicated that binding affinity of DMIs at pH 7.1 is higher than at pH 6.2 (Fig. 5). Therefore, a comparison of the rate of CO displacement of test compounds was mainly carried out at pH 7.1. Results indicated that the CO displacement of the test compounds with a relatively small *N*-1 substituent did correlate with their fungitoxicity (Table 1, Fig. 5A), suggesting that the binding affinity of DMIs to the apoprotein of cytochrome P450 isozymes was at least partly responsible for the toxicity of these compounds. The situation for DMIs with a large *N*-1 substituent was different. Ketoconazole was displaced at a slower rate than imazalil although being less toxic. Itraconazole, which was also less toxic than imazalil, showed a similar displacement rate as imazalil. This was observed at both pH 7.1 and 6.2 (Fig. 5). Lack of correlation between the ease of DMI displacement by CO and DMI toxicity has also been observed for itraconazole and ketoconazole, and for itraconazole and fluconazole with microsomal cytochrome P450 isozymes from *A. fumigatus*, respectively (13, 14). An explanation for such discrepancies may be that microsomes contain different types of P450s varying in affinity to some of the DMIs tested. The affinity of cytochrome $P450_{14DM}$ can be masked by other isozymes with high affinity to the DMIs (20). An alternative explanation is that low level of accumulation, slow transport to the target site or higher rate of metabolic breakdown may account for the relatively low toxicity of ketoconazole and itraconazole.

Displacement of ketoconazole by CO from cytochrome P450 isozymes of *P. italicum* was slower than that of itraconazole (Fig. 5). With *C. albicans* it was just the opposite (4). This suggests a difference in amino acid sequence of the binding site of the apoprotein of cytochrome $P450_{14DM}$ in both organisms. Other explanations in terms of nature and number of the cytochrome P450 isozymes in both organisms may also be relevant.

A better judgement of the relation between DMI toxicity and affinity to cytochrome $P450_{14DM}$ can be obtained by conducting CO displacement tests with purified enzyme or by measuring the inhibitory effect of DMIs on sterol 14α-demethylation activity. The latter topic is the subject of current work.

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CHAPTER 6

Interaction of microsomal cytochrome P450 isozymes isolated from wild-type and DMI-resistant isolates of *Penicillium italicum* with DMI fungicides

J. Guan and M. A. de Waard

Department of Phytopathology, Wageningen Agricultural University,
P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

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Abstract

Microsomal cytochrome P450 isozymes were isolated from isolates of *Penicillium italicum* with various degrees of resistance to fungicides which inhibit sterol 14α-demethylation (DMIs). The yield of P450 isozymes isolated from the low-resistant isolate $E_{300\text{-}3}$ was similar as that from wild-type isolate W_5 (365 pmol g^{-1} dry weight mycelium) whereas the yield from medium- and high-resistant isolates H_{17}, I_{33} and J_4 was significantly lower (285, 301 and 223 pmol g^{-1} dry weight, respectively). The specific content of P450 isozymes per mg microsomal protein from isolates H_{17}, I_{33} and J_4 was also relatively low (50, 52 and 34 pmol mg^{-1} protein, respectively) compared with that of isolate W_5 (95 pmol mg^{-1} protein). This may be an intrinsic characteristic of these isolates, but can also be a consequence of slight modifications in the standard isolation procedure as described for isolate W_5. Maximum absorbance of the reduced carbon monoxide (CO) difference spectrum of P450 isozymes of isolate $E_{300\text{-}3}$ was at 449 nm, which is identical to that of isolate W_5. Isolates H_{17} and I_{33} had a maximum absorbance at 450 nm and isolate J_4 at 452 nm, respectively. Stability of reduced cytochrome P450 isozymes in isolates H_{17}, I_{33} and J_4 was lower than that in isolates W_5 and $E_{300\text{-}3}$. The oxidized form of the P450 isozymes of all DMI-resistant isolates interacted at equimolar concentration (10^{-7} M) with imazalil (type II binding). The affinity of the heme

iron in isolates H_{17}, I_{33} and J_4 to imazalil was slightly lower than for isolates W_5 and $E_{300\text{-}3}$ but can not account for the mechanism of resistance. In CO displacement tests imazalil, itraconazole and ketoconazole were more readily displaced from P450 isozymes of isolates H_{17}, I_{33} and J_4 than from isozymes of isolates W_5 and $E_{300\text{-}3}$. These results suggest that one of the cytochrome P450 isozymes from medium- and high-resistant isolates, possibly cytochrome $P450_{14DM}$, may have an altered apoprotein, resulting in decreased affinity to DMIs. However, this conclusion should be regarded with caution because of possible differences in quality of microsomal P450 preparations of the isolates (e.g. protein content, stability, cytochrome P450 composition and contamination with cytochrome oxidase) between wild-type and medium- and high-resistant isolates. These differences may affect the CO-displacement rate and therefore hamper a proper comparison of affinity of cytochrome $P450_{14DM}$ to DMIs.

Introduction

Sterol demethylation inhibitors (DMIs) are systemic fungicides which specifically inhibit cytochrome P450-dependent sterol 14α-demethylase (cytochrome $P450_{14DM}$) activity in ergosterol biosynthesis in fungi (1, 2). Various mechanisms of resistance to DMIs have been described (3-7). Resistance based on changes of the site of action of DMIs, cytochrome $P450_{14DM}$, has only been studied for some budding fungi (*Candida albicans, Ustilago maydis* and *Saccharomyces cerevisiae*). Various types of changes at the site of action have been described. First of all resistance may be due to a lack of target enzyme. This has been reported for mutants of *C. albicans* with resistance to polyene antibiotics. These mutants show cross resistance to DMIs (8). A second possibility is that the enzyme is present but non-functional. This has been reported for mutants of *S. cerevisiae* resistant to both polyene antibiotics and DMIs (9). A third possibility is that mutations which cause resistance to DMIs, do not or hardly affect the catalytic activity of the enzyme but only reduce affinity of the enzyme to DMI fungicides. This mechanism of resistance has been suggested for certain mutants of *C. albicans* (10, 11). The first two mechanisms mentioned above do not seem to be relevant for filamentous fungi since DMI-resistant isolates of *Aspergillus nidulans* (12), *Botrytis cinerea* (13), *Cladosporium cucumerinum* (14), *Cercospora beticola* (15), *Penicillium italicum* (16) and *Rhynchosporium secalis* (17) contain ergosterol as the major sterol. The same statement holds true for DMI-resistant isolates of powdery mildews such as *Erysiphe graminis* f. sp. *hordei*, *Sphaerotheca fuliginea* and *Podosphaera*

leucotricha which form another related desmethyl sterol, ergosta-5,24(28)-dienol (18). However, the third mechanism may still be relevant. Studies on the affinity of cytochrome P450 isozymes to DMIs may elucidate the validity of this hypothesis. So far, these studies were not carried out due to difficulties in obtaining microsomal fractions containing proper cytochrome P450 isozymes.

Recently, a method for the isolation of microsomal cytochrome P450 isozymes from the filamentous plant pathogen *Penicillium italicum* has been developed (19). The present paper describes the isolation of these isozymes from DMI-resistant isolates of the fungus. Spectrophotometric studies were conducted to compare type II and reduced CO difference spectra. In addition, the CO displacement rates of imazalil, itraconazole and ketoconazole from isozymes of the wild-type isolate and isolates with various degrees of resistance to DMIs were compared.

Materials and methods

Fungus and toxicity tests. *P. italicum* wild-type isolate W_5 and isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 with increasing degrees of resistance to DMIs were used (20, 21). The isolates were maintained on malt extract agar. Toxicity of imazalil sulphate (imazalil), itraconazole and ketoconazole (gifts from Janssen Pharmaceutica, Beerse, Belgium) to various isolates was tested on malt extract agar medium pH 7 according to methods described previously (16). Imazalil was used as a 1000× concentrated solution in water and itraconazole and ketoconazole in dimethyl sulfoxide.

Isolation of microsomal fractions. Microsomal fractions of *P. italicum* isolates were obtained according to the method described previously for the wild-type isolate (19). In order to obtain P450 isozymes from DMI-resistant isolates with a quality comparable as much as possible to that of the wild-type isolate, the following modifications in the method were made. a) The growth period of isolate J_4 was 16 h instead of 11 h; b) The amount of NovoZym used for the lytic treatment of mycelia of isolates H_{17} and I_{33} was doubled (2 mg ml^{-1} standard mycelial suspension); c) During disruption of mycelium of isolates H_{17}, I_{33} and J_4 the number of strokes with the teflon pestle of the Potter-type homogenizer was reduced to 15 instead of 30 strokes for wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$. After the first and the second 5 strokes, 7 and 13 ml of glycerol (88% purity) were added, respectively. The final glycerol concentration remained the same as that for wild-type (20%, v/v).

Spectrophotometric studies. Spectrophotometric studies of cytochrome P450 isozymes were carried out according to the method described previously for the wild-type isolate (19). Reduced CO difference spectra were recorded at pH 7.1. Microsomal fractions (pH 7.4), containing 100 pmol cytochrome P450 isozymes ml^{-1} from DMI-resistant isolates were titrated with imazalil. The resulting type II difference spectra were recorded. CO displacement of imazalil, itraconazole and ketoconazole from the P450 isozymes were carried out at pH 7.1. Protein content of microsomal samples were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Veenendaal, the Netherlands) with bovine γ-globulin as standard.

Results

Properties of cytochrome P450 isozymes of DMI-resistant isolates. Reduced microsomal fractions of DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 showed CO difference spectra (Fig. 1A). The maximum absorbance of the spectrum for low resistant isolate $E_{300\text{-}3}$ was at 449 nm. This is identical to that of the wild-type isolate W_5. The maximum absorbance for isolates H_{17} and I_{33} was at 450 nm and for isolate J_4 at 452 nm. The spectra of isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 showed a second absorbance maximum in the 420 nm region. The size of the absorbance peak increased in the order of $E_{300\text{-}3}$, H_{17}, I_{33} and J_4. Upon prolonged incubation at 20°C the maximum absorbance at 450 nm decreased in time while absorbance at 420 nm increased. Absorbance increase at 420 nm was much larger than absorbance decrease at 450 nm (Fig. 2). The changes in absorbance were much more significant in the spectra of isolates H_{17}, I_{33} and J_4 than in those of isolates W_5 and $E_{300\text{-}3}$ and hence seemed inversely correlated with the degree of resistance.

The yield of P450 isozymes isolated from isolate $E_{300\text{-}3}$ was similar to that of isolate W_5, while the yield from isolates H_{17}, I_{33} and J_4 was significantly lower (Table 1). Similarly, the specific P450 content mg^{-1} protein of the microsomal fractions of isolate $E_{300\text{-}3}$ was nearly the same as that of isolate W_5 and much lower for isolates H_{17}, I_{33} and J_4. The specific P450 content of isolate J_4 was the lowest.

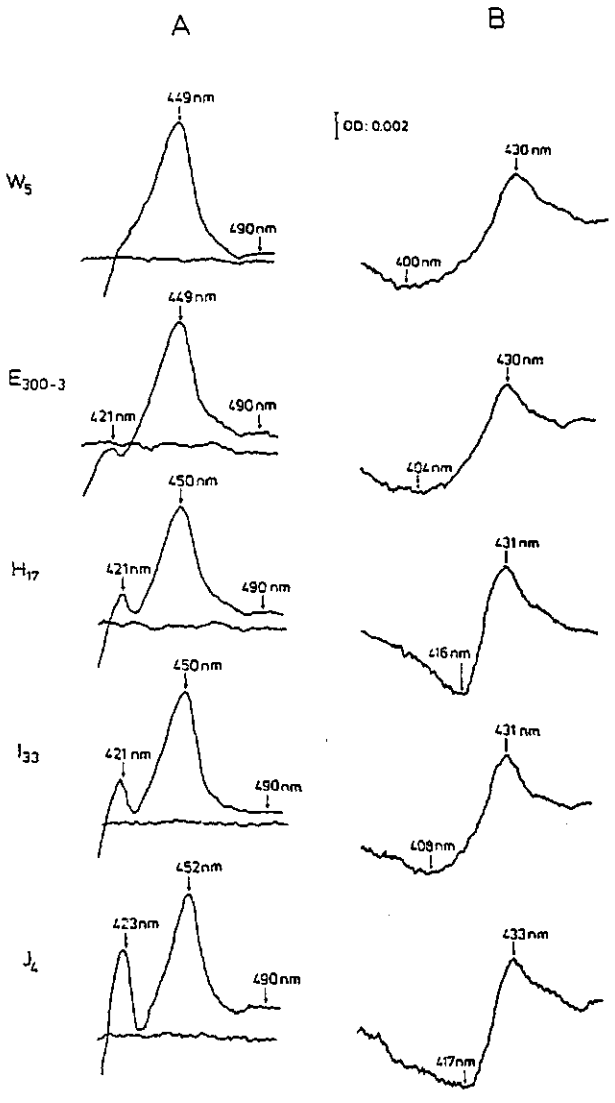

Fig. 1. A. Reduced carbon monoxide difference spectra of microsomal cytochrome P450 isozymes from wild-type isolate W_5 and DMI-resistant isolates E_{300-3}, H_{17}, I_{33} and J_4 of *Penicillium italicum* at pH 7.1. Spectra were recorded 2 min after CO treatment. B. Type II difference spectra obtained upon addition of imazalil (10^{-7} M) to microsomal cytochrome P450 isozymes (10^{-7} M) from wild-type isolate W_5 and DMI-resistant isolates E_{300-3}, H_{17}, I_{33} and J_4 of *Penicillium italicum* at pH 7.4.

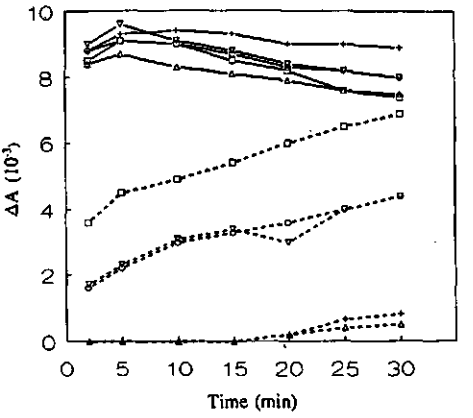

Fig. 2. Changes in absorbance of the reduced CO difference spectra of microsomal cytochrome P450 isozymes from wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○), I_{33} (▽), and J_4 (□) of *Penicillium italicum* at pH 7.1 during incubation at 20°C in time. ΔA is the difference in absorbance between maximum at 449-452 and 490 nm (——), and between maximum at 421-423 and minimum at 427-430 nm (----). Compare spectra in Fig. 1A.

Table 1. Yield of microsomal cytochrome P450 isozymes isolated from wild-type W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *Penicillium italicum* and cytochrome P450 content in these microsomal fractions.

Isolate	Yield[1]	P450[2]
W_5	365 ± 33	95 ± 18
$E_{300\text{-}3}$	350 ± 25	88 ± 12
H_{17}	285 ± 20[3]	50 ± 8[3]
I_{33}	301 ± 13[3]	52 ± 11[3]
J_4	223 ± 26[3]	34 ± 9[3]

[1] Pmol P450 per g dry weight of mycelium and standard deviation (n = 5).

[2] Pmol per mg microsomal protein and standard deviation (n = 5).

[3] Significantly different from W_5 (P = 0.05).

Various modifications of the standard isolation procedure were necessary for successful isolation of cytochrome P450 isozymes from DMI-resistant isolates. Growth of isolate J_4 in liquid cultures was much slower. Therefore, the growth period of isolate J_4 was extended to 16 h in order to obtain germlings with the same length of germ tubes as those of isolate W_5 (250 μm). The lytic activity of NovoZym (1 mg ml^{-1} mycelial suspension) was much lower for mycelium of isolates H_{17} and I_{33} than for isolates W_5, $E_{300\text{-}3}$ and J_4. Microsomal fractions of isolates H_{17} and I_{33} obtained after such an ineffective lytic treatment showed a high absorbance in the 420 nm region in the CO difference spectrum (results not shown). Incubation of mycelia of isolates H_{17} and I_{33} with NovoZym at a concentration of 2 mg ml^{-1} minimized this problem. A similar problem occurred for isolates H_{17}, I_{33} and J_4 with the standard disruption procedure. A more gentle disruption of mycelium in the Potter-type homogenizer with a total of 15 strokes did minimize this problem. After ultracentrifugation of the cell-free extracts of isolates H_{17}, I_{33} and J_4 the formation of a distinct loose pellet on top of a solid pellet as observed with isolates W_5 and $E_{300\text{-}3}$ was not clear. Still, only the top layer of the pellet was collected since this layer contained a relatively low protein content and showed a relatively low absorbance in the 420 nm region in the CO difference spectrum compared with that of the bottom layer.

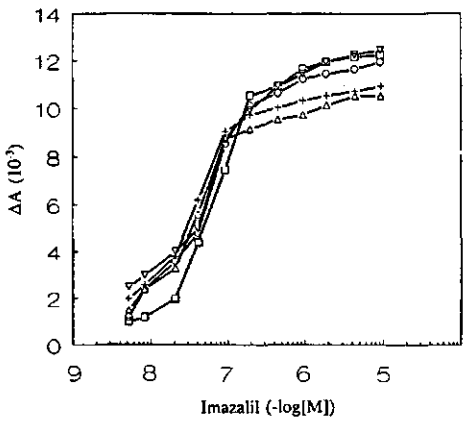

Fig. 3. Relation between imazalil concentrations and the magnitude of type II difference spectra of microsomal cytochrome P450 isozymes (10^{-7} M) from wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○), I_{33} (▽), and J_4 (□) of *Penicillium italicum* at pH 7.4. The magnitude of type II difference spectra (ΔA) is the difference between its maximum (430-433 nm) and minimum (400-417 nm). Compare spectra in Fig. 1B.

Type II binding. Oxidized cytochrome P450 isozymes from all DMI-resistant isolates interacted with imazalil (10^{-7} M) at pH 7.4 resulting in type II difference spectra. Maximum and minimum absorbance of the spectrum for isolate $E_{300\text{-}3}$ were at wavelengths close to that of the wild-type isolate W_5 while those for isolates H_{17}, I_{33} and J_4 shifted to higher wavelengths (Fig. 1B). Concentrations of imazalil necessary for inducing a saturation response in type II difference spectra for isolates H_{17}, I_{33} and J_4 were slightly higher than for isolates W_5 and $E_{300\text{-}3}$ (Fig. 3). IC_{50} values of imazalil, inducing a half saturation response in the type II difference spectra for isolates H_{17}, I_{33} and J_4 were slightly higher than that of isolates W_5 and $E_{300\text{-}3}$ (Table 2).

Table 2. IC_{50} values of imazalil which induced half of the saturation response in type II difference spectra of cytochrome P450 isozymes from wild-type isolate W_6 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *Penicillium italicum* at pH 7.4. Cytochrome P450 content was 100 pmol ml^{-1}.

Isolate	$IC_{50} \pm$ SEM (M)[1]
W_5	$4.0 \pm 1.2 \times 10^{-8}$
$E_{300\text{-}3}$	$4.2 \pm 2.1 \times 10^{-8}$
H_{17}	$5.1 \pm 2.7 \times 10^{-8}$ [2]
I_{33}	$5.8 \pm 2.3 \times 10^{-8}$ [2]
J_4	$8.0 \pm 3.2 \times 10^{-8}$ [2]

[1] Mean concentration of imazalil which induces half saturation response in type II difference spectrum and standard deviation (n = 5).

[2] Significantly different from W_6 (P = 0.05).

CO displacement. CO displacement of imazalil, itraconazole and ketoconazole from cytochrome P450 isozymes of different isolates was carried out at pH 7.1 after incubation of the oxidized form of the isozymes (10^{-7} M) with the fungicides at a concentration of 10^{-6} M and reduction with sodium dithionite. This concentration was chosen since at a lower concentration (10^{-7} M) imazalil and itraconazole were almost instantaneously displaced by CO and no differences in displacement rate between isolates could be observed (results not shown). CO displacement of imazalil from isozymes of isolate $E_{300\text{-}3}$ was at a similar rate as that of the wild-type isolate W_5. CO displacement of imazalil from isozymes of

isolates H_{17}, I_{33} and J_4 occurred at a significantly higher rate than that of isolate W_5 (Fig. 4A). A similar increase in CO displacement rate was also observed for itraconazole and ketoconazole (Fig. 4B and C). For all isolates ketoconazole was less readily displaced by CO than imazalil and itraconazole. The mean displacement efficiency of the compounds from the P450 isozymes of different isolates 30 min after CO treatment is shown in Table 3. In these experiments it was noticed that the fungicides could enhance absorbance at around 420 nm as compared to control treatments (Table 4). The effect was generally only significant for isolates W_5 and $E_{300\text{-}3}$.

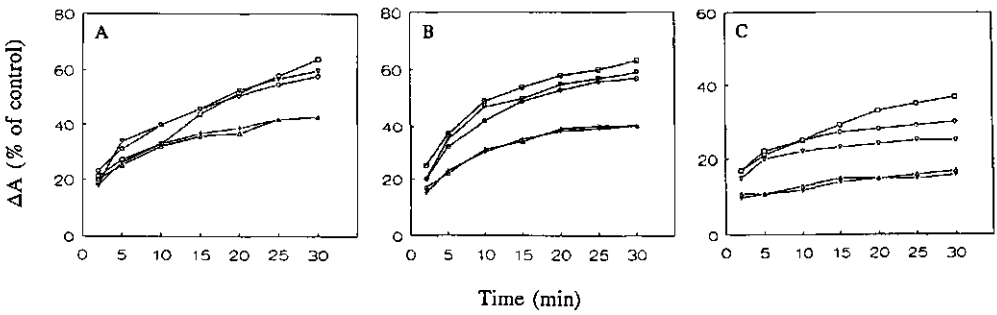

Fig. 4. Carbon monoxide displacement of imazalil (A), itraconazole (B) and ketoconazole (C) from microsomal cytochrome P450 isozymes of wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○), I_{33} (▽) and J_4 (□) of *Penicillium italicum* at pH 7.1. ΔA is the difference in absorbance between maximum at 449-452 and 490 nm of the CO difference spectrum given as a percentage of controls after CO treatment in time.

Cross resistance. DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 showed in general increasing levels of resistance to imazalil, itraconazole and ketoconazole (Table 5). The resistance level to ketoconazole was highest in all isolates. Imazalil was the most toxic DMI tested.

Table 3. CO displacement efficiency of imazalil, itraconazole and ketoconazole (10^{-6} M) from cytochrome P450 isozymes (10^{-7} M) of wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17},I_{33} and J_4 of *Penicillium italicum* 30 min after CO treatment at pH 7.1.

Isolate	CO displacement ± SEM (% of control)[1]		
	Imazalil	Itraconazole	Ketoconazole
W_5	43 ± 6	40 ± 5	16 ± 5
$E_{300\text{-}3}$	43 ± 9	39 ± 5	17 ± 4
H_{17}	58 ± 9[2]	55 ± 7[2]	30 ± 7[2]
I_{33}	60 ± 8[2]	59 ± 5[2]	25 ± 4[2]
J_4	64 ± 11[2]	63 ± 9[2]	37 ± 6[2]

[1] Mean values and standard deviation (n = 5).
[2] Significantly different from W_5 (P = 0.05).

Table 4. Effect of imazalil, itraconazole and ketoconazole on absorbance in 420 nm region of CO difference spectra of P450 isozymes of wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *Penicillium italicum* 30 min after incubation with the fungicide.

Fungicide	Absorbance difference (10^{-3})[1,2]				
	W_5	$E_{300\text{-}3}$	H_{17}	I_{33}	J_4
Control	0.6 ± 0.5	0.5 ± 0.1	2.4 ± 2.0	4.0 ± 0.7	6.8 ± 0.9
Imazalil	1.7 ± 0.3[3]	2.1 ± 0.8[3]	3.7 ± 2.1	6.0 ± 1.1	7.0 ± 2.0
Itraconazole	1.6 ± 0.8[3]	1.8 ± 0.3[3]	2.5 ± 1.4	4.3 ± 0.4	nd[4]
Ketoconazole	1.3 ± 0.3[3]	1.6 ± 0.3[3]	3.4 ± 1.5	6.1 ± 4.2[3]	5.9 ± 0.4

[1] Difference in absorbance between the maximum at 421-423 nm and the minimum at 427-430 nm (Fig. 1A).
[2] Mean values and standard deviation (n = 3).
[3] Significantly different from W_5 (P = 0.05).
[4] Not determined.

Table 5. Sensitivity of *Penicillium italicum* isolates W_6, $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 to imazalil, itraconazole and ketoconazole in radial growth tests on malt agar.

Isolate	EC_{50} (M)[1]		
	Imazalil	Itraconazole	Ketoconazole
W_5	1.4×10^{-8}	6.0×10^{-8}	5.0×10^{-8}
$E_{300\text{-}3}$	4.5×10^{-8} (3)[2]	5.0×10^{-7} (8)	6.8×10^{-7} (14)
H_{17}	6.3×10^{-7} (45)	1.0×10^{-6} (17)	4.2×10^{-6} (84)
I_{33}	8.3×10^{-7} (59)	1.3×10^{-6} (22)	4.8×10^{-6} (96)
J_4	5.8×10^{-7} (41)	2.5×10^{-6} (41)	5.7×10^{-6} (114)

[1] Concentration of DMIs which inhibits growth for 50%.
[2] Data in brackets: degree of resistance.

Discussion

Cytochrome P450 isozymes could be demonstrated in microsomal fractions of all DMI-resistant isolates of *P. italicum*. The isolation procedure for P450 isozymes from these isolates differed slightly from that described for the wild-type isolate (19). Therefore, differences in quality of microsomal fractions with respect to protein content, stability, P450 composition and contamination with cytochrome oxidase may be a consequence of the changed isolation procedure. However, the fact that the standard isolation procedure had to be modified for resistant isolates H_{17}, I_{33} and J_4 is in itself an indication that these resistant isolates may differ in cell wall, membrane and mitochondrial composition compared to the wild-type isolate. The results can not be ascribed to differences in sterol composition of these isolates since they all contain similar sterols (16). Several lines of evidence suggest that the quality of the microsomal fractions containing cytochrome P450 isozymes is inversely correlated with the degree of resistance in these isolates. This regards the specific P450 content of the microsomal fractions (Table 1) and the stability of the isozymes. Resistant isolates showed a higher conversion of P450 into P420 isozymes (Fig. 1 and 2). This conversion is generally regarded as a denaturation process (22). It is of interest to note that the fungicides tested, significantly enhanced the formation of P420 in microsomal fractions of isolates W_5 and $E_{300\text{-}3}$ while such an effect for isolates H_{17} and J_4 was generally not obvious (Table 4). A similar effect of DMI fungicides was observed with

cytochrome $P450_{SG1}$ purified from demethylation defective mutant of *S. cerevisiae* (9). The reason for this effect is not known.

The maximum absorbance of CO difference spectra of P450 isozymes of isolates H_{17}, I_{33} and J_4 showed a shift to higher wavelengths (Fig. 1A). This shift is not related to a deficiency in sterol 14α-demethylation since these isolates do synthesize ergosterol (16). In addition, mutations leading to deficiency in sterol 14α-demethylation often caused a shift in the maximum absorbance of the CO difference spectrum to lower wavelengths (9, 22). Cytochrome P450 isozymes of DMI-resistant mutants of *C. albicans* which retained the capacity to synthesize ergosterol, showed a shift in the maximum absorbance of the CO difference spectrum to a higher wavelength. The mutants were suggested to have an altered apoprotein of one of the major isozymes (probably cytochrome $P450_{14DM}$) which resulted in reduced affinity to DMIs (10, 11). Since similar spectral changes were observed for P450 isozymes of isolates H_{17}, I_{33} and J_4 of *P. italicum,* the same explanation may also be applicable. An alternative explanation for the shift of the maximum in CO difference spectrum of P450 isozymes may be the presence of cytochrome oxidase which often interferes with spectral analysis (23). Cytochrome oxidase of *P. italicum* has an absorbance maximum and minimum at 430 and 446 nm, respectively (result not shown). It is clear that the presence of a high amount of cytochrome oxidase in the microsomal fractions will not only shift the maximum absorbance of the CO difference spectrum to a higher wavelength but will also result in an asymmetric absorbance peak. However, the CO spectra obtained (Fig. 1A) were all symmetric and therefore a major contamination of the microsomal fractions with cytochrome oxidase was probably not the case. Still, a low amount of cytochrome oxidase may have been present in microsomal fractions of isolates H_{17}, I_{33} and J_4. This is because in type II binding studies, the absorbance difference at saturation concentrations of imazalil for isolates H_{17}, I_{33} and J_4 was higher than for isolates W_5 and $E_{300\text{-}3}$ (Fig. 3). IC_{50} values of imazalil in type II spectra for resistant isolates H_{17}, I_{33} and J_4 were also higher than for wild-type isolate W_5. The difference was less than two-fold (Table 2). Therefore, the difference in affinity of the heme iron to imazalil can probably not account for the high level of resistance to the test compounds (Table 5). Maximum and minimum in the type II dif-ference spectra of P450 isozymes of resistant isolates H_{17}, I_{33} and J_4 shifted to slightly higher wavelengths (Fig. 1B). This may be related to an alteration in the configuration of the heme environment (9, 14), and account for the slightly higher IC_{50} values.

Since binding of the heterocyclic nitrogen of DMIs to the heme iron of cytochrome P450 isozymes does not play a major role in differential toxicity (Table 2), the affinity of the *N*1-substituent of the fungicides to the apoprotein of the isozymes may be more relevant. Therefore, CO displacement tests may give more

valid information in this respect (2, 25, 26). The results of the tests showed no difference in CO displacement rate for wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$ (Fig. 4). Therefore, it may be concluded that the mechanism of resistance in isolate $E_{300\text{-}3}$ is not due to decreased affinity of the apoprotein of any of the P450 isozymes. This is in agreement with the hypothesis that resistance in this isolate is caused by an increased energy-dependent efflux of DMIs (4, 27). The relatively high CO displacement rate of the test fungicides from cytochrome P450 isozymes of isolates H_{17}, I_{33} and J_4 suggest that the mechanism of resistance in these isolates may be due to decreased affinity of one of the major P450 isozymes (probably cytochrome $P450_{14DM}$). These results may be in line with the changes in spectral characteristics of the CO and type II difference spectra (Fig. 1). However, this conclusion should be regarded with caution for the following reasons. First of all, one should realize that the displacement tests reflect the additive affinity of all the isozymes present in the microsomal fractions to the test fungicides and the target enzyme cytochrome $P450_{14DM}$ is only one of them. Therefore, the affinity of $P450_{14DM}$ to DMIs can be masked by other P450 isozymes with different affinity to DMIs (28). Secondly, P450 isozymes of isolates H_{17}, I_{33} and J_4 were less stable than that of isolate W_5 (Fig. 2). It is not known which types of P450 isozymes did denature during the CO-displacement tests. Thirdly, the P450 content of isolates H_{17}, I_{33} and J_4 may be underestimated due to the presence of a trace amount of cytochrome oxidase in the microsomal fractions. In consequence, the ratio between P450 isozymes and the fungicides may be relatively high and result in a faster CO displacement rate. Furthermore, the relatively high protein content of microsomal fractions of isolates H_{17}, I_{33} and J_4 (Table 1) may increase aspecific partition of the DMIs into the microsomal phase. This can result in less DMIs available for binding to P450 isozymes (29) and cause a higher CO displacement rate. All these factors complicate a direct comparison of the results from CO displacement studies. Therefore, a definite conclusion in this respect should be drawn from CO displacement tests with purified cytochrome $P450_{14DM}$ or from tests in which the *in vitro* sensitivity of sterol 14α-demethylase to DMIs is determined.

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CHAPTER 7

Metabolism of imazalil by wild-type and DMI-resistant isolates of *Penicillium italicum*

J. Guan[1], L. Van Leemput[2] and M. A. de Waard[1]

[1]Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

[2]Janssen Pharmaceutica, Turnhoutseweg 30, B-2340 Beerse, Belgium.

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Abstract

Metabolism of imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole) in *Penicillium italicum* isolates with a wild-type sensitivity and with various degrees of resistance to sterol demethylation inhibitors was studied in liquid cultures. A putative metabolite, 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243), was detected in the culture filtrate after prolonged incubation. The metabolism occurred in the propenyl side chain of imazalil probably through epoxidation and hydratation. This is the first report of such a conversion of imazalil in fungi. R42243 was much less toxic to *P. italicum* than imazalil. Therefore, the metabolism can be regarded as a detoxification step. Both wild-type and resistant isolates

metabolized imazalil, but metabolism by resistant isolates was faster than by the wild-type isolate. This is probably caused by a relatively strong inhibition of growth of the wild-type isolate by the fungicide. Results indicate that the detoxification of imazalil does not operate as a mechanism of resistance. This conclusion was confirmed by the fact that resistant isolates showed cross-resistance to miconazole and R42243, which had a similar structure as imazalil except for the propenyl side chain.

Additional keywords: DMIs, detoxification, mechanism of resistance, metabolism, miconazole

Introduction

Differential metabolism can either be responsible for selective fungitoxic action between target and non-target fungi or for acquired resistance in a specific target organism (Lyr, 1987; Dekker, 1987). This statement also holds true for fungicides which inhibit sterol 14α-demethylation (DMIs) in ergosterol biosynthesis. For instance, detoxification of triforine was supposed to be responsible for insensitivity of *Aspergillus niger, Colletotrichum atramentarium* and *Stemphylium radicinum* (Gasztonyi and Josepvits, 1975). Insensitivity to triadimefon of *Stemphylium radicinum* and *Saccharomyces cerevisiae* (Gasztonyi and Josepovits, 1984), *A. niger* (Deas and Clifford, 1981) and some others fungi (Fuchs, 1988) can be due to a reduced rate of activation into triadimenol of which certain stereoisomers have a relatively higher fungitoxicity. So far, resistance based on metabolism of DMIs has only been observed in resistant strains of *Cladosporium cucumerinum* (Fuchs and De Vries, 1984) and *Nectria haematococca* (Kalamarakis *et al.*, 1986). The resistant strains of the fungi showed a slower rate of transformation of triadimefon to more toxic isomers of triademenol than the sensitive strains.

The aim of the present work was to investigate whether *Penicillium italicum* isolates with a wild-type sensitivity and with resistance to DMIs metabolize imazalil and if so, whether the metabolism operates as a mechanism of resistance.

Materials and methods

Fungal isolates, culture methods and toxicity tests. Wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *P. italicum* were used in the experiments (De Waard *et al.*, 1982; De Waard and Van Nistelrooy, 1990). The

fungus was maintained on malt extract agar medium. Toxicity of imazalil, 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243) and miconazole (gifts from Janssen Pharmaceutica, Beerse, Belgium) to various isolates was tested on malt extract agar medium pH 7.0 according to the method described previously (Guan *et al.* 1989).

Metabolism of imazalil. Metabolism of [^{14}C]imazalil (gift from Janssen Pharmaceutica, Beerse, Belgium) was studied in liquid malt medium pH 7 (Guan *et al.*, 1989). Liquid cultures were prepared by inoculating the medium (100 ml) in flasks (300 ml) with 1 ml spore suspension (10^8 conidia ml^{-1}) collected from 7 to 10 days old agar plates. The flasks were incubated at 26°C in an orbital shaker at 200 rpm for 10 h. [^{14}C]imazalil (sp. act. 0.56 and 0.028 GBq mmol^{-1} in methanol) was added to the mycelial suspension to final concentrations of 0.005 and 0.1 μg ml^{-1}, respectively. The mycelial suspensions were further incubated in duplicate for 16 and 24 h,respectively.

Extraction of imazalil and metabolite. For each experiment, mycelium was harvested from liquid cultures (100 ml) by filtration on a Büchner funnel under vacuum. Mycelium was extracted twice with methanol (100 ml) by shaking on a reciprocal shaker for 10 and 5 h, respectively. The methanol extracts were combined and water in the extracts was removed by adding 2 g of anhydrous sodium sulphate. The methanol extracts were evaporated under vacuum at 40°C and the residues dissolved in chloroform (2 ml). Culture filtrates (100 ml) were adjusted to pH 11 with 10 M sodium hydroxide and extracted three times with equal volumes of chloroform. Water in the combined chloroform extracts was removed by adding 5 gram of anhydrous sodium sulphate and the extracts were evaporated under vacuum at 40°C. The residues were dissolved in chloroform (2 ml). Samples of 20 μl (duplicate) from mycelial extracts and culture filtrates were taken for determining radioactivity in a liquid scintillation counter (Beckman LS 2800). The chloroform was dried down under nitrogen and the residues were stored at -20°C.

Thin layer chromatography (TLC). Residues of extracts of mycelium and culture filtrates were dissolved in chloroform (100 μl) and spotted on TLC plates (Silicagel F254, 0.25 mm thick, Merck, Germany). The plates were developed in a solvent system of diethylether/chloroform/methanol/ammonium hydroxide (100:85:15:1, v/v/v/v). Two authentic metabolites of imazalil detected in animals, 1-[2(2,4-dichlorohenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243)

and 1-[2(2,4-dichlorophenyl)-2-(hydroxy)ethyl]-1*H*-imidazole (R14821) (gifts from Janssen Pharmaceutica, Beerse, Belgium) were used as references. Radioactive areas on the plates were located with a thin layer scanner (Berthold, Wildbad, Germany). The identification of imazalil and a metabolite was tentatively made by comparison of R_f values with those of reference compounds which could be detected under UV light. Silicagel containing radioactivity was scraped off and counted in a liquid scintillation system.

Radio-HPLC. The radio-HPLC equipment consisted of two Waters 6000 A pumps, a Waters 680 gradient controller, a Waters WISP 710 automatic injector and a Rheodyne 2 ml loop injector for manual injections. A stainless steel column (30 × 0.46 cm), packed with Hypersil C-18 (5 μm, Shandon) by a balanced density slurry procedure using a Haskel DSTV 122-C pump at 7×10^7 Pa, was used for separation. Residues of extracts of mycelium dissolved in dimethyl sulfoxide (500 μl) and culture filtrates (without further processing) were used for radio-HPLC analysis. The amount of radioactivity injected ranged from 834 to 11,000 dpm. The column was eluted with a linear gradient from 70% solvent A (0.1 M ammonium acetate pH 8 and 30% solvent B (1.0 M ammonium acetate pH 8/methanol/acetonitrile, 10/45/45, v/v/v) to 15% solvent A and 85% solvent B over a 40 min period. The latter solvent composition was held for 5 min before a short gradient to 100% solvent B was applied for 1 min. Solvent flow rate was 1 ml min^{-1}. UV detection was carried out at 230 nm by a Varian Varichrom spectrophotometric detector. On-line radioactivity detection was carried out with a Berthold Radioactivity Monitor LB 504, equipped with a 800 μl flow-through cell. The elutes were mixed with Pico-Fluor 30 (Packard) as scintillation cocktail, delivered by a FMI LB 5031 pump at a flow rate of 4 ml min^{-1}. Imazalil and a metabolite were identified by comparing their retention times with those of reference data by means of the UV detector.

Results

Incubation with [^{14}C]imazalil and extraction of radioactivity. Metabolism of imazalil in *P. italicum* was studied at 0.005 and 0.1 μg imazalil ml^{-1}, respectively. The lowest concentration inhibited growth of the wild-type isolate W_5 for about 25%. The highest concentration severely inhibited growth of

isolates W_5 and low-resistant isolate $E_{300\text{-}3}$ but still allowed growth of medium- and high-resistant isolates H_{17}, I_{33} and J_4 (Table 1). Radioactivity recovered after 24 h of incubation with 0.005 and 0.1 μg imazalil ml^{-1} was in the order of 76 to 97% of total radioactivity added. Radioactivity recovered after 16 h of incubation was in the same order of magnitude (results not shown).

Table 1. Metabolism of [^{14}C]imazalil by wild-type and DMI-resistant isolates of *Penicillium italicum* in a liquid malt extract medium pH 7 during 24 h of incubation.

Isolate	Imazalil (μg ml^{-1})	Growth[1]	Recovery of radioactivity[2]				
			Total	Mycelium		Culture filtrate	
				Imazalil	Metabolite	Imazalil	Metabolite
W_5	0.005	75	89.1	42.7	0	28.2	18.2
$E_{300\text{-}3}$	0.005	100	93.5	45.0	0	31.2	17.3
H_{17}	0.005	100	79.4	37.7	0	10.8	30.9
I_{33}	0.005	100	97.3	35.7	0	28.0	33.6
J_4	0.005	100	90.4	30.2	0	29.2	31.0
W_5	0.1	0	91.4	40.3	0	51.1	0
$E_{300\text{-}3}$	0.1	25	76.3	39.5	0	46.8	0
H_{17}	0.1	100	97.6	37.4	0	36.5	24.0
I_{33}	0.1	100	91.0	33.1	0	34.6	30.3
J_4	0.1	100	94.1	35.2	0	30.5	28.4

[1] Percentage of control.

[2] Percentage of total radioactivity added.

TLC. Scans of radioactivity in TLC plates with extracts from mycelium and culture filtrates of all isolates obtained after 16 h of incubation with 0.005 μg imazalil ml^{-1} revealed only one peak of which the R_f value was identical to

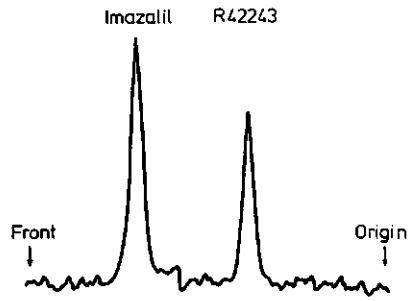

Fig. 1. TLC radioscans of extracts of culture filtrates of *Penicillium italicum* W_5 incubated for 24 h with [^{14}C]imazalil (0.005 μg ml^{-1}). TLC solvent, diethylether/chloroform/methanol/ammonium hydroxide (100:85:15:1, v/v/v/v).

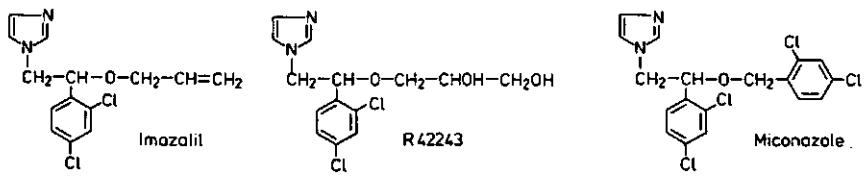

Fig. 2 Chemical structures of imazalil, 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243) and miconazole.

imazalil (results not shown). The same result was found for mycelial extracts of all isolates incubated with 0.005 μg imazalil ml^{-1} for 24 h (Table 1). Scans of radioactivity in TLC plates with extracts of culture filtrates of wild-type isolate W_5 incubated with 0.005 μg imazalil ml^{-1} for 24 h showed two peaks. One had a R_f value identical to imazalil and the other one had the same R_f value as the authentic metabolite R42243 (Fig. 1). Similar results were observed with resistant isolates. However, the amount of metabolite detected in extracts of culture filtrates of isolates H_{17}, I_{33} and J_4 was higher than in those of isolates W_5 and $E_{300\text{-}3}$ (Table 1). When the mycelia were incubated with 0.1 μg imazalil ml^{-1} the

metabolite was only detected in the culture filtrates of medium- and high-resistant isolates H_{17}, I_{33} and J_4, respectively (Table 1). Incubation of 24-h-old culture filtrates of all isolates with 0.005 μg [^{14}C]imazalil ml^{-1} for another 24 h did not result in production of any metabolites (results not shown).

Radio-HPLC. Mycelial suspensions were incubated with 0.1 μg [^{14}C]imazalil ml^{-1} for 24 h. Analysis of the mycelial extracts of isolates W_5 and J_4 showed only a single peak with a retention time of 44.3 min, which is identical to the one of imazalil. Analysis of the culture filtrate of isolate W_5 gave the same result. Analysis of the culture filtrate of isolate J_4 showed two peaks with retention times of 44.3 and 26.8 min, respectively. The retention time of the latter compound is identical to the one of R42243. The relative peak size area of both compounds was 54.3 and 45.7%, respectively.

Cross resistance. In order to study whether metabolism of imazalil in the propenyl side chain was important for resistance, toxicity of miconazole and R42243 (Fig. 2) to various isolates was tested. Results indicate that the DMI-resistant isolates of *P. italicum* were also cross-resistant to miconazole and R42243 although the toxicity of the latter compound was relatively low (Table 2).

Table 2. Inhibition of imazalil, R42243 and miconazole on radial growth of wild-type and DMI-resistant isolates of *Penicillium italicum* on malt extract medium at pH 7.

Isolate	EC_{50} values (μg ml^{-1})[1]		
	Imazalil	R42243	Miconazole
W_5	0.005	32	0.05
$E_{300\text{-}3}$	0.015 (3)[2]	130 (4)	0.27 (6)
H_{17}	0.21 (45)	330 (10)	0.70 (15)
I_{33}	0.27 (59)	600 (19)	1.20 (26)
J_4	0.19 (41)	590 (18)	1.70 (37)

[1] Concentration which inhibits radial growth by 50%

[2] Between brackets: degree of resistance (EC_{50} resistant isolate : EC_{50} wild-type isolate).

Discussion

After prolonged incubation of *P. italicum* with imazalil one metabolite could be detected (Fig. 1). The putative identity of the metabolite was suggested to be R42243 by TLC and radio-HPLC. It has been reported that R42243 is one of the first metabolites of imazalil formed in animals. This was ascribed as epoxidation and hydratation processes in the propenyl side chain (Heykants, 1978). The same metabolic mechanism may be involved in *P. italicum*. The present paper is the first report that a fungus has such a capacity. The low toxicity of R42243 (Table 2) indicates that the metabolism is a detoxification step. The fact that R42243 was only detected in the incubation medium and the culture filtrate did not have the potency to metabolize imazalil suggest that imazalil was metabolized intercellularly and that the metabolite was secreted into the culture medium. Metabolism of imazalil has also been studied in plants, such as in apple (Cano *et al.*, 1987) and cucumber (Vonk and Dekhuijzen, 1979). In cucumber leaves, imazalil was metabolized to non-identified polar products. However, the metabolic process occurred at a much slower rate possibly through hydrolysis of the ether bond in the molecule (Vonk and Dekhuijzen, 1979).

At least three arguments support the statement that detoxification of imazalil does not operate as mechanism of resistance. Firstly, the metabolite could only be detected after prolonged incubation (24 h) while resistance to imazalil already became apparent within 8 h (Guan *et al.*, 1989). Secondly, if resistance was based on metabolism of the propenyl side chain of imazalil, no cross resistance should be observed to the related DMI-fungicide miconazole and to metabolite R42243 which have identical structures as imazalil except for the propenyl side chain (Fig. 2). This was however, not the case (Table 2). In addition, cross resistance is also present to structurally non-related DMIs (De Waard *et al.*, 1982; De Waard and Van Nistelrooy, 1990). Thirdly, the rate of metabolism of imazalil in various isolates was concentration-dependent and differed with isolates. The slightly lower detoxification rate by isolates W_5 (wild-type) and $E_{300\text{-}3}$ (low resistant) is probably the consequence of less biomass caused by growth inhibition during the incubation period (Table 1).

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CHAPTER 8

Characterization of energy-dependent efflux of imazalil and fenarimol in isolates of *Penicillium italicum* with a low, medium and high degree of resistance to DMI fungicides

J. Guan[1], J. C. Kapteyn[1], A. Kerkenaar[2,3], and M. A. de Waard[1]

[1]Department of Phytopathology, Wageningen Agricultural University
P.O. Box 8025, 6700 EE Wageningen, The Netherlands.

[2]Netherlands Organization of Applied Scientific Research,
TNO Institute of Applied Chemistry,
P.O. Box 108, 3700 AC Zeist, The Netherlands

[3]Present address: Denka International B. V.,
Hanzeweg 1, 3771 Barneveld, The Netherlands

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Abstract

Differential accumulation of imazalil and fenarimol by wild-type W_5 and DMI-resistant isolates of *Penicillium italicum* of different mycelial age was studied at various pH values. At pH 7 and 8 the low resistant isolate $E_{300\text{-}3}$ accumulated 22% and 35% less imazalil than the wild-type isolate W_5, respectively. No further differential accumulation of imazalil between low ($E_{300\text{-}3}$), medium (H_{17}) and high resistant isolates (I_{33}) was observed. The reduced accumulation in isolate $E_{300\text{-}3}$ may account for its relatively low level of resistance. Differential accumulation of fenarimol between wild-type W_5 and resistant isolate $E_{300\text{-}3}$ was observed as described before (De Waard and Van Nistelrooy, 1984). No difference in accumulation of fenarimol by isolates $E_{300\text{-}3}$, H_{17} and I_{33} was noticed. These results suggest that reduced accumulation of DMIs is responsible only for resistance in

isolate $E_{300\text{-}3}$ and that additional mechanism of resistance may operate in isolates with a medium and high degree of resistance. Accumulation of fenarimol by all isolates was energy-dependent. This was less obvious for imazalil, which may relate to the cationic nature of the fungicide.

The plasma membrane potential of resistant isolates may be significantly lower than that of the wild-type isolate. Various test compounds among which ATPase inhibitors, ionophoric antibiotics, calmodulin antagonists and KCl affected the membrane potential and reduced or enhanced the accumulation of imazalil and fenarimol. However, no obvious correlation between the accumulation level of these fungicides and the magnitude of the membrane potential of these isolates could be observed. Therefore, it was concluded that the membrane potential does not mediate the efflux of DMI fungicides.

Additional keywords: ATPase inhibitors, calmodulin antagonists, ionophoric antibiotics, sterol 14α-demethylation inhibitors, plasma membrane potential

Introduction

Sterol biosynthesis inhibitors (SBIs) are systemic fungicides used in agriculture to control plant diseases. The primary mode of action of most SBIs (e.g. derivatives of imidazoles, triazoles and pyrimidines) is based on inhibition of cytochrome P450-dependent sterol 14α-demethylation (Kato, 1986). Therefore, these SBIs are referred to as demethylation inhibitors (DMIs).

Resistance to DMIs has been reported for a number of fungi in both laboratory and field (Köller and Scheinpflug, 1987; Brent and Hollomon, 1988). Studies with various fungi suggested that a large number of potential mechanisms of resistance can be involved (Deas, 1986; Hitchcock *et al.*,1986; Portillo and Gancedo, 1985; Smith and Köller, 1990; Taylor *et al.*, 1983; Vanden Bossche *et al.*, 1990; Walsh and Sisler, 1982; Watson *et al.*, 1988; Weete, 1986). A well documented mechanism is energy-dependent efflux of DMI fungicides from mycelium. Increased efflux from fenarimol-resistant mutants leads to secretion of DMIs into the external medium and hence prevents intracellular accumulation. As a consequence the target enzyme becomes less readily inhibited and the mutants less sensitive (De Waard and Van Nistelrooy, 1979; 1984; 1987; 1988). The ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) and the plasma membrane ATPase inhibitor sodium orthovanadate inhibited efflux and caused increased accumulation of all

DMIs tested. This might be a consequence of dissipation of the electrochemical proton gradient ($\Delta\tilde{\mu}_{H}+$) maintained by plasma membrane ATPase across plasma membranes (De Waard and Van Nistelrooy, 1987). The $\Delta\tilde{\mu}_{H}+$ is composed of a proton gadient ($\Delta_{H}+$) and a plasma membrane potential ($\Delta\psi$) and is known to drive transport processes of various xenobiotics such as benzoic and sorbic acid (Warth, 1977) and aminoglycoside antibiotics (Eisenberg *et al.*, 1984), respectively . Accumulation of the aminoglycoside antibiotic gentamycin by *Staphylococcus aureus* was also found to be enhanced by DCCD (Gilman and Saunders, 1986). DMI-resistant isolates of *Aspergillus nidulans* showed cross-resistance to the aminoglycoside antibiotic neomycin (Van Tuyl, 1977). These results suggest that a similar mechanism may mediate the accumulation of DMIs and aminoglycosides. In addition, resistance to aminoglycoside antibiotics in *Saccharomyces cerevisiae* and *Escherichia coli* was found to correlate with decreased intracellular accumulation and was ascribed to a defect plasma membrane potential (Perlin *et al.*, 1988; Damper and Epstein,1981).

Accumulation of fenarimol and other DMIs (De Waard and van Nistelrooy, 1987) and of imazalil (De Waard and Van Nistelrooy, 1988) by wild-type and fenarimol-resistant isolates of *P. italicum* which were low-resistant to imazalil, differed in various aspects. The main difference was that the differential accumulation by low-resistant isolates was obvious for fenarimol but absent for imazalil. Therefore, fenarimol and imazalil were selected as test compounds in the present study. The study describes the accumulation of these fungicides not only in a low-resistant isolate of *P. italicum* but also in isolates with a medium and high degree of resistance under various conditions. In order to investigate the impact of membrane potentials on DMI accumulation, membrane potentials were determined for all isolates, and the effect of compounds with known membrane-interfering properties on membrane potentials was compared with their effect on the accumulation of fenarimol and imazalil.

Materials and methods

Fungal isolates. *P. italicum* wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance, respectively, were used. The isolates have been described previously

(De Waard *et al.*, 1982; De Waard and Van Nistelrooy, 1990). The fungus was maintained on malt extract agar medium.

Culture methods and preparation of mycelial suspensions. Mycelium was grown in liquid malt extract medium (De Waard and Van Nistelrooy, 1984). Flasks (300 ml) with medium (100 ml) were inoculated with 1 ml of spore suspension (10^9 conidia ml^{-1}) collected from 7 to 10 days old agar plates. The flasks were incubated at 26°C on an orbital shaker at 200 rpm for 11 h (fungicide accumulation experiments) or 16 h (plasma membrane potential experiments). Mycelium was harvested on a sieve (0.21 mm pores) and collected on a second sieve (0.02 mm pores) by intensive washing with tap water. Mycelium was washed once with 25 mM potassium phosphate buffer pH 7.0, containing 0.1 mM calcium chloride and 1% (w/v) glucose. Standard mycelial suspensions with an average dry weight of about 2 mg ml^{-1} were made by resuspending 1 g wet weight of mycelium in 50 ml of the same buffer.

Fungicides and chemicals. [^{14}C]imazalil and imazalil sulphate (imazalil) were gifts from Janssen Pharmaceutica (Beerse, Belgium); [^{14}C]fenarimol from Lilly Research Center Ltd (Erl Wood Manor, England).[^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP^+, sp. act. 17.3 MBq $mmol^{-1}$) was purchased from Amersham, (UK), TPP^+ from ICN Pharmaceuticals Inc. (New York, USA); carbonyl cyanide 3-chlorophenylhydrazone (CCCP), calmidazolium, chloropromazine, diethylstilbestrol (DES), gramicidin-S, nigericin and trifluorperazine (TFP) from Sigma (Deisenhofen, Germany). Valinomycin and sodium orthovanadate (vanadate) from Janssen Chimica (Beerse, Belgium). Fungicides and chemicals were prepared as 100-fold concentrated solution in methanol.

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Experiments were carried out according to the method previously described (De Waard and Van Nistelrooy, 1984). Standard mycelial suspensions were shaken on a reciprocal shaker at 26°C for 30 min. Concentrations of [^{14}C]imazalil (sp. act. 11.1 MBq $mmol^{-1}$) and [^{14}C]fenarimol (sp. act. 5.6 MBq $mmol^{-1}$) in the mycelial suspensions were 10 and 90 μM, respectively. The final methanol concentration in the mycelial suspension was below 1%.

Determination of plasma membrane potential. Standard mycelial susensions (70 ml) in flasks (300 ml) were incubated on a reciprocal shaker at 26°C for 30 min. [^{14}C]TPP$^+$ was added to a final concentration of 10 μM. At time intervals, samples (5 ml) were taken and filtered on a Whatman GF/A glass filter using a Millipore sampling manifold apparatus. Mycelial pellets were washed 5 times with 5 ml of water in 30 sec. Mycelium-associated radioactivity was extracted with scintillation liquid (Aqua luma plus) overnight and counted in a liquid scintillation spectrometer (Beckman 2800 LS). The magnitude of $\Delta\psi$ was estimated with the Nernst equation (Rottenberg, 1979). The intercellular volume of *P. italicum* was taken to be 2.3 μl per mg dry mycelium (De Waard and Van Nistelrooy, 1988). Effects of various chemicals on accumulation of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ were determined by addition of these agents from 500-fold concentrated stock solutions in methanol before and after the addition of the labeled fungicides. The correspondent amount of methanol was added to the controls.

Results

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Accumulation of [14C]imazalil by 11-h-old mycelium of wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$ of *P. italicum* was pH-dependent and gradually reached its steady level in about 50 min (Fig. 1). Accumulation of the fungicide increased at higher pH, except at pH 8. Accumulation at pH 5 was the same for both isolates, but at pH 7 and 8 accumulation by isolate $E_{300\text{-}3}$ was 22 and 35% less compared to the wild-type isolate (Fig. 1). Accumulation of [^{14}C]fenarimol by isolates W_5 and $E_{300\text{-}3}$ was not significantly affected by changes of extracellular pH (results not shown). For all following experiments pH 7 was selected as a standard pH. Results in Fig. 2 show that accumulation of [^{14}C]imazalil by the wild-type isolate W_5 was higher than by the resistant isolates. No differential accumulation of [^{14}C]imazalil among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was noticed. Differential accumulation of [^{14}C]imazalil between wild-type isolate W_5 and resistant isolate $E_{300\text{-}3}$ was not observed when 16-h-old mycelium was used for the experiments (result not shown).

Accumulation pattern of [^{14}C]fenarimol by 11-h-old mycelium of wild-type isolate W_5 at pH 7 was similar to the typical transient accumulation curve of [^{14}C]fenarimol demonstrated previously by De Waard and Van Nistelrooy (1984), who used 16-h-old mycelium. A minor difference was that in the present experiments equilibrium in accumulation was obtained less readily. No

differential accumulation of [^{14}C]fenarimol among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was noticed (Fig. 3).

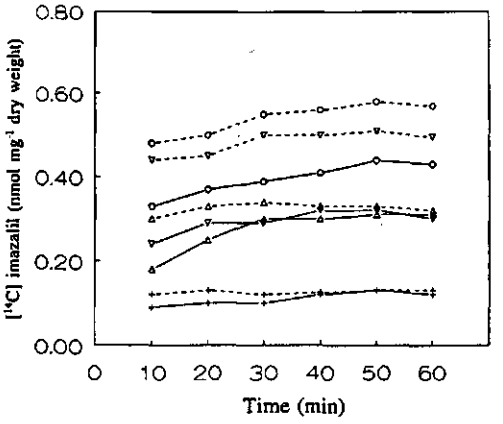

Fig. 1. Effect of external pH on accumulation level of [^{14}C]imazalil (10μM) by wild-type isolate W_5(---) and DMI-resistant isolate $E_{300\text{-}3}$ (—) of *Penicillium italicum* in 23.4 mM potassium phosphate, containing 0.1 mM calcium chloride and 1% (w/v) glucose at pH 5 (+), 6 (△), 7 (○) and 8 (▽).

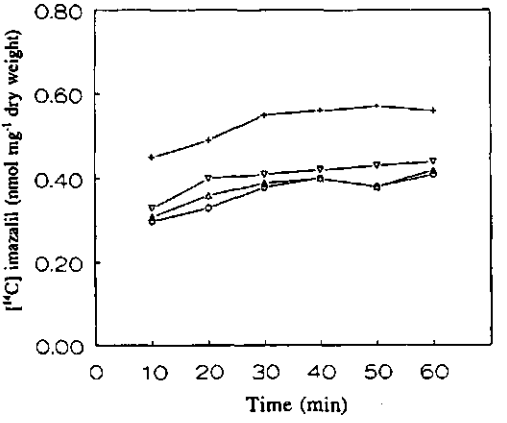

Fig. 2. Accumulation of [^{14}C]imazalil (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○) and I_{33} (▽) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

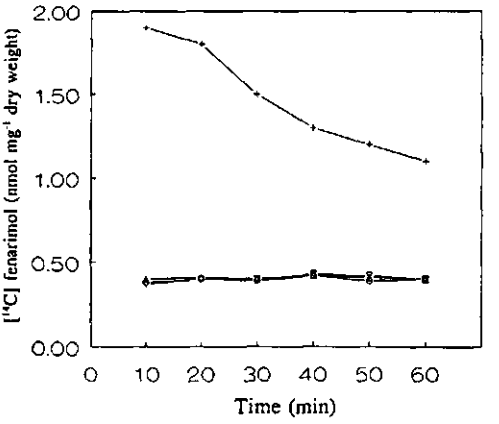

Fig.3. Accumulation of [^{14}C]fenarimol (90 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○) and I_{33} (▽) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

Plasma membrane potential. The accumulation level of [^{14}C]TPP$^+$ in 11-h-old mycelium was similar for all isolates (0.55 to 0.58 nmol mg^{-1} dry weight of mycelium). With 16-h-old mycelium accumulation by all resistant isolates was about 20% less than by the wild-type isolate W_5 (Fig. 4). The magnitude of the plasma membrane potential ($\Delta\psi$) under equilibrium conditions (60 min after addition of [^{14}C]TPP$^+$) for isolates W_5, $E_{300\text{-}3}$, H_{17} and I_{33} was calculated to be 96.3 $\pm$ 4.5, 86.6 $\pm$ 4.2 , 85.0 $\pm$ 4.1 and 86.2$\pm$ 3.0 mV, respectively (n = 5). The background absorption of [^{14}C]TPP$^+$ to mycelium was measured after various treatments such as boiling of mycelium, or incubation with cetylpyrimidine bromide. The results were highly dependent on the method used (results not shown). Therefore, the $\Delta\psi$ values were not corrected for background absorption and hence should be regarded as apparent membrane potentials.

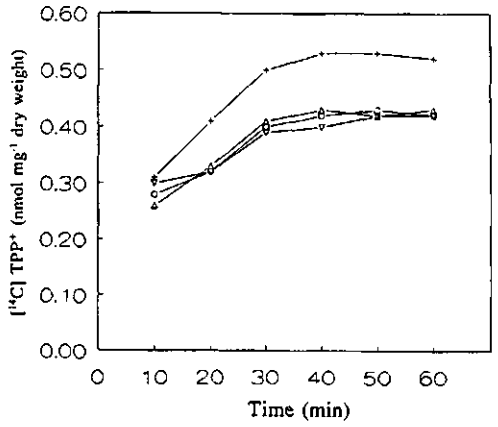

Fig. 4. Accumulation of [^{14}C]TPP$^+$ (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○) and I_{33} (▽) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

Table 1. Effect of KCl (0.2 M) on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP$^+$ (10 μM) in *Penicillium italicum* isolates W_5 and $E_{300\text{-}3}$[1].

Isolate	Accumulation level in controls[2] (nmol mg^{-1} dry weight)			Relative effect of KCL on accumulation level[3]		
	Imazalil	Fenarimol	TPP$^+$	Imazalil	Fenarimol	TPP$^+$
W_5	0.59±0.05	1.05±0.06	0.51±0.04	1.0	2.3	0.46
$E_{300\text{-}3}$	0.47±0.05	0.42±0.04	0.39±0.04	1.0	1.0	0.58

[1] Mycelial age in experiments on accumulation of fungicides and of TPP$^+$ was 11 and 16 h, respectively.

[2] Average of 3 values measured after 60 min of incubation.

[3] Ratio between accumulation level in presence of KCl : accumulation level in controls.

Effect of various compounds on accumulation of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$. Differential accumulation of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ was observed between wild-type W_5 and low-resistant isolate $E_{300\text{-}3}$. Therefore, only these two isolates were selected for

Table 2. Effect of various test compounds on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP$^+$ (10 μM) by mycelium of *Penicillium italicum* W_5 at pH 7.0.

Fungal isolate	Compound[1]	Concentration (μM)	Relative effect of chemicals on accumulation[2,3]		
			Imazalil	Fenarimol	TPP$^+$
W_5[5]	Calmidazolium	10	0.75[2]	2.73	0.90
	,,	25	0.80	3.21	0.97
	,,	50	-[4]	1.42	0.85
	CCCP	50	1.84	4.51	0.95
	,,	100	1.17	5.23	0.59
	Chloropromazine	10	0.50	1.42	0.92
	,,	50	0.72	3.90	0.86
	,,	100	0.62	2.94	0.90
	DES	10	0.92	2.23	1.05
	,,	50	1.31	4.71	1.12
	,,	100	1.39	3.04	1.64
	TFP	10	-	1.82	-
	,,	50	0.72	5.02	1.10
	,,	100	0.76	2.90	1.36
W_5[6]	Gramicidin-S	100	-	1.30	0.64
	Nigericin	100	-	2.71	1.08
	Valinomycin	100	-	2.03	0.85
	Vanadate	30000	-	2.03	0.85

[1] Abbreviations used: DES (diethylstilbestrol), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), TFP (trifluorperazine), TPP$^+$ (tetraphenylphosphonium bromide).

[2] Average accumulation levels of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ in untreated mycelium 40 min after adding the radiolabeled compounds were 0.58 ± 0.05, 1.10 ± 0.07 and 0.50 ± 0.04 ng mg^{-1} dry weight mycelium, respectively.

[3] Ratio between accumulation level in treatments and controls.

[4] Not tested.

[5] Experiments carried out with 11-h-old mycelium.

[6] Experiments carried out with 16-h-old mycelium.

most of the following experiments. Pre-incubation of mycelium with KCl (0.2 M) for 1 h inhibited accumulation of [^{14}C]TPP$^+$ by both isolates indicating a decrease in $\Delta\psi$ (Table 1). KCl did not affect accumulation of [^{14}C]imazalil in

Table 3. Effect of various test compounds on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP$^+$ (10 μM) by mycelium of *Penicillium italicum* isolate $E_{300\text{-}3}$ at pH 7.0.

Fungal isolate	Compound[1]	Concentration (μM)	Relative effect of chemicals on accumulation[2,3]		
			Imazalil	Fenarimol	TPP$^+$
$E_{300\text{-}3}$[5]	Calmidazolium	10	0.84	4.62	0.95
	,,	25	0.80	6.51	0.88
	,,	50	0.85	4.11	0.97
	CCCP	50	1.66	7.92	0.94
	,,	100	1.45	-[4]	0.82
	Chloropromazine	10	0.66	3.02	0.91
	,,	50	0.85	7.84	0.85
	,,	100	0.54	1.13	0.98
	DES	10	1.02	1.14	0.98
	,,	50	1.23	7.74	1.16
	,,	100	1.32	7.12	1.58
	TFP	10	-	4.81	-
	,,	50	0.72	12.72	1.10
	,,	100	0.55	9.24	1.50
$E_{300\text{-}3}$[6]	Gramicidin-S	100	-	3.81	0.97
	Nigericin	100	-	14.62	1.29
	Valinomycin	100	-	5.73	0.96
	Vanadate	30000	-	1.62	0.75

[1] Abbreviations used: DES (diethylstilbestrol), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), TFP (trifluorperazine), TPP$^+$ (tetraphenylphosphonium bromide).

[2] Average accumulation levels of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ in untreated mycelium 40 min after adding the radiolabeled compounds were 0.40 ± 0.05, 0.42 ± 0.04 and 0.4 ± 0.03 ng mg^{-1} dry weight mycelium, respectively.

[3] Ratio between accumulation level in treatments and controls.

[4] Not tested.

[5] Experiments carried out with 11-h-old mycelium.

[6] Experiments carried out with 16-h-old mycelium.

both isolates but caused an obvious increase in accumulation of [^{14}C]fenarimol by isolate W_5 only.

Other chemicals tested were selected because of their known effects on respiration or membrane processes. After 10 min of incubation DES, TFP and nigericin caused no or only a slight increase in accumulation of [^{14}C]TPP$^+$ in both isolates, depending on the concentration used (Table 2 and 3). All other chemicals tested slightly decreased [^{14}C]TPP$^+$ accumulation. Gramicidin-S and CCCP had a relatively stronger effect on [^{14}C]TPP$^+$ accumulation by isolate W_5 than by isolate $E_{300\text{-}3}$. Accumulation of [^{14}C]fenarimol strongly increased upon incubation with all compounds tested. The relative effect of most test compounds on accumulation of [^{14}C]fenarimol by isolate $E_{300\text{-}3}$ was higher than by isolate W_5 (Table 2 and 3). The effect of these compounds on accumulation of [^{14}C]imazalil was not comparable with that of [^{14}C]fenarimol. Only CCCP and DES caused a slight increase in accumulation of [^{14}C]imazalil while all other compounds tested even led to a slightly lower accumulation level. It was also noticed that in isolates H_{17}, I_{33} and J_4, CCCP, DES and TFP had similar effects on accumulation of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ as in isolate $E_{300\text{-}3}$ (results not shown).

Discussion

Energy-dependent efflux of a number of DMIs from DMI-resistant isolates of *A. nidulans* and *P. italicum* has been well documented (De Waard and Van Nistelrooy, 1979; 1984; 1987; 1988). The efflux of DMIs in resistant isolate $E_{300\text{-}3}$, which was high-resistant to fenarimol and low-resistant to imazalil, is probably responsible for a lower accumulation by which the DMIs do not readily saturate the target enzyme in sterol biosynthesis. Imazalil was the only exception within DMIs tested since no differential accumulation between sensitive and resistant isolates was observed (De Waard and Van Nistelrooy, 1988). However, the present study indicates that an obvious difference in accumulation could be observed if relatively young mycelium was used (11 h old) and the pH of the external medium was 7.0 or higher (Fig. 1). Older mycelium probably accumulates more aspecifically-bound imazalil than younger mycelium and may, therefore, mask small differences. Imazalil has a pK_a value of 6.5. Hence, in a medium with pH 7.0 it is mainly in a neutral form and therefore the accumulation process of imazalil may not be affected by the presence of its protonated form as compared with pH 6.0 used in previous studies (De Waard and Van Nistelrooy, 1988). It may be possible that the difference in accumulation of imazalil by wild-type isolate W_5 and

low-resistant isolate $E_{300\text{-}3}$ as observed with young mycelium at pH 7.0, is responsible for the relatively low level of resistance to this fungicide. A similar result was also found for resistance to imazalil in *Aspergillus nidulans* (Siegel and Solel, 1981).

Isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance to DMIs, respectively, did not show differential accumulation of fenarimol and imazalil (Fig. 2 and 3). Hence, the mechanism of resistance in isolates with medium and high degrees of resistance remains unexplained. It might be that a relatively large amount of aspecifically bound fungicides masks any further decrease in accumulation in medium and high-resistant isolates. An alternative explanation is that the resistance mechanism in these isolates is based on other mechanisms.

Increase in accumulation of imazalil by respiration inhibitors, like CCCP (uncoupler) and DES (plasma membrane ATPase inhibitor) (Table 2 and 3), suggests that imazalil uptake might also be mediated by a mechanism of energy-dependent efflux as described for other DMIs (De Waard and Van Nistelrooy, 1987; 1988). However, all other compounds tested decreased accumulation. There is no easy explanation for this. In contrast, fenarimol accumulation strongly increased upon incubation with an uncoupler (CCCP), membrane ATPase inhibitors (DES and vanadate), calmodulin antagonists (calmidazolium, chloropromazine and TFP) and ionophoric antibiotics (gramicidin-S, nigericin and valinomycin). This suggests that the mechanism involved in efflux of fenarimol differs from that of imazalil. However, no explanation for this difference can be given. It is also not clear why the time-course accumulation pattern of fenarimol (and other DMIs) and imazalil are different (Fig. 2 and 3). The differences might be related to the cationic nature of imazalil.

Calculation of the plasma membrane potential ($\Delta\psi$) by measuring the overall TPP^+ uptake in whole cells has been studied in various fungi (Boxmann *et al.* 1982; Höfer and Kenemund, 1984; Prasad and Höfer, 1986) and proved to reflect the true value of the $\Delta\psi$ of cells as measured by microelectrodes (Lichtenberg *et al.*, 1986). In the present study we also used the overall TPP^+ uptake in mycelium of *P. italicum* to calculate the $\Delta\psi$ of the various isolates. All DMI-resistant isolates had a lower $\Delta\psi$ than the wild-type isolate (Fig. 4). However, the lower $\Delta\psi$ may not be causally related with the lower accumulation of imazalil and fenarimol in these isolates. Various arguments are in favour of this supposition. Firstly, depolarization of the $\Delta\psi$ of mycelium by KCl was not accompanied by a decrease in accumulation of

either imazalil or fenarimol (Table 1). Secondly, manipulation of the $\Delta\psi$ with test compounds did not result in a correlation between the magnitude of $\Delta\psi$ and the accumulation of either fenarimol or imazalil (Table 2 and 3). In particular, accumulation of fenarimol can be enhanced by test compounds which hardly influenced $\Delta\psi$ (Table 2 and 3). Hence, the hypothesis that DMI accumulation is mediated in one way or another by the $\Delta\psi$ does not seem to be valid. An alternative explanation for the relatively low membrane potential in resistant isolates may be the reduced competitive fitness of these isolates. This results in slower spore germination (De Waard *et al.*, 1982; De Waard and Van Nistelrooy, 1990). Hence, mycelium harvested after 11 h of incubation may be of a different physiological age and this may reflect the difference in $\Delta\psi$. In this context it should also be mentioned that an overall TPP^+ uptake may not be a good parameter for quantitatively measuring $\Delta\psi$ since accumulation of TPP^+ in yeast cells was suggested to be a complex process which depends on multiple factors and not solely on plasma membrane potential (Eraso *et al.*, 1984). This should be of concern in following studies.

Energy-dependent efflux of DMI fungicides in fungi resembles in several aspects multi-drug resistance (MDR) in mammalian cancer cells (Bradley *et al.*, 1988). Firstly, these MDR cells are resistant to structurally non-related drugs. In fungi, cross-resistance not only to DMIs but also to unrelated fungitoxicants has been reported (Van Tuyl, 1977; Krämer *et al.*, 1987). Secondly, in both type of organisms the resistance is based on reduced accumulation of compounds mediated by energy-dependent efflux. Thirdly, the accumulation of both anticancer drugs and DMI fungicides can be reversed by calmodulin antagonists, like TFP, chloropromazine and calmidazolium (Table 2 and 3; Endicott and Ling, 1989). In addition, cycloheximide which reversed drug accumulation in MDR cells (Gottesman and Pastan, 1988) also increased accumulation of fenarimol in *A. nidulans* (unpublished results). These lines of similarity suggest that DMI-resistance in fungi may be based on a similar mechanism as present in MDR mammalian cancer cells. This topic will be the subject of future studies.

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CHAPTER 9

General discussion

Isolates of *Penicillium italicum* H_{17}, I_{33} and J_4 with different levels of resistance to imazalil and other DMI-fungicides stepwise selected from fenarimol-resistant isolate $E_{300\text{-}3}$ (De Waard and Van Nistelrooy, 1990) were used throughout the study. Isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 exhibit increasing levels of resistance to imazalil. The resistance level remained stable in course of the experimental period for isolates $E_{300\text{-}3}$, H_{17} and I_{33} and decreased to a limited extent for isolate J_4 (Chapters 2 and 6). This may relate to the polygenic nature of DMI resistance.

Sterol analyses showed that both wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *P. italicum* contained ergosterol as the major sterol (95.6% to 98.3% of total sterols identified). The DMI fungicide imazalil inhibited ergosterol biosynthesis and led to accumulation of 24-methylenedihydrolanosterol in all isolates. Effects of imazalil on synthesis of other sterols was not significant. However, in high-resistant isolates H_{17}, I_{33} and J_4 much higher concentrations of imazalil were necessary to perform the inhibitory effect than in wild-type isolate W_5 (Chapter 2). These results indicate that the same target site for imazalil is present in all isolates. Hence, resistance to DMIs in *P. italicum* is not due to a lack of the target enzyme, cytochrome P450-dependent sterol 14α-demethylase (cytochrome $P450_{14DM}$) or the presence of a non-functional one, as observed in mutants of *Ustilago maydis* and *Saccharomyces cerevisiae* (Walsh and Sisler, 1982; Aoyama *et al.*, 1983). These mutants contained C14-methylsterols instead of ergosterol as the major sterols. The normal sterol composition of DMI-resistant isolates of *P. italicum* corroborates with the observation that they have an almost normal saprophytic fitness and virulence as the wild-type isolate (De Waard and Van Nistelrooy, 1990). The results also suggest that a mechanism of resistance based on tolerance of abnormal sterols like C14-methylsterols (Weete and Wise, 1987) or detoxification of these abnormal sterols (Vanden Bossche *et al.*, 1987) is unlikely. The fact that a relatively high concentration of imazalil was needed to inhibit ergosterol biosynthesis in the resistant isolates as in the wild-type again suggests that absence of the target enzyme or the presence of a non-functional one is not

involved as a mechanism of resistance. Among other potential mechanisms of resistance, ample attention was paid to study the hypothesis that reduced affinity of sterol 14α-demethylase to DMIs in DMI-resistant isolates would be involved. The reason for this strategy is that such a mechanism of resistance is in general the most common one in cases of fungicide resistance (Dekker, 1985).

Table 1. Relation between toxicity of DMIs to radial growth of wild-type isolate W_6 of *Penicillium italicum*, sensitivity of sterol 14α-demethylase and affinity of cytochrome P450 isozymes to DMIs.

Compound	EC_{50} (M)[1]	IC_{50} (M)[2]	I_{50} (M)[3]	CO (%)[4]
Imazalil	$1.4\pm0.2\times10^{-8}$	$1.6\pm0.4\times10^{-8}$	$4.0\pm1.2\times10^{-8}$	42±8
Itraconazole	$6.0\pm0.2\times10^{-8}$	$1.0\pm0.3\times10^{-8}$	$3.7\pm1.0\times10^{-8}$	44±6
Ketoconazole	$5.0\pm0.4\times10^{-8}$	$6.5\pm0.5\times10^{-9}$	$5.0\pm1.1\times10^{-8}$	15±2
Penconazole	$4.0\pm0.2\times10^{-8}$	$1.7\pm0.7\times10^{-8}$	$4.2\pm1.3\times10^{-8}$	94±8
Propiconazole	$2.2\pm0.3\times10^{-8}$	$1.0\pm0.2\times10^{-8}$	$4.1\pm1.1\times10^{-8}$	80±6
R14821	$6.6\pm0.4\times10^{-6}$	$6.0\pm0.7\times10^{-6}$	$7.0\pm0.8\times10^{-8}$	99±3
R42243	$9.7\pm0.4\times10^{-5}$	$4.0\pm0.3\times10^{-5}$	$1.2\pm0.9\times10^{-7}$	101±3

[1] Concentration which inhibits radial growth on malt agar pH 7.0 by 50% (Chapter 5, Table 1).

[2] Concentration which inhibits incorporation of radioactivity into C4-desmethyl sterols in cell-free extracts by 50% (Chapter 3, Table 1).

[3] Concentration which induces half saturation response of type II difference spectra (Chapter 5, Table 2).

[4] Displacement of test compounds (10^{-6} M) from P450 isozymes as a percentage of control treatment 30 min after bubbling with CO (Chapter 5, Fig. 5).

An assay to study cell-free ergosterol biosynthesis in *P. italicum* and its sensitivity to DMIs was developed for wild-type isolate W_5 (Chapter 3). The relatively high percentage of ergosterol synthesized in cell-free extracts (25.6% of total non-saponifiable lipids) from [2-^{14}C]mevalonate and its differential sensitivity to the DMI fungicides imazalil, itraconazole, ketoconazole, penconazole and propiconazole, and the less-toxic DMI compounds R42243 and R14821 (Table 1) indicated that this method was suitable to study the sensitivity

of sterol 14α-demethylase activity of this fungus to DMIs. This is an important achievement in the methodology of studying *in vitro* enzymes involved in sterol biosynthesis of filamentous plant pathogenic fungi. The assay developed is an improvement of the one described for the mammalian pathogen *Aspergillus fumigatus* (Ballard *et al.*, 1990a). Using the same test method, cell-free extracts of DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} could be prepared. These cell-free extracts contained a slightly higher protein content as compared with that of the wild-type isolate. In addition, ergosterol synthesized as percentage of total non-saponifiable lipids produced in cell-free extracts of isolates H_{17} and I_{33} was slightly higher than that of the wild-type isolate. Nevertheless, IC_{50} values (concentrations which inhibit incorporation of radioactivity into ergosterol by 50%) of imazalil for cell-free synthesis of ergosterol in different isolates did not vary significantly (Table 2). Hence, the present data indicate that decreased affinity of sterol 14α-demethylase to imazalil will not play a major role as a mechanism of resistance in *P. italicum* (Chapter 4).

Table 2. Relation between toxicity of imazalil to wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *Penicillium italicum*, sensitivity of sterol 14α-demethylase activity and affinity of cytochrome P450 isozymes of these isolates to imazalil.

Isolate	EC_{50} (M)[1]	IC_{50} (M)[2]	I_{50} (M)[3]	CO (%)[4]
W_5	$1.4\pm0.2\times10^{-8}$	$1.6\pm0.4\times10^{-8}$	$4.0\pm1.2\times10^{-8}$	43 ± 6
$E_{300\text{-}3}$	$4.5\pm0.3\times10^{-8}$	$1.7\pm0.5\times10^{-8}$	$4.2\pm2.1\times10^{-8}$	43 ± 9
H_{17}	$6.3\pm0.2\times10^{-7}$	$2.0\pm0.5\times10^{-8}$	$5.1\pm2.7\times10^{-8}$	58 ± 9
I_{33}	$8.3\pm0.5\times10^{-7}$	$2.1\pm0.4\times10^{-8}$	$5.8\pm2.3\times10^{-8}$	60 ± 8
J_4	$5.8\pm0.4\times10^{-7}$	nd[5]	$8.0\pm3.2\times10^{-8}$	64 ± 11

[1] Concentration which inhibits radial growth on malt agar pH 7.0 by 50% (Chapter 6, Table 5).

[2] Concentration of imazalil which inhibits incorporation of radioactivity into C4-desmethyl sterols in cell-free extracts by 50% (Chapter 4, Table 2).

[3] Concentration of imazalil which induces half saturation response of type II difference spectra (Chapter 6, Table 2).

[4] Displacement of imazalil (10^{-6} M) from P450 isozymes as a percentage of control treatment 30 min after bubbling with CO (Chapter 6, Table 3).

[5] Not determined.

In addition, microsomal cytochrome P450 isozymes were isolated to study the affinity of cytochrome $P450_{14DM}$ by means of difference spectrophotometry. Studies with wild-type isolate W_5 were described in Chapter 5. After mechanical disruption of protoplasts and differential centrifugation of homogenates, cytochrome P450 isozymes could be demonstrated in microsomal fractions. The yield of these microsomal cytochrome P450 isozymes was high (365 $\pm$ 33 pmol per g dry mycelium) and the quality of the isozymes with respect to specific activity and stability was good. Next to *A. fumigatus* (Ballard *et al.*, 1990b), this is a second case of successful isolation of cytochrome P450 isozymes from a filamentous fungus. Interaction of the P450 isozymes with the DMI fungicides imazalil, itraconazole, ketoconazole, penconazole and propiconazole, and the less-toxic DMI compounds R42243 and R14821 was demonstrated by formation of type II spectra upon addition to the oxidized form of the isozymes. Only slight differences in I_{50} values (concentrations which induce half saturation response in the type II difference spectra) of compounds which differed significantly in toxicity were observed (Table 1). Therefore, it was concluded that the interaction between the heme iron of the isozymes and the heterocyclic nitrogen of the test compounds does not play a major role in determining affinity of the isozymes to DMIs. This is in agreement with proposals of Vanden Bossche *et al.* (1988) that affinity of cytochrome P450 isozymes to DMIs is largely dependent on the interaction between the apoprotein of the isozymes and the *N*1-substituent of the DMIs. Therefore, the ease of removal of DMIs from P450 isozymes will largely depend on the interaction between the apoprotein of the isozymes and the *N*1-substituent of the DMIs. This can be determined by measuring the ability of carbon monoxide (CO) to displace DMIs from the isozymes (Vanden Bossche, 1987). Results showed that the ease of CO to displace the DMI fungicides and the less-toxic DMI analogues (R14821 and R42243) from the P450 isozymes did correlate to some degree with their fungitoxicity (Table 1). Exceptions were observed with DMIs with a relatively large *N*1-substituent, such as ketoconazole and itraconazole. This may be due to the possibility that a relatively large *N*1-substituent hampers their accumulation in mycelium and transport to the target site and hence reduces fungitoxicity. Another explanation is that microsomes may contain P450s varying in affinity to the DMIs tested. A way to avoid such interfering factors may be the use of purified cytochrome $P450_{14DM}$ in similar tests. However, this research strategy was not pursued due to experimental difficulties.

Cytochrome P450 isozymes were also isolated from DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} and J_4 (Chapter 6). The isolation procedure for isolate $E_{300\text{-}3}$

was the same as that of the wild-type isolate whereas the procedure for isolates H_{17} and I_{33} and J_4 had to be modified in order to obtain proper P450 isozymes. The ease of CO to displace imazalil, itraconazole and ketoconazole from P450 isozymes of DMI-resistant isolates was determined and compared with that from the wild-type isolate W_5. Results showed that the DMIs tested were more readily displaced from the P450 isozymes of resistant isolates H_{17} and I_{33} and J_4 than from those of wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$ (Table 2). This suggests that the affinity of the P450 isozymes to DMIs is lower than that of the wild-type isolate. This conclusion contradicts with the results obtained from the cell-free bioassay in which the sensitivity of sterol 14α-demethylase activity of all isolates proved to be similar (Chapter 4). However, the reliability of the conclusion is challenged by a number of factors which hamper a proper interpretation of the results. These factors include the necessary modification of the standard isolation procedure of P450 isozymes from resistant isolates H_{17}, I_{33} and J_4, the possible difference in quality of the isozymes in wild-type and resistant isolates and a possible underestimation of the concentration of the isozymes in microsomal preparations of the resistant isolates H_{17}, I_{33} and J_4 (Chapter 6). Furthermore, data obtained are also only of a semi-quantitative nature. Hence, conclusions derived from the spectrophotometric assays may not be reliable to compare affinity of cytochrome $P450_{14DM}$ to DMIs in different isolates. Consequently, these results do not provide sound evidence to reject the conclusion of chapter 4 that sterol 14α-demethylase of wild-type and DMI-resistant isolates have a similar sensitivity to DMIs.

Another potential mechanism of resistance studied is metabolism of DMIs. Fungicide metabolism may theoretically either be a detoxification or an activation step. Since imazalil itself is already extremely toxic (Chapter 2), activation of the fungicide in the fungus is hardly possible. So far, resistance to DMIs based on activation has only been observed with resistant strains of *Cladosporium cucumerinum* (Fuchs and De Vries, 1984) and *Nectria haematococca* (Kalamarakis *et al.*, 1986). In both cases, resistant isolates failed to activate triadimefon into triadimenol. In *P. italicum*, imazalil was metabolized only after prolonged incubation (24 h) to a less-toxic compound, which was putatively identified as the 2,3-dihydroxypropyloxy analogue (R42243). R42243 was detected in both wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4, although in resistant isolates higher amount of R42243 was detected (Chapter 7). This can be explained by the fact that growth of resistant isolates was not inhibited. Hence, more biomass was available to perform the metabolism. DMI-resistant isolates also showed cross resistance to another DMI-

fungicide miconazole, which has a similar structure as that of imazalil except for the side chain and hence can not be metabolised in a similar way as imazalil. In addition, the resistant isolates showed cross resistance to structurally unrelated DMIs (De Waard *et al.*, 1982; De Waard and Van Nistelrooy, 1990). These data confirm that metabolism of the side chain of imazalil is not involved as a mechanism of resistance.

The results of sterol analyses, studies on the sensitivity of the sterol 14α-demethylase activity to DMIs and metabolism of imazalil in wild-type and DMI-resistant isolates suggest that the mechanism of resistance in *P. italicum* relates to factors which prevent the fungicide from reaching the target site. Therefore, accumulation of imazalil by wild-type and DMI-resistant isolates was studied (Chapter 8). Fenarimol was used as a reference compound. It was observed that the low-resistant isolate $E_{300\text{-}3}$ accumulated a significantly lower amount of imazalil than the wild-type isolate. High-resistant isolates H_{17} and I_{33} accumulated a similar level of imazalil as the low-resistant isolate $E_{300\text{-}3}$. Similar results were also observed for fenarimol. This may suggest that the reduced accumulation of imazalil and fenarimol is only responsible for the relatively low level of resistance in isolate $E_{300\text{-}3}$ to the fungicides.

Enhancement of accumulation of fenarimol upon treatment with ATPase and respiration inhibitors suggests that accumulation of fenarimol in all isolates is mediated by a mechanism of energy-dependent efflux. An increased energy-dependent efflux of fenarimol may also operates in high-resistant isolates as proposed for the low-resistant isolate $E_{300\text{-}3}$ (De Waard and Van Nistelrooy, 1984, 1988). However, energy-dependent efflux of imazalil is less obvious. This may relate to the cationic nature of the fungicide.

High-resistant isolates H_{17}, I_{33} and J_4 probably contain additional genes for resistance to DMIs as compared with isolate $E_{300\text{-}3}$ (De Waard and Van Nistelrooy, 1990). A crucial question is whether these genes still code for the same mechanism of resistance as present in isolate $E_{300\text{-}3}$. There are reasons to believe that this may be the case. Arguments to support this hypothesis are based on the observations that different genes in *Aspergillus nidulans* which cause resistance to imazalil and other DMIs (*imaA* and *imaB*) both code for decreased accumulation of fenarimol. Many other DMI-resistant isolates of *A. nidulans* were not genetically characterized but also showed, without exception, decreased accumulation (De Waard and Van Nistelrooy, 1979). These isolates probably also contained some of the other genes for DMI resistance identified in this fungus (Van Tuyl, 1977). Apparently, all genes code for energy-dependent efflux of DMIs. Various mutants of *Monilinia fructicola* with different degrees of

resistance to DMIs also showed a lower accumulation level of various DMIs tested than the wild-type isolate (Ney, 1988). Also in case of *Nectria haematococca* var. *cucurbitae* several genes involved in DMI resistance were found to control a permeability barrier to DMIs. Isolates containing two genes for resistance showed a higher degree of resistance and accumulated a lower level of DMIs than the ones with a single gene (Kalamarakis *et al.*, 1991). These data suggest that high-resistant isolates of *P. italicum* may also contain additional genes coding in a similar way for energy-dependent efflux of DMIs. However, the difference in amount of fungicide released from low-, and high-resistant isolates may be very low in comparison to the background adsorption of the fungicides to mycelium and therefore, may not be detectable with the test method used. Hence, the possibility that a high degree of resistance to DMIs is caused by an additional energy-dependent efflux should, as yet, not be excluded.

DMI resistance in *P. italicum* and other filamentous fungi resembles in some way multidrug resistance of mammalian cells (Chapter 8). In such drug-resistant cancer cells, a number of multigene-coded transport proteins may be responsible for the energy-dependent efflux of different drugs. It may be possible that similar drug transport proteins are also present in *P. italicum* and can be responsible for differential accumulation of various DMIs. Further studies should be designed to explore this hypothesis.

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SUMMARY

The fungitoxic action of azole fungicides is based on inhibition of cytochrome P450-dependent sterol 14α-demethylase (cytochrome $P450_{14DM}$) activity. This thesis presents results of studies on various potential mechanisms of resistance to these sterol 14α-demethylation inhibitors (DMIs) in DMI-resistant isolates of the fungal plant pathogen *Penicillium italicum*. In chapters 2, 4, 6, 7 and 8 results of studies on four potential mechanisms of resistance are described. In chapters 3 and 5 two important developments in experimental procedures which were essential to test one of the potential mechanisms of resistance, decreased affinity of the target enzyme, are presented.

In chapter 2, sterol composition of wild-type isolate W_5 and isolates $E_{300\text{-}3}$ (low resistance), H_{17} (medium resistance), I_{33} and J_4 (high resistance) is described. Results showed that in all isolates ergosterol was the major sterol. Inhibition of ergosterol biosynthesis in W_5 was observed at a low concentration of imazalil (0.01 $\mu g\ ml^{-1}$), while in the resistant isolates much higher concentrations were necessary to achieve the inhibitory effect. Inhibition of ergosterol biosynthesis was accompanied by accumulation of 24-methylenedihydrolanosterol. These results proved that the same target site for imazalil is present in all isolates and that the mechanism of resistance in this fungus is not related to absence of the target enzyme or the presence of a non-functional one.

Chapter 3 describes an important achievement in studying *in vitro* enzymes involved in sterol biosynthesis in filamentous plant pathogenic fungi. A novel method to obtain a cell-free extract of *P. italicum* which was active in synthesizing ergosterol from [^{14}C]mevalonate is presented and may serve as a model to study similar processes in other filamentous plant pathogens. The *in vitro* synthesis of ergosterol in cell-free extracts of *P. italicum* accounted for about 26% of total non-saponifiable lipids synthesized. The inhibitory effect of various test compounds (DMI fungicides and less-toxic imazalil analogues) on *in vitro* ergosterol biosynthesis was investigated. IC_{50} values (concentrations which inhibit incorporation of radioactivity into ergosterol by 50%) of the highly toxic DMI fungicides imazalil, itraconazole, ketoconazole, penconazole and propiconazole ranged from $6.5 \pm 0.5 \times 10^{-9}$ to $1.7 \pm 0.7 \times 10^{-8}$ M. This indicates that DMI fungicides are very potent inhibitors of sterol 14α-demethylase activity in cell-free extracts of the fungus. Less-toxic imazalil analogues had much higher IC_{50} values, suggesting that these compounds have

a significantly lower potency to inhibit sterol 14α-demethylase activity. The test method used to study ergosterol biosynthesis in the wild-type isolate W_5 could also be applied to DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33}. It was noted that the quality of cell-free extracts of wild-type and resistant isolates differed slightly. Nevertheless, IC_{50} values of imazalil for inhibition of *in vitro* ergosterol biosynthesis were not significantly different in wild-type and all DMI-resistant isolates (Chapter 4). This indicates that sterol 14α-demethylase of sensitive and resistant isolates has a similar sensitivity to imazalil. Therefore, reduced affinity of the target enzyme to DMIs in resistant isolates will not play a major role as a mechanism of resistance.

In Chapter 5, a method to isolate cytochrome P450 isozymes from wild-type isolate W_5 is described. Interaction between the heterocyclic nitrogen atom of the test compounds (DMI-fungicides and less-toxic imazalil analogues) and the oxidized heme iron atom of the isozymes could be demonstrated by difference spectrophotometry (type II spectra). However, the magnitude of the type II spectra did not correlate with toxicity of the test compounds. A difference in the ability of carbon monoxide (CO) to displace the test compound from reduced cytochrome P450 isozymes was observed. It appeared that the less-toxic imazalil analogues were immediately displaced while the displacement of various DMI fungicides gradually occurred in time. This suggests that the binding affinity of the test compounds to cytochrome P450 isozymes did correlate to some degree with fungitoxicity of the test compounds. However, inconsistent results were observed for DMI fungicides with a large *N*1-substituent, like itraconazole and ketoconazole.

Cytochrome P450 isozymes were also isolated from DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 (Chapter 6). However, the procedure for isolate H_{17}, I_{33} and J_4 had to be slightly modified in order to isolate proper P450 isozymes. The necessary modification of the isolation procedure suggests that the resistant isolates H_{17}, I_{33} and J_4 differ in some way, possibly in cell wall composition, from that of the wild-type isolate. Maximum and minimum absorbance in type II spectra of P450 isozymes of isolates H_{17}, I_{33} and J_4 shifted to higher wavelengths as compared with those of the wild-type isolate W_5 and low-resistant isolate $E_{300\text{-}3}$. Maximum absorbance in CO spectra of P450 isozymes of isolates H_{17}, I_{33} and J_4 also shifted to higher wavelengths. Imazalil, itraconazole and ketoconazole were more readily displaced by CO from reduced P450 isozymes of isolates H_{17}, I_{33} and J_4 than from those of wild-type isolate W_5 and isolate $E_{300\text{-}3}$ (Chapter 6). These results suggest that P450 isozymes of isolates H_{17}, I_{33} and J_4 had a relatively lower affinity to DMI

fungicides. However, due to various factors which hamper a proper interpretation of the results, conclusions derived from spectrophotometric assays may not be reliable enough to compare affinity of cytochrome $P450_{14DM}$ to DMIs in different isolates. Hence, these results do not provide sound evidence to reject the conclusion that sterol 14α-demethylase of wild-type and DMI-resistant isolates have a similar sensitivity to DMIs (Chapter 4).

In Chapter 7, results of experiments on metabolism of imazalil in wild-type and DMI-resistant isolates are presented. It appeared that all isolates metabolized imazalil to its putative 2,3-dihydroxypropyloxy analogue (R42243), which was much less fungitoxic. DMI-resistant isolates showed cross resistance to miconazole which has a similar structure as imazalil except for the propenyloxy side chain. Therefore, it was concluded that metabolism of imazalil in *P. italicum* is not involved as a mechanism of resistance.

Fungicide accumulation studies indicated that the low-resistant isolate $E_{300\text{-}3}$ accumulated a significantly lower level of imazalil and fenarimol than the wild-type isolate W_5 (Chapter 8). The low level of accumulation of the fungicides may be responsible for the relatively low degree of resistance to these fungicides in this isolate. Reduced accumulation of fenarimol may be caused by an increased energy-dependent efflux of the fungicide. However, this mechanism is less obvious for imazalil. Accumulation of both fungicides is probably not mediated by the plasma membrane potential. Medium- and high-resistant isolates H_{17}, I_{33} and J_4 accumulated similar levels of imazalil and fenarimol as the low-resistant isolate $E_{300\text{-}3}$. This suggests that the mechanism of resistance in medium- and high-resistant isolates is not caused by differential accumulation of the DMI fungicides as compared to the low-resistant isolate $E_{300\text{-}3}$. However, it might be that differential accumulation of the DMIs between low- and high-resistant isolates is present, but masked by a relatively high background adsorption of the fungicides to mycelium and therefore, not detected with the test method used.

From the results of the studies summarized above, it is concluded that the mechanism of resistance in *P. italicum* to DMIs relates to factors which prevent the fungicides from reaching the target site. Further studies should elucidate which mechanism is relevant in this respect.

SAMENVATTING

De fungitoxische werking van azool-fungiciden is gebaseerd op remming van cytochroom P450-afhankelijke aktiviteit van sterol 14α-demethylase (cytochroom $P450_{14DM}$). Deze dissertatie beschrijft de resultaten van studies naar verschillende potentiële resistentiemechanismen tegen sterol 14α-demethyleringsremmers (DMIs) in DMI-resistente isolaten van de plant-pathogene schimmel *Penicillium italicum*. In de hoofdstukken 2, 4, 6, en 8 worden resultaten van studies naar vier potentiële resistentiemechanismen beschreven. In de hoofdstukken 3 en 5 worden twee belangrijke ontwikkelingen in experimentele methoden die essentieel zijn voor het testen van één van de potentiële resistentiemechanismen, verminderde affiniteit van cytochroom $P450_{14DM}$, besproken.

In hoofdstuk 2 wordt de sterol samenstelling van het wild-type isolaat W_5 en de isolaten $E_{300\text{-}3}$ (laag resistent) en H_{17} (middel resistent), I_{33} en J_4 (hoog resistent) beschreven. De resultaten toonden aan dat ergosterol in alle isolaten het belangrijkste sterol is. Remming van de biosynthese van ergosterol in W_5 werd reeds bij een lage imazalil concentratie (0,01 $\mu g\ ml^{-1}$) waargenomen, terwijl in resistente isolaten veel hogere concentraties nodig waren om het remmende effect te kunnen bereiken. Remming van de ergosterol biosynthese ging gepaard met accumulatie van 24-methyleendihydrolanosterol. Deze resultaten demonstreren dat dezelfde aangrijpingsplaats, cytochroom $P450_{14DM}$, aanwezig is in alle isolaten en dat het resistentiemechanisme dus niet is gebaseerd op afwezigheid van de aangrijpingsplaats of de aanwezigheid van een niet-functioneel 14α-demethyleringsenzym.

Hoofdstuk 3 beschrijft een belangrijke vooruitgang bij de *in vitro* bestudering van enzymen die bij de sterol biosynthese in filamenteuze, plant-pathogene schimmels betrokken zijn. Er wordt een nieuwe methode in beschreven voor de bereiding van celvrije extracten van *P. italicum* die in staat zijn om vanuit [^{14}C]mevalonaat ergosterol te synthetiseren. Dit systeem kan tevens als model dienen voor de bestudering van dezelfde biosyntheseweg in andere filamenteuze schimmels. De *in vitro* synthese van ergosterol in celvrije extracten van *P. italicum* bedroeg 26% van de totale hoeveelheid gesynthetiseerde, niet-verzeepbare lipiden. De remming van de *in vitro* ergosterol-biosynthese door verschillende teststoffen (DMI fungiciden en weinig-toxische imazalil-analogen) werd onderzocht. IC_{50} waarden

(concentraties die de incorporatie van radioaktiviteit voor 50% remmen) van DMI fungiciden met een relatief hoge fungitoxiciteit varieerden van $6.5 \pm 0.5 \times 10^{-9}$ tot $1.7 \pm 0.7 \times 10^{-8}$ M. Dit duidt er op dat DMI fungiciden zeer sterke remmers zijn van sterol 14α-demethylase aktiviteit in celvrije extracten van de schimmel. De minder toxische imazalil-analogen hadden veel hogere IC_{50} waarden, hetgeen suggereert dat deze verbindingen sterol 14α-demethylase aktiviteit veel minder sterk remmen. De methode die werd gebruikt om de ergosterol biosynthese in het wild-type isolaat W_5 te bestuderen, kon ook worden toegepast op de DMI-resistente isolaten $E_{300\text{-}3}$, H_{17} en I_{33}. De kwaliteit van de celvrije extracten van wild-type en resistente isolaten verschilde wel enigszins. Desalniettemin waren de IC_{50} waarden van imazalil voor remming van de *in vitro* ergosterol biosynthese in het wild-type en alle DMI-resistente isolaten niet significant verschillend (Hoofdstuk 4). Dit duidt er op dat het sterol 14α-demethylase van de verschillende isolaten een gelijke gevoeligheid heeft voor imazalil. Verminderde affiniteit van cytochroom $P450_{14DM}$ speelt daarom geen beduidende rol als resistentiemechanisme tegen DMIs in resistente isolaten.

In hoofdstuk 5 wordt een methode beschreven om cytochroom P450 isozymen te isoleren uit het wild-type W_5. De interactie tussen het heterocyclische stikstof atoom van de teststoffen (DMI fungiciden en weinig toxische imazalil analogen) en het geoxideerde haem ijzer atoom van de isozymen kon met behulp van verschilspectrofotometrie (type II spectra) worden gedemonstreerd. De grootte van de type II spectra correleerde echter niet met de toxiciteit van de teststoffen. Er werd een verschil waargenomen in het vermogen van koolmonoxyde (CO) om de teststoffen van de gereduceerde cytochroom P450 isozymen te vervangen. De weinig toxische imazalil-analogen werden onmiddellijk vervangen, terwijl de vervanging van verschillende DMI fungiciden geleidelijk verliep in de tijd. Dit suggereert dat de bindingsaktiviteit van de teststoffen voor cytochroom P450 isozymen enigszins met hun fungitoxiciteit correleerde. Er werden echter afwijkende waarden gevonden voor DMI fungiciden met een grote *N*1-substituent zoals itraconazool en ketoconazool.

Cytochroom P450 isozymen werden ook geïsoleerd uit de DMI-resistente isolaten $E_{300\text{-}3}$, H_{17}, I_{33} en J_4 (Hoofdstuk 6). De methode moest voor isolaat H_{17}, I_{33} en J_4 echter enigszins worden gewijzigd teneinde uit deze isolaten P450 isozymen van goede kwaliteit te kunnen isoleren. De noodzakelijke wijziging in de isolatieprocedure suggereert dat de resistente isolate H_{17}, I_{33} en J_4 in bepaalde opzichten, zoals in samenstelling van de celwand, zouden

kunnen verschillen van het wild-type isolaat. De maximum en minimum absorptie in de type II spectra van P450 isozymen van isolaten H_{17}, I_{33} en J_4 verschoof naar hogere golflengten in vergelijking met die van het wild-type isolaat W_5 en het laag-resistente isolaat $E_{300\text{-}3}$. De maximum absorptie in CO spectra van P450 isozymen verschoof ook naar hogere golflengten. Imazalil, itraconazool en ketoconazool werden sneller door CO vervangen bij P450 isozymen van isolaten H_{17}, I_{33} en J_4 dan bij P450 isozymen van het wild-type isolaat W_5 en isolaat $E_{300\text{-}3}$ (Hoofdstuk 6). Deze resultaten suggereren dat P450 isozymen van isolaten H_{17}, I_{33} en J_4 een relatief lagere affiniteit voor DMI fungiciden zouden hebben. Er zijn echter verschillende factoren die een goede interpretatie van de resultaten bemoeilijken. De conclusies van de spectrofotometrische testen worden daarom niet voldoende betrouwbaar geacht om de affiniteit van cytochroom $P450_{14DM}$ voor DMIs in verschillende isolaten te vergelijken. De resultaten vormen dus geen betrouwbaar bewijs om de conclusie te verwerpen dat sterol 14α-demethylase van het wild-type en de DMI-resistente isolaten een gelijke gevoeligheid hebben voor DMIs (Hoofdstuk 4).

In hoofdstuk 7 worden resultaten van experimenten over metabolisme van imazalil in het wild-type en in DMI-resistente isolaten beschreven. Het bleek dat alle isolaten imazalil waarschijnlijk omzetten in de 2,3-dihydroxypropyloxy analoog (R42243), die veel minder fungitoxisch is. DMI-resistente isolaten bezaten kruisresistentie tegen miconazool dat een structuur heeft die vergelijkbaar is met die van imazalil met uitzondering van de propenyloxy zijketen. Er werd daarom geconcludeerd dat metabolisme van imazalil in *P. italicum* niet opereert als een resistentiemechanisme.

Accumulatie proeven met fungiciden toonden aan dat het laag-resistente isolaat $E_{300\text{-}3}$ een aanzienlijk lagere hoeveelheid imazalil en fenarimol accumuleerde dan het wild-type isolaat W_5 (Hoofdstuk 8). De lage accumulatie kan verantwoordelijk zijn voor de lage graad van resistentie tegen deze fungiciden in dit isolaat. De verminderde accumulatie van fenarimol kan een gevolg zijn van een toename in energie-afhankelijke efflux. Dit mechanisme is echter minder duidelijk voor imazalil. De plasma membraan potentiaal is waarschijnlijk niet bij de accumulatie van beide fungiciden betrokken. Middel- en hoog-resistente isolaten H_{17}, I_{33} en J_4 accumuleerden de fungiciden tot hetzelfde lage niveau als in het laag-resistente isolaat $E_{300\text{-}3}$. Dit suggereert dat het resistentiemechanisme in hoog-resistente isolaten niet veroorzaakt wordt differentiële accumulatie van de DMI fungiciden in vergelijking met het laag-resistente isolaat $E_{300\text{-}3}$. Het mag echter niet worden uitgesloten dat

differentiële accumulatie van DMIs tussen laag- en hoog-resistente isolaten toch aanwezig is, maar gemaskeerd wordt door een relatief hoge achtergrond-adsorptie van deze fungiciden aan het mycelium en daardoor met de gebruikte testmethode niet kan worden waargenomen.

Op grond van de resultaten van hierboven samengevatte studies, wordt geconcludeerd dat het resistentiemechanisme van *P. italicum* voor DMIs verband houdt met factoren die voorkomen dat deze fungiciden hun aangrijpingsplaats in het mycelium bereiken. Verdere studies moeten inzicht verschaffen in het mechanisme dat hierbij betrokken is.

CURRICULUM VITAE

Jiansheng Guan was born at July 30, 1955 in Liaoning Province, China. He graduated from the Department of Plant Protection, Shenyang Agricultural University in China in 1983. In 1985 he came to the Netherlands and continued his study at the Department of Phytopathology of the Wageningen Agricultural University. In the final part of his study he did research subjects in phytopathology, entomology and virology. He passed his ingenieurs-examination in 1987. From 1987 till 1991 he worked as a research assistant (AIO) at the Department of Phytopathology of the Wageningen Agricultural University under the supervision of Dr. Ir. M. A. de Waard. Results of research work in this period are the basis of this thesis.