Biological activity of triazole fungicides towards *Botrytis cinerea*

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Biological activity of triazole fungicides towards *Botrytis cinerea* / Christiane Stehmann. -[S.l.: s.n.] (Wageningen : Ponsen & Looijen)
Thesis Landbouw Universiteit Wageningen. - With ref. - With summary in Dutch.
ISBN 90 - 5485 - 367 - 0
Subject headings: fungicides / sterol biosynthesis inhibitors / *Botrytis cinerea*.

Cover design: Niels Nolta, Waddinxveen

Financial support: Bayer AG, Leverkusen, Germany
Deutscher Akademischer Austauschdienst, Bonn - Bad Godesberg, Germany
Helmuth Aurentz Stiftung, Stuttgart - Hohenheim, Germany
Sandoz AG, Basel, Switzerland
Wageningen Agricultural University, Wageningen, the Nether-

Christiane Stehmann

Biological activity of triazole fungicides towards *Botrytis cinerea*

Proefschrift
ter verkrijging van de graad van doctor
in de Landbouw- en Milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 12 mei 1995
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Promotor: Dr. Ir. P.J.G.M. de Wit
Hoogleraar in de moleculaire fytopathologie

Co-promotor: Dr. Ir. M.A. de Waard
Universitair hoofddocent in de fytofarmacie

Stellingen

1 The cell-free sterol synthesis assay is a useful biochemical screen for inhibitors of sterol biosynthesis.
This thesis.

2 DMI antifungals interfere also with P_{450}-dependent enzymes other than fungal sterol 14α-demethylase.
This thesis.

3 Relatively low sensitivity of subpopulations and marginal application rates recommended contribute to the limited field performance of triazole fungicides towards grey mould diseases.
This thesis.

4 Compounds inhibiting energy-dependent efflux of DMI fungicides can synergize DMI activity.
This thesis.

5 The phytopathological definitions of *in vitro* and *in vivo* are confusing and should be changed.

6 Although members of the class *Oomycetes* are characterized by fungal-like morphology, they are more correctly placed in the kingdom *Protista*.
Griffith et al., 1992. In: Target Sites of Fungicide Action, Köller (ed.), CRC Press, Boca Raton, pp. 69-100.

7 The difference between public and private organisations is the same as that between work and power: the factor time.

8 The best parameter for the success of Women's Liberation is the percentage of fathers taking parental leave.

9 Activity of phosphonates towards *Oomycetes* is the combination of direct and indirect modes of action.
Guest & Grant, 1991. Biol. Rev. **66**, *159-187.*

10 Nature is often unpredictable since its complexity exceeds human imagination.

11 Ein Stehmann steht immer seinen Mann.
(Z.Z.)

12 Voetbal remt de verbetering van de Nederlands - Duitse relatie.

13 To be is to do. *Sokrates.*
To do is to be. *Sartre.*
Do be do be do. *Sinatra.*

Stellingen behorend bij het proefschrift

"Biological activity of triazole fungicides towards Botrytis cinerea",

te verdedigen op 12 mei 1995 te Wageningen.

Christiane Stehmann

"If a question can be put at all,
then it can also be answered."
L. Wittgenstein
Tractus logico philosophicus 6.5

Für Rai und Jule

Table of Contents

Glossary

AF	antagonism factor: ratio between EC_{50} of a test compound on B5-agar in the presence and absence of a putative antagonist
AI	active ingredient
ATP	adenosine 5'-triphosphate
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DMI	sterol 14α-demethylation inhibitor
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC_{50}	concentration of a compound inhibiting fungal growth by 50%
EDTA	ethylene diamine tetraacetic acid
HPLC	high performance liquid chromatography
IC_{50}	concentration of a compound inhibiting incorporation of [2-^{14}C] mevalonate into C4-desmethyl sterols by 50%
In vitro	experiments performed with cell-free assays and in Petri dishes
In vivo	experiments performed with intact plants or detached plant parts
log P	logarithm of the octanol/H_2O partition coefficient
MCE	ß-mercapto ethanol
NAA	nicotinic acid amide
N-AC	*N*-acetyl cysteine
NAD^+	nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
NSL	non-saponifiable lipid
PDA	potato dextrose agar
$P450_{14DM}$	cytochrome P450-dependent sterol 14α-demethylase
R_{EC50}	ratio between EC_{50} of test and reference compound
R_{IC50}	ratio between IC_{50} of test and reference compound
r_s	Spearman's ranking coefficient
SBI	sterol biosynthesis inhibitor
TLC	thin-layer chromatography
TLH	tomato leaf homogenate
transfer factor	ratio between EC_{50} of a compound determined in field and glasshouse trials
transfer ratio	ratio between EC_{50} of a compund determined in *in vivo* and radial growth tests
VF	variation factor: ratio between lowest and highest EC_{50} detected in a pathogen population
Q-value	ratio between EC_{50} of resistant mutant and sensitive wild-type isolate

Outline of this thesis

Botrytis cinerea Pers. ex Fr., the causal agent of grey mould, is one of the most ubiquitous plant pathogens.[1] The fungus is of high economic importance in various major crops and during transport and storage of agricultural products. Protectant fungicides such as chlorothalonil, dichlofluanid, folpet or thiram are widely used for disease control. Since their introduction in the 1960s/1970s, systemic fungicides such as the benzimidazoles or dicarboximides have been used extensively. However, their effectivity is severely hampered by a rapid development of resistance to these fungicides.[2-6]

Antifungal activity of N1-substituted azoles was discovered in the late 1960s. Since then, a large number of azole derivatives have been developed as agricultural fungicides and antimycotics. The mode of action of these azoles is based on inhibition of the cytochrome P450-dependent sterol 14α-demethylase ($P450_{14DM}$), an enzyme of the sterol pathway.[7-9] By now, sterol demethylation inhibitors (DMIs) comprise about 35 commercial products and represent the most important group of systemic fungicides. DMI fungicides are commonly applied in control of rusts, powdery mildews and scabs. Only few of them are registered for control of *B. cinerea*.[10-12] This is ascribed to a limited field performance for which the reasons are not evident. A replacement of dicarboximides or benzimidazoles by DMI fungicides would be attractive, since DMIs have a number of advantages over other fungicides including a relatively low resistance risk.[13-15]

The aim of the study described in this thesis is to identify factors involved in the limited field performance of DMI fungicides towards *B. cinerea*. The study is restricted to the largest group of DMIs, the triazoles. Before presenting results obtained in this study a literature review on the biology and control of *B. cinerea*, the mode of action and mechanisms involved in selective fungitoxicity of DMI fungicides, and factors responsible for discrepancies in laboratory and field pesticide performance is given (*chapter 1*). Biological activity of triazoles towards *B. cinerea* was investigated *in vitro* with cell-free assays (*chapters 3 - 4*) and toxicity assays (*chapters 3 - 7*) and *in vivo* on different hosts (*chapter 5*).

The first step in the research presented in this thesis was the development of a cell-free assay for sterol synthesis from the model fungus *Penicillium italicum* (*Moniliaceae*) according to a method described for *Aspergillus fumigatus*[16] (*chapter 2*). Subsequently, the method developed was adopted for *Botrytis cinerea* (*chapter 3*). This assay was used to study the relationship between chemical structure and biological activity of commercial and experimental triazoles and stereoisomers of cyproconazole, SSF-109 and tebuconazole towards *B. cinerea* (*chapter 4*). On basis of

these experiments intrinsic inhibitory activity of triazoles towards $P450_{14DM}$ of the target pathogen was determined. In following experiments, factors which influence *in vivo* activity or field performance were investigated. *In vivo* activity of triazole fungicides towards *B. cinerea* was tested on foliar-sprayed tomato plants and dip-treated grape berries, and compared with that of selected benzimidazoles and dicarboximides (*chapter 5*). In this context was also studied whether biological compounds could specifically antagonize activity of triazoles (*chapter 5*). Variation in triazole sensitivity of the pathogen population was studied for field isolates (121) of *B. cinerea* collected during 1970 - 1992 in Europe and Israel (*chapter 6*). In this survey less sensitive populations were detected. A putative mechanism of resistance to DMI fungicides in field isolates with a relatively low sensitivity to DMIs was studied and compared with that operating in laboratory-generated DMI-resistant mutants (*chapter 7*). Effects of inhibitors of mitochondrial respiration[17-19] and multisite-inhibiting fungicides on accumulation of tebuconazole were tested to evaluate their potency as candidate compounds in synergistic mixtures with DMIs (*chapter 7*). The development of synergistic mixtures may improve biological activity of DMI fungicides in control of *B. cinerea*.

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Chapter 1

General Introduction

The pathogen *Botrytis cinerea*

The fungus

Botrytis cinerea Pers. ex Fr. belongs to the class of *Fungi Imperfecti*, order *Moniliales* and family *Moniliaceae*. The name of the asexual (anamorph) state is derived from the ancient Greek *Βοτρυς*, meaning a bunch of grapes, since the oval blastoconidia are arranged on the conidiophores in a racemose pattern. The perfect (teleomorph) state is *Botryotinia fuckeliana* (de Bary) Whetzel.[1] Production of apothecia by *B. cinerea* under laboratory conditions was reported for the first time by Groves and Drayton (1939).[2] Reports on apothecia production in the field are rare.[3-6] Therefore, the fungus is still referred to as *B. cinerea* in literature, although the name of the perfect stage should be used. The name of the anamorph is also used in this thesis.

The anamorph is commonly known as grey mould, since the fungus is characterized by grey-brown conidia, which are disseminated by wind. In addition to conidia, *B. cinerea* produces *in vivo* and *in vitro* black sclerotia as a principal survival structure. The sclerotial state was described as *Sclerotium* Tode.[1,7] Depending on the origin of an isolate and cultural conditions, microconidia can be obtained *in vitro*. The sexual function of microconidia under laboratory conditions is widely accepted.[2,8-11] However, their relevance in sexual reproduction under field conditions remains to be confirmed.

Botrytis spp. belong to the most ubiquitous plant pathogens and saprophytes.[12] Geographically they occur wherever their host crops are grown, ranging from cool temperate zones of Alaska and Canada to subtropical areas like Egypt. Grey mould is the most widely spread and economically important disease on grapes, reducing quality and quantity of grapes.[13] Vines produced from rotten grapes have a reduced quality due to depletion of monosaccharides (glucose, fructose), and accumulation of metabolites (glycerol, gluconic acid) and enzymes catalyzing oxidation of phenolic compounds. These wines are also unsuitable for aging, since they are susceptible to oxidation and bacterial contamination.[14,15] Although *B. cinerea* causes severe damage in wine production (Sauerfäule, pourriture grise, bunch rot), it can lead under certain circumstances (Edelfäule, pourriture noble, noble rot) to heavy sweet vines of high quality such as the Sauternes (France), Tokays (Hungary) and Trockenbeerenauslesen (Germany, Austria).[14,15] Only infection of undamaged, fully mature berries during a dry period shortly before harvest increases quality of grape berries, as enhanced transpiration of infected berries results in exceptionally high sugar concentrations.

Infection and pathogenesis

The host-range of *B. cinerea* comprises about 235 plant species.[12] Additionally, there are about 25 other *Botrytis* spp., which have a smaller host range, such as *B. allii* (syn. *B. aclada*) infecting four *Allium* spp., *B. gladioli* infecting gladiolus and iris and *B. tulipae* infecting tulip and crocus. *Botrytis* spp. and especially *B. cinerea* are important pathogens of grape vine (*Vitis vinifera* L.) and other *Vitis* spp., small fruits, vegetables, bulbous monocotyledons, forest tree seedlings and glasshouse crops. Moreover *Botrytis* spp. cause severe post-harvest losses in stored and transported products. Probably, these post-harvest rots are due to latent infections in the field, which escape detection and develop only under special post-harvest conditions.

B. cinerea is primarily a saprophyte, present on moribund plant tissues.[16] The pathogen is known as a weakness parasite, initially establishing on senescing, stressed, weakened or dead plant parts and subsequently spreading into adjacent healthy tissue.[17-20] *B. cinerea* is also a secondary invader, attacking plants already infected by other pests.[20,21] Direct penetration of germ tubes via natural openings[19,22] or through the cuticle into undamaged tissues has also been observed.[23-26] This can be achieved by mechanical pressure, formation of appressoria-like structures and/or cutinase activity.[19,22,27] Edlich *et al.* (1989) associated formation of activated oxygen with the infection process.[28]

The most common symptoms of *B. cinerea* infections are decay, development of necrotic lesions, flecking or rotting. In this context, various enzymes degrading cell-walls (*e.g.* polygalacturonases, pectin lyases, cellulases)[29-31] or membranes (*e.g.* phospholipases, lipases)[32] and potential toxins (*e.g.* polysaccharides, organic acids)[33,34] may play a role during pathogenesis.

Epidemiology

Epidemics of *B. cinerea* depend mainly on production of conidia and their dispersal by wind, air currents (glasshouses), water droplets and insects.[35,36]

Conidia of *Botrytis* spp. are able to germinate and grow in the dark. Still, light affects various growth phases, such as conidia production, which is induced by near-uv light.[37] Conidia of *B. cinerea* have a broad temperature optimum for germination ranging between 12 and 22°C. Even at 0 - 5°C germination has been reported.[12] Relative humidity and availability of free water are important factors influencing the infection process. The requirement of a high relative humidity (93 - 100%) for germination relates to the low water content of conidia (ca. 17% of fresh weight).[16] Consequently, infection of hosts is generally higher, the longer conidia are exposed to surface wetness.[20] Presence of plant extracts or sugars and phosphates enhances germination, whereas the germination percentage in plain water is low.[16] As reported for other fungi, germination of conidia of *Botrytis* spp. is inhibited when the

concentration of conidia exceeds 10^5 or 10^6 ml^{-1}. This phenomenon is ascribed to self-inhibition.[16] However, germination of conidia at high concentrations increases when nutrients are added. This suggests, that competition for nutrients rather than self-inhibition suppresses germination of conidia.

Control

Control of *B. cinerea* is onerous, as the pathogen is able to attack virtually all parts of a crop at almost any stage of growth and agricultural products during transport and storage. Furthermore, the pathogen occurs on cultivated and wild hosts and can persist saprophytically on plant debris and organic material or as sclerotia and conidia. Disease control methods being explored or applied are breeding for host-resistance, cultural practices, biological and chemical control.

Breeding for resistance against *B. cinerea* is difficult, as major gene resistance against this pathogen is not known. This is probably due to the variability of infection processes and pathogenesis.[38] In some cultivars morphological properties (*e.g.* thickness of cuticula, hairy epidermis) or differences in growth (*e.g.* rapid ripening, density of grapes or leaves) may result in escape from grey mould infection. These characteristics are polygenically controlled, which hampers introduction of these genes into cultivars. Transformation of tobacco cells with a stilbene synthase gene from grape was reported to enhance resistance against *B. cinerea*.[39]

Cultural practices can reduce inoculum levels and create environmental conditions, which are less favourable for infection. These include:

- Hygienic measures, which reduce the amount of inoculum or infection sites, such as removal of dead, decaying or infected plant tissue. Wounding of plant tissue should be avoided, since it creates infection sites.
- Mulching reduces the dispersal of inoculum.
- A relatively low crop density creates a less favourable microclimate. This can be achieved by adequate spacing of the crop or removal of leaves.
- Foliar treatments with calcium nitrate delay senescence and result in plants less susceptible to grey mould infection.
- Exposure of conidia to surface wetness is minimized by ventilation or heating (glasshouses).

Cultural methods have mainly an ameliorative value and should be combined with other methods of grey mould control.[38,40,41]

Since the 1970s, biological control of *Botrytis* spp. with antagonists as *Aureobasidium* spp., *Bacillus subtilis*, *Gliocladium* spp. and *Trichoderma* spp. has been investigated. Most research has been performed with the fungal genus *Trichoderma*, a mycoparasite.[42] A general problem of biological control with antagonists is their

narrow temperature optimum of activity as compared to that of *B. cinerea*. *T. harzianum* has been tested successfully in Israel, Europe, South Africa and New Zealand on grapevine, cucumber, tomato, strawberry, bean, carrot and kiwi fruit.[42] Despite numerous positive results, application of biological agents is not yet a major strategy in grey mould control. Only in combination with other methods satisfactory grey mould control is obtained in a range of crops.

Chemical control of grey mould depends mainly on the availability of fungicides for certain crops and the current status of resistance development. Non-systemic fungicides with a multisite action such as chlorothalonil, dichlofluanid, dicloran, mancozeb, maneb, tolylfluanide and thiram are already for a long time available for grey mould control, as their activity has not been endangered by resistance development.[41,43] Their application is limited, because they act only preventively and their use is severely restricted. Substantial improvement of grey mould control was obtained by the introduction of benzimidazole fungicides in the late 1960s and dicarboximide fungicides in the early 1970s. After a few years these fungicides lost their initial high activity due to selection for resistant populations.[44-48] This resistance development reduced the use of benzimidazoles and dicarboximides. Despite a decreased selection pressure benzimidazole-resistant populations still persist whereas dicarboximide-resistant isolates occur less frequently, but also still persist. Diethofencarb, which showed initially good activity against benzimidazole-resistant strains (negative cross-resistance) also selected rapidly for resistant strains.[49] Sterol biosynthesis inhibiting fungicides (SBIs), which have a broad spectrum of antifungal activity, were introduced from the 1970s onwards. However, only few SBIs show activity towards *B. cinerea* under field conditions. These compounds are tebuconazole and prochloraz. Tebuconazole is marketed in mixture with dichlofluanid for control of grey mould and other pathogens in grapevine and prochloraz is applied against *B. cinerea* in tulip.[43,50] In general, the use of SBIs against *Botrytis* spp. is uncommon.[43,51,52] New fungicides with activity towards *B. cinerea* are the phenylpyrroles fenpiclonil and fludioxanil and the anilino pyrimidine mepanipyrim.[53-55]

Sterol 14α-demethylation inhibitors (DMIs)

Fungal sterol synthesis

Sterols are essential for all eukaryotes as membrane components (maintaining optimal membrane fluidity and integrity) and regulatory compounds (*e.g.* steroid hormone synthesis).[56-58] The first function of sterols mentioned is often referred to as the 'bulk' function. Large quantities are needed and structural requirements are low.

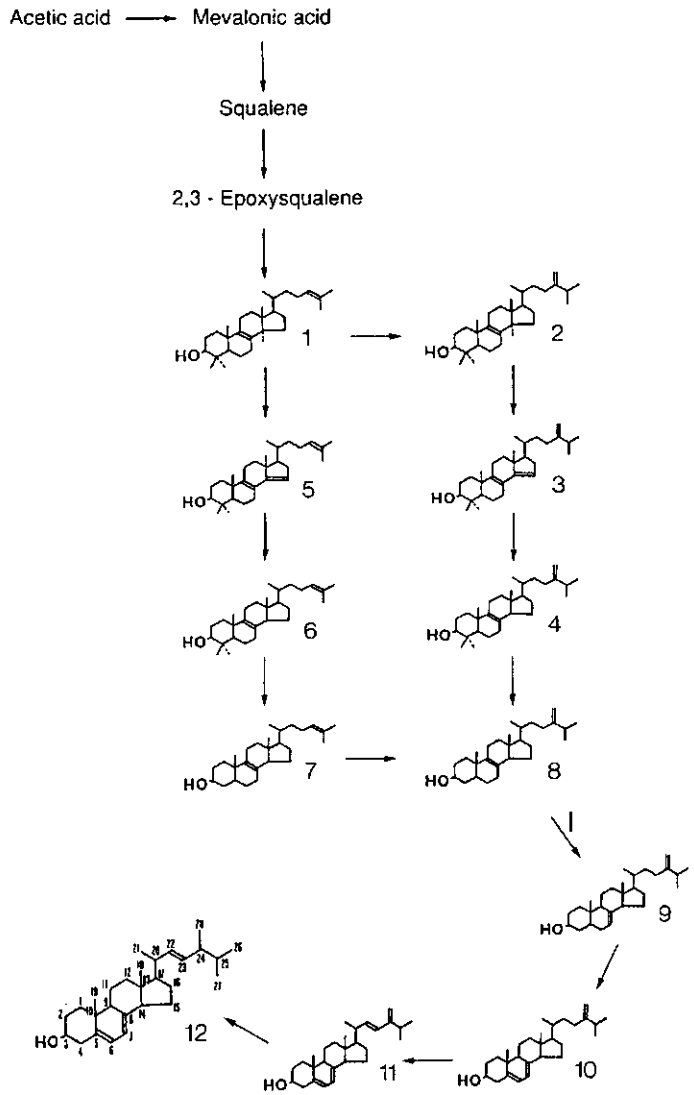

Fig. 1.1 Ergosterol biosynthesis pathway in fungi. (1) Lanosterol, (2) 24-Methylene dihydrolanosterol, (3) 4,4-Dimethyl-ergosta-8,14,24(28)-trienol, (4) 4,4-Dimethyl-ergosta-8,24(28)-dienol, (5) 4,4-Dimethyl-cholesta-8,14,24-trienol, (6) 4,4-Dimethyl-cholesta-8,24-dienol, (7) Cholesta-8,24-dienol (Zymosterol), (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. From: Kato (1986)[67]

The latter, so-called 'sparking' function requires only small amounts of a particular sterol and possesses high structural requirements.[59,60] Sterols demethylated in C14-position, such as ergosterol, seem to satisfy both functions in fungi.[58,61,62]

Ergosterol (ergosta-5,7,22-trienol) occurs widely in most higher fungi such as *Ascomycetes*, *Basidiomycetes* and *Fungi Imperfecti* and is also characteristic for *Zygomycetes*.[63] Ergosterol is not present in lower fungi such as the *Chytridiomycetes* and *Oomycetes*. *Oomycetes* are classified by their ability to synthesize sterols from mevalonate.[64] *Pythiaceae* can not synthesize sterols, but are able to produce squalene and transform exogenously added sterols. *Chytridiomycetes* and sterol synthesizing *Oomycetes* produce mainly fucosterol (stigma-5,24(28)-dienol) and cholesterol (cholesta-5-enol).

Sterol biosynthesis is part of the highly conserved isoprenoid pathway, which starts from acetyl-CoA and occurs in animals, plants and bacteria (hopanoid synthesis).[58,65] The pathway of sterol synthesis in fungi, summarized in Fig. 1.1, has been extensively reviewed.[65-67] The condensation of C_5 units (mevalonic acid) leads to the triterpene lanosterol (C_{30}), the first cyclization product of fungal sterol synthesis, or other terpenoids such as gibberellic acid (C_{20}) and carotenoids (C_{40}). Intermediates of the isoprenoid pathay are also precursors in synthesis of cytokinines, chlorophyll, heme and ubiquinones. In filamentous fungi, lanosterol is methylated in C24 position by Δ^{24}-methyltransferase. In yeasts zymosterol (cholesta-8,24-dienol) is believed to be the substrate for Δ^{24}-methyltransferase (Fig. 1.1). The enzyme uses *S*-adenosyl-methionine as methyl donor and is probably located in mitochondria. The previous steps and all subsequent reactions are believed to take place in the cytoplasm.[58,66] According to the different substrates of Δ^{24}-methyltransferase, removal of the C14-methyl group of the sterol core proceeds from lanosterol in yeasts and from 24-methylene dihydrolanosterol (eburicol) in filamentous fungi. Subsequent reactions include removal of the two methyl groups in C4-position and re-arrangements of double bonds in the sterol core and side chain (Fig. 1.1).

14α-Demethylation of sterols

The 14α-methyl group of eburicol or lanosterol is oxidized to a 14α-hydroxymethyl group, which is subsequently oxidized to a 14α-formyl group and eliminated as formate.[65] The enzyme involved belongs to the superfamily of cytochrome P450 mixed function oxidases (P450s) hydroxylating lipophilic substrates. P450s are heme containing proteins which show after reduction with dithionite and in the presence of carbon monoxide (CO) a maximal absorption at 450 nm.[68] In contrast to cytochromes involved in electron transfer, P450s are regarded as enzymes because they possess a substrate binding site. P450-dependent enzymes can be divided into two groups according to their biological function. (i) Catabolic enzymes metabolizing

xenobiotics. They are inducible and characterized by a low substrate specificity. (ii) Substrate specific enzymes involved in synthesis of endogenous lipophilic substrates (steroids, fatty acids, secondary metabolites).[69-71] Besides oxygenations, P450s catalyze reactions such as N-, S-, O-dealkylations, deaminations, dehalogenations, desulfurizations, epoxidations and isomerizations.[68] The total number of P450 species is not known, but is assumed to amount up to 200.[72]

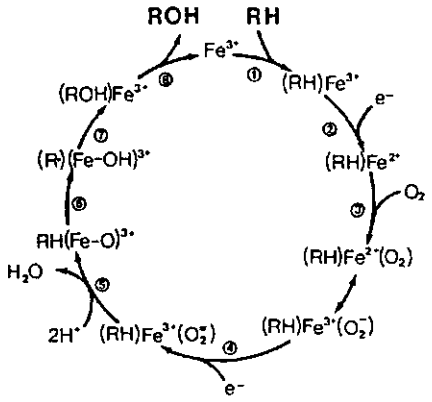

Fig. 1.2 Proposed scheme for the mechanism of action of cytochrome P450 in hydroxylation reactions. (RH) Substrate, (ROH) corresponding product, (Fe) heme iron atom at the active site of P450. From: Black & Coon (1988)[68]

The hydroxylation of lipophilic substrates by P450s requires molecular oxygen and NADPH and is characterized by the following equation:

$$RH + O_2 + NADPH + H^+ \rightleftharpoons ROH + NADP^+ + H_2O$$

The individual steps of the reaction, summarized in Fig. 1.2, are:

(1) binding of the substrate (RH) mainly by hydrophobic interactions with parts of the apoprotein moiety of the P450,

(2) transfer of the first electron, donated from NADPH, to NADPH-cytochrome P450 reductase leading to a ferrous P450 (Fe^{2+}),

(3) binding of molecular oxygen, the first step of oxygen activation,

(4) transfer of the second electron, which is donated by P450-reductase or cytochrome b_5,

(4) splitting of the oxygen-oxygen bond resulting in activated oxygen,
(5,6) incorporation of one oxygen atom into the substrate and the other one into $2H^+$ leading to formation of product alcohol (ROH) and H_2O,
(7,8) dissociation of the product (R-OH) and regeneration of the ferric P450 (Fe^{3+}).[68]

The P450 superfamily is divided into 11 families. According to the nomenclature the enzyme is referred to as P450 LI[69] and the encoding gene as *CYP51*.[72] In literature the enzyme is usually described with the trivial names $P450_{14DM}$ or 14α-DM. The heme iron Fe^{3+}, the prosthetic group of $P450_{14DM}$, is linked with four of its six coordination sites to the pyrrole nitrogens of the heme system and its fifth coordination site to the thiolate of cysteine in the apoprotein.[73] The amino acid sequence around this cysteine residue is highly conserved in various classes of P450s.[74] The sixth coordination site of the central Fe^{3+} is occupied by the nucleophile of the substrate eburicol (Fig. 1.3A).

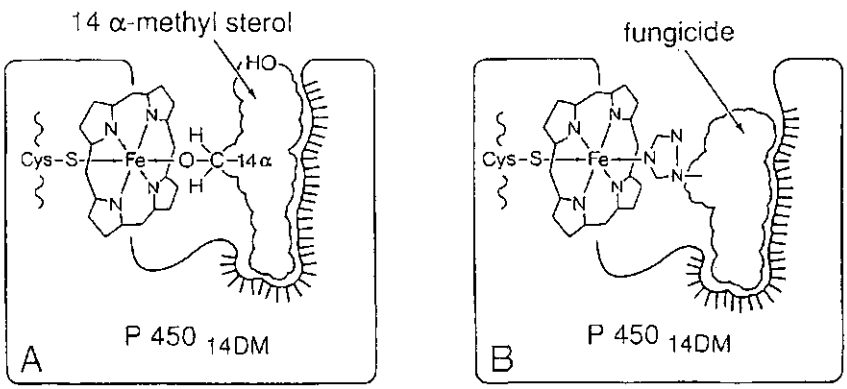

Fig. 1.3 Assumed structures of cytochrome P450-sterol 14-demethylation transition state complex **(A)** and cytochrome P450-fungicide complex **(B)**. Adapted from: Buchenauer (1987)[76]

Primary and secondary modes of action of DMIs

In the late 1960s, the excellent antifungal activity of azoles was discovered by researchers from Bayer AG (Germany), Dow Elanco (USA) and Janssen Pharmaceutica (Belgium). As a result of more than 20 years research and development about 40 DMIs have been developed for agricultural and clinical use.[66,75] Initially, these structurally diverse compounds were not regarded as biochemically related. However, they possess a common feature, the N-heterocycle containing a basic nitrogen. By now, it

is clear that these compounds have a common mode of action, inhibition of fungal $P450_{14DM}$. Spectrophotometrical and enzymatic studies have confirmed the molecular model of the inhibitory action of DMIs.[75-79] Spectral changes (type II spectra) indicated an interaction of the free electron pair of the N-heterocycle with the sixth coordination site of the heme iron (Fig. 1.3B). These studies also indicated a strong influence of the N1-substituent on inhibitory activity. It is believed, that the hydrophobic N1-substituent interacts with the apoprotein pocket, which is normally occupied by the substrate of the enzyme, lanosterol or eburicol. The structural flexibility of DMIs implies that the apoprotein pocket can be occupied by a variety of structures. Inhibition of $P450_{14DM}$ activity leads to accumulation of C14-methyl sterols as eburicol and lanosterol and rapid depletion of ergosterol.[58,66,80]

Inhibitory action of DMIs is clearly not restricted to fungal $P450_{14DM}$ only. Side effects of DMIs on hosts plants, which can lead to beneficial plant growth retardation (PGR) or to undesirable phytotoxic effects, are frequently reported.[81-85] Phytotoxic effects can be due to strong PGR activity, inhibition of plant sterol synthesis or direct membrane damage. PGR effects are often associated with inhibition of gibberellin synthesis (*ent*-kaurene oxidation). Such effects are reversed by exogenous supply of gibberellic acid.[84,85] PGR and phytotoxic effects have also been shown to parallel inhibition of plant sterol biosynthesis (obtusifoliol-14-demethylase).[81,83,86] Synergistic and antagonistic effects of DMI fungicides on activity of pyrethroid insecticides and phosphorothiolate fungicides, respectively, are ascribed to interaction of DMIs with P450-dependent mixed function oxidases involved in detoxification or activation of the respective pesticide.[87,88] Vanden Bossche (1992) reviewed various interactions of DMI pharmaceuticals with P450-dependent reactions from mammalian cells, such as (i) the 17α-hydroxylase involved in androgen synthesis, (ii) mitochondrial P450s involved in synthesis of *e.g.* cortisol or cholesterol, (iii) $P450_{AROM}$ involved in conversion of androgens to estrogens, (iv) P450-enzymes involved in thromboxane and prostacyclin synthesis and (v) P450-dependent reactions in the skin involved in metabolism of the vitamins A and D.[71] Interference of bifonazole, clotrimazole and triadimefon with fungal enzymes other than P450s, such as HMG-CoA reductase, have also been reported.[66] Secondary effects of DMIs on integrity of fungal and plant membranes due to interaction with desaturated fatty acids and other membrane lipids are supposed to contribute to phytotoxic and fungitoxic effects of these fungicides.[89-92]

Natural insensitivity and resistance to DMIs

DMI fungicides are characterized by their selective activity.[93] Selective action of DMI fungicides can be due to various mechanisms. Low sensitivity of fungal and plant species to triadimefon was attributed to the enantiomeric composition of triadimenol produced during metabolism of triadimefon.[94-96] Selective activity of triforine was

reported to depend on differences in either accumulation or metabolic breakdown of the fungicide.[97] Mechanisms described above prevent accumulation of the fungicide at the target site. In contrast, the DMI-insensitive fungus *Mucor rouxii* accumulated C14-methyl sterols, indicating that quantitative or qualitative differences in sterol requirements account for DMI-insensitivity of the fungus.[98] Natural insensitivity may also be due to differences in sensitivities of $P450_{14DM}$s from fungal species to DMI fungicides.[99,100]

Table 1.1 Mechanisms of acquired resistance to DMIs. Adapted from: De Waard (1994)[105]

Mechanism	Fungus	Reference
Circumvention of formation of toxic sterols	*Cercospora beticola*	110
	Saccharomyces cerevisiae	111
Defect in sterol 14α-demethylation	*Candida albicans*	112
	Ustilago maydis	113
Deposition in cell compartments	*Ustilago avenae*	114
Mutation of the gene encoding $P450_{14DM}$	*Nectria haematococca*	109
Overproduction of $P450_{14DM}$	*Saccharomyces cerevisiae*	115
Reduced uptake	*Aspergillus nidulans*	116
	Candida albicans	117
	Nectria haematococca	102
	Penicillium italicum	118

Since DMI fungicides possess a specific mode of action, they are more prone to resistance development than conventional multisite inhibitors. Strains resistant to DMIs are readily isolated under laboratory conditions.[101,102] However, the risk for development of DMI-resistance in the field is considered to be lower than for other singlesite-inhibitors such as the benzimidazoles and dicarboximides.[103,104] At present eroding field performance of DMI fungicides due to resistance development has been reported for a number of pathogens.[105] It is generally accepted that resistance to DMI fungicides is polygenic, implying that highly resistant strains develop by stepwise selection or hybridization of first step mutants.[106,107] In laboratory mutants these mutations appear to be pleiotropic, resulting in reduced saprophytic fitness and pathogenicity of resistant isolates.[101,102] In laboratory-generated mutants of *Nectria haematococca*, triadimenol resistance was found to be due to a highly mutable gene coding for high levels of resistance, which did not result in reduced

fitness resistant isolates.[108,109] Resistance in these mutants is ascribed to a mutation of the gene encoding $P450_{14DM}$.

Several mechanisms of resistance to DMIs, described in the past, are reviewed in Table 1.1. Research was mostly performed with laboratory-generated mutants. The relevance of these mechanisms for natural insensitivity or acquired resistance to DMI fungicides in field isolates remains to be established.

Apparent discrepancies between laboratory and field performance of pesticides

The development of new agricultural chemicals requires several years of research, which needs large quantities of the compound and a variety of tests, such as *in vitro* and *in vivo* screens, field trials and risk assessments (environment, farmer, consumer, residue analyses).[119,120] A major factor in the process of discovery and development of a new compound for registration are the costs involved (Table 1.2).

Table 1.2 Costs for the development of an agricultural chemical. Adapted from: Giles (1989)[121]

Development phase		Compounds per registered product	Total costs 10^3 US $
Research	First synthesis and glasshouse testing	22,000	80,000
	Resynthesis and first field experiments	150	2,000
Development	Synthesis optimization, large scale field experiments and product safety	7.5	12,000
	Further synthesis optimization, full field development, product safety and registration	1.5	15,000
Registered product (approximately)		1	109,000

More than 50% of the chemicals screened come from random sources, such as compounds synthesized for other biological targets, or are derived from secondary metabolites of fungi and bacteria. Key to the development of new active compounds is a standardized approach to discover and evaluate biological activity of candidate compounds (screen).[121] Primary screens show whether or not a compound has activi-

Table 1.3 Comparison of screening procedures used in greenhouse and field trials. Adapted from: Gold *et al.* (1994)[124]

Parameter	Greenhouse	Field	Consequence for active ingredient
Fungus			
Inoculum	One isolate	Many isolates	Level and spectrum of activity
Infection time relative to treatment	Defined, constant Single massive infection period	Variable Numerous infection periods	Spectrum of activity
Genetic variation	Sensitive population	Mixed populations	Spectrum of activity
Host plant			
Age, condition	Young plants	Variable	Retention, penetration, transport, metabolism
	Intact epidermis	Weathered epidermis	Retention, penetration, transport, metabolism
Morphology	Constant	Variable	Retention, penetration, transport
Environment			
Light	Artificial light	Natural light	Photolytic breakdown
Temperature	Defined	Variable	Volatility, penetration, metabolism
Relative humidity	Defined	Variable	Penetration
Wind	None	Variable	Volatility, penetration
Rain	None	Variable amounts and durations	Redistribution, wash-off, resolubilization
Chemical, technical			
Formulation	Organic solvent and emulsifier in water	Various formulations in water	Solubility, penetration
Spray volume	500 - 2000 l ha^{-1}	100 - 500 l ha^{-1}	Coverage
Spray concentration	Low	High	Solubility, penetration
Spray deposit	Complete coverage of plants	Incomplete coverage of plants	Level of activity
Duration of experiments	1 - 3 weeks	1 - 3 months	Residual activity

ty. Therefore, a single or in some cases two or more doses are applied to a selected range of target species (between 5 and 17 species in a fungicide screening).[120,121] In secondary screens the level of activity is defined and compared to that of structural analogues. In addition, compounds are selected for characteristics such as systemic activity, activity rate and persistence. Activity, expected from these glasshouse experiments, is then evaluated under field conditions (field screens). These tests reveal whether compounds should be candidates for expensive risk assessment studies. The reproducibility and correlation of results obtained from laboratory or greenhouse tests is generally not a problem. In contrast, the dosage required to control a particular disease under glasshouse conditions or in the field may vary up to a factor of 2000.[120] Such high factors often create doubts about the relevance of laboratory and greenhouse tests. However, the apparent discrepancy between glasshouse and field tests, the so-called 'transfer factor', should be recognized as the result of a complex interaction between environmental, chemical and biological factors within the field situation.[122,123]

The biological activity of a pesticide in any test system is the product of various factors: the amount of active ingredient reaching the target site, the intrinsic inhibitory potency of the active ingredient and the biological importance of the target functioning in the epidemic of an organism.[124] Any parameter affecting one of these factors will therefore influence biological activity of the active ingredient, regardless of the biological complexity of experiments (*in vitro*, greenhouse, field). The differences in test procedures for greenhouse and field screenings are numerous and involve biological, chemical, environmental and technical parameters (Table 1.3). The altered fate of the active ingredient within the target fungus, the host plant or the environment may then result in the high 'transfer factors' mentioned above. Therefore, the screening process should be regarded as a stepwise assessment and understanding of biological activity of test compounds.

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Chapter 2

Ergosterol Biosynthesis in a Cell-Free Preparation of *Penicillium italicum* and its Sensitivity to DMI Fungicides

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Pesticide Biochemistry and Physiology **42** (1992) 262-270

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-methylene dihydrolanosterol. Inhibition of ergosterol synthesis by fungicides which inhibit sterol 14α-demethylation (DMIs) led to accumulation of 24-methylene dihydrolanosterol 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 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-methylene dihydrolanosterol.[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 on sterol 14α-demethylation can be tested, is essential. The assay can also be helpful in optimizing 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 (R 14821), 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*N*-imidazole (R 42243), ketoconazole, and itraconazole were gifts from Janssen Pharmaceutica (Beerse, Belgium); penconazole and propiconazole from Ciba Geigy AG (Basel, Switzerland). Imazalil and the other compounds were used in 1000 x 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 mM). 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 (5 M) to pH 7.0 and 6.7, respectively.

Fungus and culture conditions

Wild-type isolate W5 of *P. italicum* was maintained on malt extract agar medium. Preparation of fungal cultures (11 h old) and mycelial suspensions were carried out according to methods described previously.[15]

Preparation of cell-free extract

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, Bartlessville, 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 four times for 30 s with 30 s intervals while the outer jacket around the vessel was filled with ice water. The homogenate was filtered over two layers of cheese cloth (presoaked in the buffer). Glycerol (88% purity) was gently mixed with the filtrate to a final concentration of 20% (by volume). The mixture was centrifuged twice at 3000 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 (19 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 (5 M). The volume of K_2HPO_4 solution added never exceeded 1% of the total volume. The mixture was divided into 994 μl 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). Test tubes were incubated in a reciprocal water bath shaker (80 strokes per min) at 25°C for 3 h in the dark. The caps were opened every hour. The pH of the incubation mixtures was measured again after incubation. Effect 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 $K_2HPO_4 \cdot 3H_2O$ (5 M). Experiments were carried out at least in threefold with different cell-fee 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 h. 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 by volume) 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 R_f values of different fractions with those of authentic compounds (uv) and 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 LS5800). The radioactivity recovered from TLC plates was considered as radioactivity incorporated into 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 radio-HPLC equipment consisting of a Waters 510 HPLC pump, two uv detectors (Waters 484 and 481, Millipore, Milford, MA) set at 280 and 210 nm, respectively and an on-line radioactivity monitor (Canberra Radiomatic, A-200, Radiomatic Instruments and Chemical Co., Inc., Tampa, IL) using Pico-Aqua (Packard Instrument Company, Inc., Downers Grove, IL) 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 x 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 conditions (pH 7.3) incorporation of radioactivity into non-saponifiable lipids was on average (n = 13) 23 $\pm$ 2% of total radioactivity added. Separation of the

non-saponifiable lipids by TLC revealed several distinct bands (Fig. 2.1, control treatment). Bands 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 (composed of 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 26 ± 4, 12 ± 4 and 32 ± 7% respectively (n = 13). Water and DMSO added in controls did not have any obvious effect on synthesis of sterols.

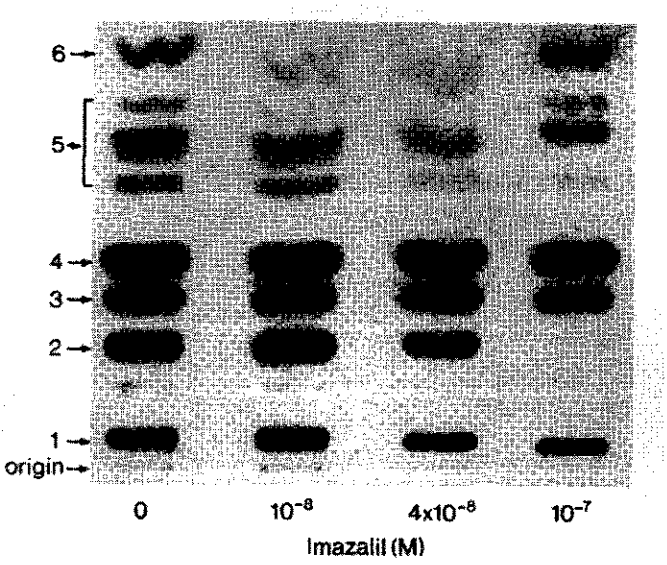

Fig. 2.1 Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C] mevalonate in cell-free bioassays of *Penicillium italicum* W5 in the absence and the presence of imazalil at pH 7.3. Bands 2, 3 and 4 contained C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl 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 ± 0.3 mg ml^{-1} (n = 13). The average protein content of cell-free extracts in assays with individual compounds (n ≥ 3) did not differ from the average value mentioned above.

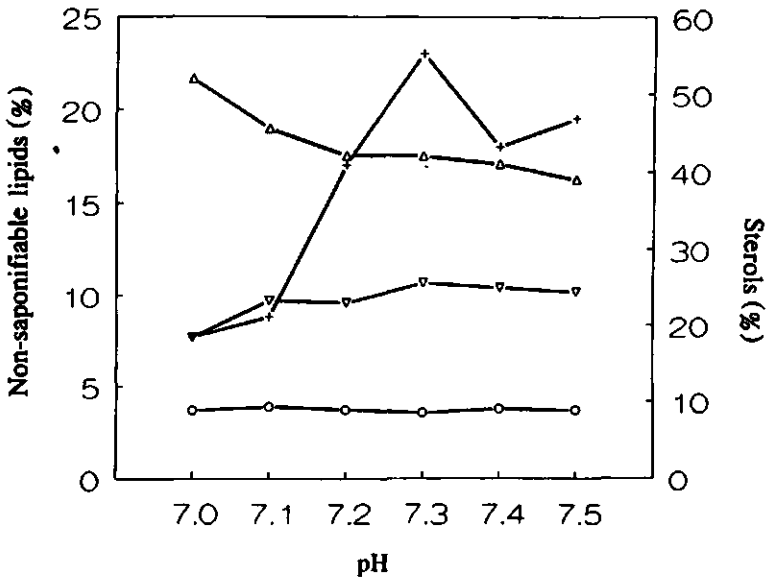

Fig. 2.2 Effect of pH on incorporation of [2-^{14}C]mevalonate into non-saponifiable lipids (+), C4,4-dimethyl sterols (△), C4-monomethyl sterols (○), and C4-desmethyl sterols (▽) in a cell-free extract of *Penicillium italicum* W5. Left ordinate: radioactivity in non-saponifiable lipids as percentage 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 during 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 relatively higher protein content. Although incorporation of radioactivity into C4,4-dimethyl sterols did occur, synthesis of C4-desmethyl sterols in these extracts was poor (data 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. 2.2). 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. 2.2). 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 the 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.

Table 2.1 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* W5 at pH 7.3.

Compound	$IC_{50} \pm SEM$[1] [M]	n[2]
Imazalil	$1.6 \pm 0.4 \times 10^{-8}$	10
Itraconazole	$1.0 \pm 0.3 \times 10^{-8}$	3
Ketoconazole	$6.5 \pm 0.5 \times 10^{-9}$	3
Penconazole	$1.7 \pm 0.7 \times 10^{-8}$	4
Propiconazole	$1.0 \pm 0.2 \times 10^{-8}$	3
R 14821	$6.0 \pm 0.7 \times 10^{-6}$	3
R 42243	$4.0 \pm 0.3 \times 10^{-5}$	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.

Inhibition studies

Fig. 2.1 shows an autoradiogram of a TLC plate on which total non-saponifiable lipids extracted from the 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 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 these bands was only slightly affected by imazalil (Fig. 2.1).

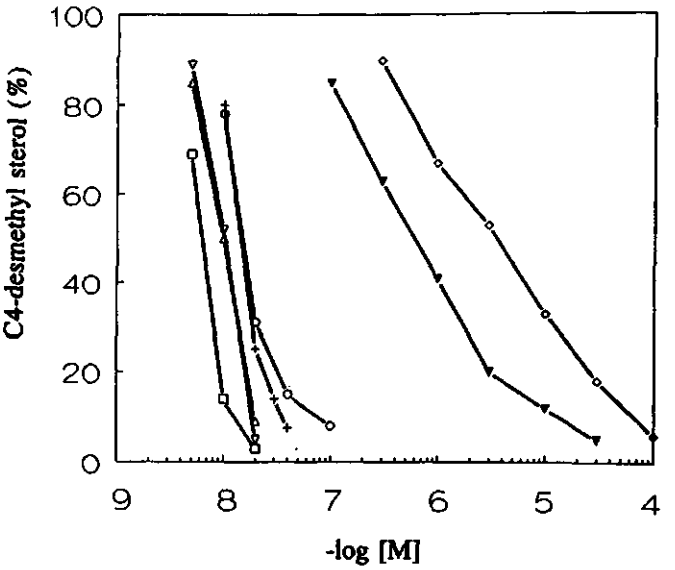

Fig. 2.3 Inhibition of incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols in cell-free bioassays of *Penicillium italicum* W5 at pH 7.3 by (+) imazalil, (▽) itraconazole, (□) ketoconazole, (○) penconazole, (△) propiconazole and imazalil analogues (▾) R 14821 and (◇) R 42243. Abscis: concentration of test compounds. Ordinate: radioactivity in C4-desmethyl sterols as percentages of control treatment.

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. 2.3. 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, R 14821 and R 42243, also showed an inhibitory effect but only at much higher concentrations (Fig. 2.3, Table 2.1).

Identification of radiolabelled sterols

Non-saponifiable lipids were analyzed with radio-HPLC. In control samples, five 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 (peak 5), respectively (Fig. 2.4A). Peak 3 and 4 had retention times identical to those of authentic ergosterol and lanosterol, respectively (data not shown). In imazalil-treated samples (10^{-7} M), only four major peaks were observed. Compared with control samples, peak 3 disappeared and peak 5 increased significantly (Fig. 2.4B). 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 2.2) suggests that the identities of peak 3, 4 and 5 are most likely ergosterol, lanosterol and 24-methylene dihydrolanosterol, respectively. The identity of other lipids was not studied any further.

Table 2.2 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* W5.

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-methylene dihydrolanosterol
5	Unknown	Not recovered	
6	Squalene	Not recovered	

[1] See Fig. 2.1.
[2] See Fig. 2.4A.

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. 2.2). 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 2.2; Fig. 2.4). These data suggest that ergosterol is the only C4-desmethyl sterol synthesized in the cell-free preparations. Other sterols identified in the non-saponifiable lipids were

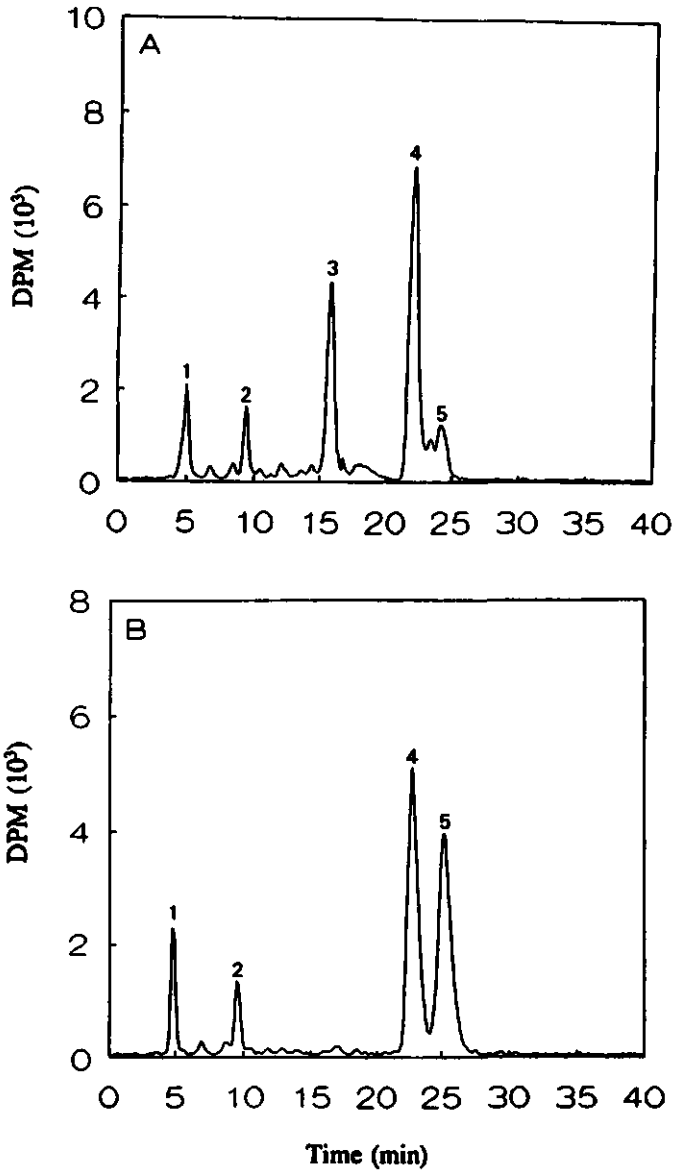

Fig. 2.4 Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from control (A) and imazalil-treated (10^{-7} M) (B) cell-free bioassays of *Penicillium italicum* W5 at pH 7.3. The identities of peak 3, 4 and 5 are ergosterol, lanosterol and 24-methylene dihydrolanosterol, respectively.

lanosterol and 24-methylene dihydrolanosterol (Table 2.2; Fig. 2.4). 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 2.2 and Fig. 2.4). The tentative identification of 24-methylene dihydrolanosterol 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-methylene dihydrolanosterol.[17] Mass spectrometry of the compound in peak 5 demonstrated the presence of 24-methylene dihydrolanosterol (Vanden Bossche, pers. comm.). Intact mycelium of *P. italicum* contains ergosterol and only a trace amount of 24-methylene dihydrolanosterol.[16] Therefore, synthesis of a large amount of lanosterol and a trace amount of 24-methylene dihydrolanosterol in cell-free extracts suggests a rate limiting step in ergosterol biosynthesis. This is probably the C24 side chain alkylation of lanosterol and may result from hampered transport of lanosterol into mitochondria.[14] Incubation of cell-free extracts with imazalil led to accumulation of 24-methylene dihydrolanosterol (Fig. 2.4B). 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. 2.2). This result is different from that reported for the cell-free assay of *A. fumigatus*, which had an optimal pH sharply confined between pH 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 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-methylene dihydrolanosterol 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} to 1.7×10^{-8} M). Values for the less toxic DMI analogues (R 14821 and R 42243) were much higher (6×10^{-6} and 4×10^{-5} M) (Table 2.1). 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 with 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 DMI fungicides tested did not correlate with their fungitoxicity. For instance, imazalil which was the most toxic DMI fungicide tested[15] showed a significantly higher IC_{50} value than ketoconazole (Table 2.1). 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.

Acknowledgements

The authors are grateful to Prof. Dr. J. Dekker for critical reading the manuscript and to Janssen Pharmaceutica for financial support.

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Chapter 3

Development of a Cell-Free Assay from *Botrytis cinerea* as a Biochemical Screen for Sterol Biosynthesis Inhibitors

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Pesticide Science **40** (1994) 1-8

Abstract

An assay for measuring ergosterol synthesis in cell-free extracts of the filamentous plant pathogen *Botrytis cinerea* is described. The extracts capable of synthesizing C4-desmethyl sterols from (2-^{14}C)mevalonate were derived by mechanical disruption of young conidial germlings in a Bead-Beater apparatus. The C4-desmethyl sterol fraction consisted of three distinct compounds and totalled 39% of the non-saponifiable lipids formed. Ergosterol accounted for 63% of the C4-desmethyl sterols. Only small amounts of C4-monomethyl sterols were synthesized, while C4,4-dimethyl sterols made up 29% of the non-saponifiable lipids. The latter fraction mainly consisted of lanosterol (54%) and eburicol (28%). The cell-free system had a narrow pH optimum for synthesis of C4-desmethyl sterols of pH 7.3 - 7.4. Cell-free synthesis of C4-desmethyl sterols was inhibited by the imidazole fungicide imazalil, concomitant with an accumulation of eburicol. The IC_{50} value (concentration of fungicide inhibiting cell-free synthesis of C4-desmethyl sterols by 50%) was 9.1×10^{-9} M. These results are consistent with the hypothesis that imazalil is a potent inhibitor of the cytochrome P450-dependent sterol 14α-demethylase of *B. cinerea*. The method described may be used to screen compounds biochemically for inhibition of sterol synthesis in an agriculturally important plant pathogen.

Introduction

In the last two decades the development of resistance to benzimidazole and dicarboximide fungicides has severely hampered chemical control of *Botrytis cinerea* Pers. ex Fr. During this period, fungicides which inhibit sterol biosynthesis (SBIs) were developed. Sterol demethylation inhibitors (DMIs) constitute the largest group of SBIs. The primary target of these antifungals is the cytochrome-P450-dependent 14α-demethylase ($P450_{14DM}$).[1,2] Although DMIs have a broad antifungal spectrum only few of them show substantial biological activity towards the economically important plant pathogen *B. cinerea*.[3-6] Registration of more DMIs for control of *B. cinerea* is therefore desirable. A cell-free sterol synthesis assay from this filamentous fungus would be useful to study the quantitative structure-activity relationships of potential DMI-fungicides, so that activity of DMIs towards *B. cinerea* could be optimized. The information would aid the understanding of the selectivity of DMIs for different pathogens and could be used to screen for SBIs which inhibit sterol synthesis at sites other than the $P450_{14DM}$.

Cell-free extracts capable of synthesizing ergosterol from the sterol precursor mevalonate have been largely confined to *Saccharomyces cerevisiae* Meyer[7,8] and the yeast form of *Candida albicans*.[9-11] These assays have often been used to optimize the fungitoxicity of candidate fungicides. This is not an ideal situation, since sensitivity of

$P450_{14DM}$ activity of different fungi to a specific DMI is not necessarily the same. For a long time attempts to develop comparable assays from plant pathogenic filamentous fungi have been unsuccessful.[12] This might have been due to instability of the membrane-bound $P450_{14DM}$ during preparation of the cell-free extract or to a relatively low concentration of the enzyme in filamentous fungi. A difference in the sterol biosynthetic pathway (transmethylation of lanosterol instead of zymosterol, probably in mitochondria) might be an additional explanation.[13,14] The development of sterol 14α-demethylase assays has recently been reported for the filamentous fungi *Aspergillus fumigatus* Fres.[15] and *Penicillium italicum* Wehmer.[16] Nevertheless, a similar assay for an agriculturally important plant pathogen is still not available.

In this paper a method is described for obtaining a cell-free extract of the filamentous plant pathogen *B. cinerea* capable of synthesizing C4-desmethyl sterols. To evaluate the validity of this assay, inhibitory action of the imidazole DMI fungicide imazalil on $P450_{14DM}$ activity was studied.

Materials and methods

Chemicals

[2-^{14}C]mevalonate, dibenzethylenediamine salt in ethanol (sp. act. 1.9 GBq mmol^{-1}), was purchased from Amersham International plc (Amersham, UK). Nicotinamide-adenine dinucleotide (NAD^+), nicotineamide-adenine dinucleotide phosphate ($NADP^+$, NADPH), adenosine 5'-triphosphate (ATP), glucose-6-phosphate, L-methionine, dimethyl sulfoxide (DMSO), oxytetracycline, dithiothreitol (DTT), ß-mercapto ethanol (MCE), ergosterol, lanosterol and squalene were from Sigma (St. Louis, Mo., USA). Reduced glutathione and *N*-acetyl cysteine were from Boehringer (Mannheim, Germany). Nicotinic acid amide was from Hoffmann-La Roche (Basel, Switzerland). Imazalil hydrogen sulphate was kindly supplied by Janssen Pharmaceutica (Beerse, Belgium).

Fungus and culture conditions

The monoascospore isolate of *B. cinerea* SAS56,[17] a gift from Dr. F. Faretra (Bari, Italy), was maintained on PDA slants. Conidia of *B. cinerea* were obtained from PDA cultures (30 ml) in Petri dishes (diameter 9 cm) incubated at 20°C for 24 h in the dark and for 14 to 20 days under near-uv light. Subcultures were made every three to four days by transferring agar plugs with young sporulating mycelium to the centre of agar plates. Flasks (2 l) with liquid synthetic media (1 l) prepared according to Fritz *et al.*[18] were inoculated with washed conidia collected from these plates (initial density 2×10^6 conidia ml^{-1}). The cultures were incubated in a rotary shaker (200 rpm) in the dark at 20°C for 14 h. By then, germination percentages of conidia exceeded 90%.

A cell-free assay from B. cinerea

Germinated conidia had one to three (usually two) germ tubes. The average germ tube length of the longest germ tube was 140 ± 6 μm.

Preparation of cell-free extract

Cell-free extracts were prepared according to a modified method described by Guan *et al.*[16] A standard germling suspension of *B. cinerea* was made by passing 14 h old cultures (harvest amounted to about 3 g wet weight l^{-1} medium) through a 0.2 mm pore sieve to remove clusters of mycelium and collecting the germlings on a 0.05 mm pore stainless steel sieve. Germlings collected were washed extensively with running cold tap water and then with ice-cold potassium phosphate buffer (100 mM, pH 7.5, 2 x 250 ml). Washed germlings were resuspended in the buffer to give a ratio of 100 mg wet weight (27 mg dry weight) ml^{-1} buffer. Subsequent steps were carried out at 0 - 4°C. A 32-ml Bead-Beater vessel (Biospec Products, Bartlesville, Okl., USA) containing 15 g glass beads (0.5 mm diameter) was completely filled with germling suspension. The remaining air was removed by evacuation at -100 kPa for 5 min. The vessel was again completely filled with germling suspension. Fungal cells were disrupted four times for 30 s with 30 s intervals, while the outer jacket of the vessel was filled with ice-water. The disruptor was driven at 100 V. The resulting homogenate was filtered through two layers of gauze (Klinion, Medical Care, Untermöhlen, Utrecht, the Netherlands) presoaked in buffer. Glycerol was added immediately to the filtrate (final concentration 20 ml l^{-1}), gently mixed and centrifuged twice at 3000 g for 10 min to sediment cell debris. The resulting supernatant was used immediately for sterol synthesis assays. The protein concentration in cell-free preparations was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Veenendaal, the Netherlands) with bovine-γ-globulin as a standard. The absence of any intact cells was verified by microscopic observation and streak tests on PDA.

Sterol synthesis assay

Standard sterol synthesis assays were carried out according to a modified method of Ballard *et al.*[15] Incubation mixtures (1 ml) consisted of cell-free extract (924 μl), cofactor solution (50 μl; containing 1 μmol NADPH, 1 μmol $NADP^+$, 1 μmol NAD^+, 3 μmol glucose-6-phosphate, 5 μmol ATP, 1 μmol reduced glutathione in distilled water adjusted to pH 7.2 with 1 M KOH), L-methionine (5 μl; 2 μmol) and divalent cation solutions (10 μl containing 5 μmol $MgCl_2$ and 5 μl containing 2 μmol $MnCl_2$, both solutions adjusted to pH 7.0 with 1 M KOH). The above solutions were combined (normally 20 ml), adjusted to pH 7.4 with 5 M K_2HPO_4 (which never exceeded 1% of the total volume) and samples of 994 μl were divided into screw-capped tubes. DMSO (control treatments) or DMSO-solutions of imazalil (1 μl) were added to the incubation

mixtures. Sterol synthesis was started by addition of [2-^{14}C]mevalonate (5 μl; 18.5 kBq; 8.5 nmol). In standard experiments, mixtures were incubated in a reciprocal water bath shaker (80 strokes min^{-1}) at 20°C in the dark for 2 h. Reaction was stopped by adding 1.5 ml of freshly prepared KOH (200 g l^{-1}) in ethanol + water (90 + 10 by volume). Effects of pH on cell-free sterol synthesis were studied by adjusting incubation mixtures with KH_2PO_4 (3 M) or K_2HPO_4 (5 M) to the pH values tested. The pH of mixtures was measured again after incubation.

Saponification, sterol extraction and analysis

Saponification of incubation mixtures, extraction and analysis of the non-saponifiable lipids (NSLs) by thin layer chromatography (TLC) and radio-HPLC were carried out essentially as described by Guan *et al.*[16] In order to identify components of the C4-desmethyl, C4-monomethyl and C4,4-dimethyl sterol fractions separated on TLC plates, zones containing sterol fractions were cut out of the plates, extracted with chloroform and re-chromatographed on TLC-plates pretreated with silver nitrate as described by Kerkenaar *et al.*[19] Ergosterol, lanosterol and eburicol (24-methylene dihydrolanosterol) were identified by co-chromatography with authentic standards. Incorporation of [2-^{14}C]mevalonate into different sterols was determined by cutting radiolabel-containing zones from TLC-plates, which were then counted for radioactivity in a liquid scintillation spectrophotometer (Beckman LS 5800).

Inhibitory effects of imazalil on sterol synthesis

Effects of imazalil on sterol synthesis were investigated by incubating cell-free extracts with the inhibitor at various concentrations. Incorporation of radiolabel into C4-desmethyl sterols was calculated as a percentage of total incorporation into NSLs.[8] Subsequently, corresponding incorporation rates of fungicide treatments were calculated as percentages of control treatments (100%). Using the soft-ware program Lotus 1-2-3, percentages were plotted against fungicide concentration on a logarithmic scale, regression analysis of these inhibitor-response data was performed and the imazalil concentration which inhibited synthesis of C4-desmethyl sterols by 50% (IC_{50}) was calculated. The experiment was repeated four times.

Results

Characterization of cell-free sterol synthesis

Incorporation of [2-^{14}C]mevalonate into NSLs accounted for 27.0 $\pm$ 4.6% (n = 10) of the total radiolabel added. Separation of these NSLs by TLC resulted in a typical separation pattern as illustrated by the autoradiogram presented in Fig. 3.1 (control

lane). Upon co-chromatography with authentic samples of ergosterol and lanosterol and by comparison with literature data[8,11,15] bands 2, 3 and 4 were tentatively identified as C4-desmethyl sterols, C4-monomethyl sterols and C4,4-dimethyl sterols, respectively. Similarly, band 7 was identified as squalene. Literature data suggest that band 6 is probably 2,3-oxidosqualene.[8,20] The identities of bands 1 and 5, composed of several zones, are unknown. C4-desmethyl sterols represented the major component of the sterols formed (39.0 ± 4.4% of the NSLs); C4,4-dimethyl sterols accounted for 28.0 ± 2.9%, while only minor amounts (3.2 ± 1.1%) of C4-monomethyl sterols were synthesized (n = 10).

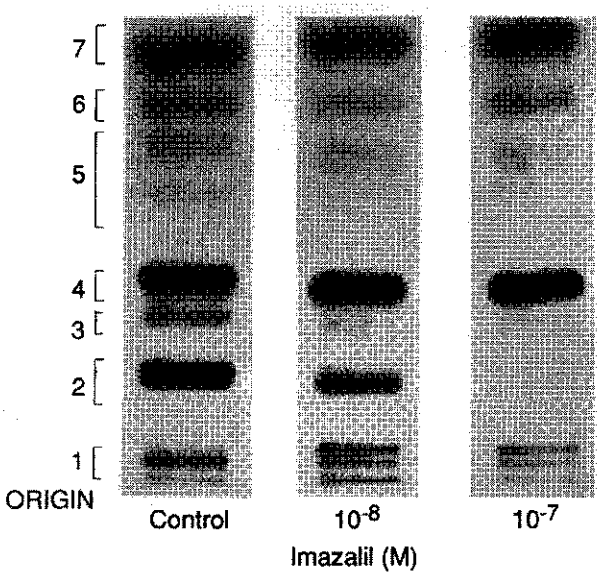

Fig. 3.1 Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C] mevalonate in cell-free assays of *Botrytis cinerea* in the absence and presence of imazalil at pH 7.4 in cyclohexane + ethyl acetate (4 + 1 by volume). Bands 2, 3, 4, 6 and 7 contained C4-desmethyl sterols, C4-monomethyl sterols, C4,4-dimethyl sterols, 2,3-oxidosqualene (tentative) and squalene, respectively. The identities of compounds in bands 1 and 5 are unknown.

Results of a typical time-course experiment (Fig. 3.2) show that incorporation of [2-^{14}C]mevalonate into NSLs was approximately linear with time up to 2 h. This was also true for synthesis of C4-desmethyl sterols. Formation of C4,4-dimethyl sterols decreased after 1 h. The amount of radiolabel incorporated into C4-monomethyl sterols was low and decreased after 1 h.

Half or double the standard [2-^{14}C]mevalonate concentration (8.5 nmol ml^{-1} incubation mixture) resulted in similar incorporation percentages of the radiolabel into NSLs and C4-desmethyl sterol fractions (data not shown), indicating that sterol synthesis was linear with the substrate concentration. The intermediate concentration of 8.5 nmol ml^{-1} incubation mixture was chosen for all other studies. In a following experiment, cell-free sterol synthesis was investigated in extracts diluted with potassium phosphate buffer to cover a range of protein concentrations from 1.1 to 9.1 mg ml^{-1}. Synthesis of NSLs and C4-desmethyl sterols proved to be more-or-less proportional to the protein content and hence the amount of enzyme present in cell extracts, except for the lowest concentration tested (Fig. 3.3). The highest protein concentration investigated led to the highest amount of C4-desmethyl sterols and to the highest ratio between C4-desmethyl sterols and total NSLs synthesized. Therefore, this protein concentration was used throughout the experiments.

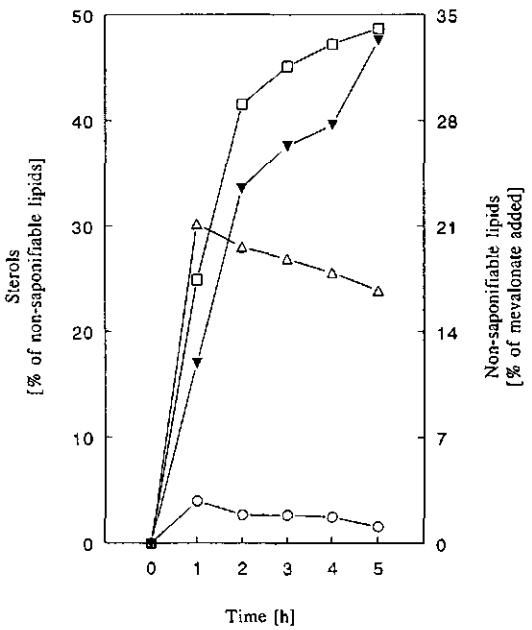

Fig. 3.2 Time course of synthesis of (▾) non-saponifiable lipids, (△) C4,4-dimethyl sterols, (○) C4-monomethyl sterols and (□) C4-desmethyl sterols in a cell-free preparation from *Botrytis cinerea*.

Stability of sterol synthetic activity did not significantly decrease upon storage of extracts at -18°C for eight days (data not shown).

A cell-free assay from B. cinerea

Cell-free extracts were not contaminated with mycelial fragments, since no cells could be detected microscopically after the second centrifugation of the cell-free extract and PDA-plates inoculated with droplets of extract did not show fungal growth, although some bacterial colonies were noticed. Addition of oxytetracycline (10 mg ml^{-1}) to incubation mixtures had no significant effect on cell-free sterol synthesis but did inhibit bacterial growth on PDA plates. Cell-free extracts heated at 80°C for 1 min incorporated only 0.2% of [2-^{14}C]mevalonate added into NSLs. Omission of either the cofactor solution or the divalent cations in the incubation mixture yielded only 0.2 and 1.0% incorporation of the radiolabel into NSLs, respectively.

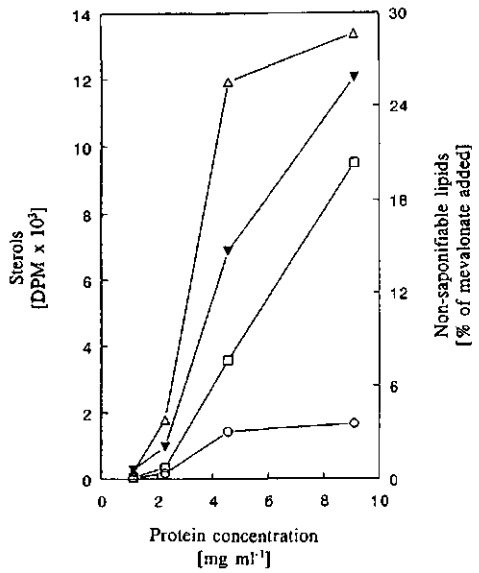

Fig. 3.3 Effect of protein concentration of cell-free extracts on synthesis of (▾) non-saponifiable lipids, (□) C4-desmethyl sterols, (○) C4-monomethyl sterols and (△) C4,4-dimethyl sterols.

Essential factors for synthesis of C4-desmethyl sterols

The intensity of disruption of the germlings was a crucial point. Microscopical examination showed that the standard procedure disrupted more than 90% of the cells. Protein concentrations of the resulting cell-free extracts were in the range of 9.0 ± 1.1 mg ml^{-1} (n = 10). Lower disruption speeds of the Bead-Beater, shorter disruption periods or the use of smaller glass beads resulted in less severe disruption of conidial germlings and yielded cell-free extracts with lower protein

concentrations. Similarly, higher disruption speeds, longer disruption periods and the use of larger glass beads increased protein concentrations of cell-free extracts and germlings were more severely disrupted than in the standard assay. Incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by these extracts was poor, although C4,4-dimethyl sterols were formed (data not shown). Similar results were obtained when glycerol was not added to the cell-free extract immediately after filtration of the germling homogenate (Table 3.1). Evacuation of the disruption vessel was also essential for synthesis of C4-desmethyl sterols.

Table 3.1 Effect of composition of the disruption buffer on sterol synthesis in cell-free extracts from *Botrytis cinerea*.

Standard composition[1]		NSLs[2]	Sterol ratio[3]		
			Eburicol	Lanosterol	C4-desmethyl sterols
None		18.5	1.0	1.2	2.0
+ MCE[5]	(14 mM)	9.3	1.0	8.7	n.d.[4]
+ DTT	(10 mM)				
+ MCE	(14 mM)	10.2	1.0	8.7	n.d.
+ DTT	(10 mM)				
+ EDTA	(10 mM)				
+ *N*-AC	(10 mM)	1.4	n.d.	n.d.	n.d.
+ NAA	(10 mM)				
- glycerol	(22%)	18.9	1.0	5.9	n.d.

[1] K_2HPO_4 - KH_2PO_4 buffer pH 7.5 supplied with 220 ml l^{-1} glycerol.
[2] Percentage of [2-^{14}C]mevalonate added.
[3] Ratio relative to eburicol; based on HPLC analysis.
[4] n.d. - Not detected.
[5] MCE: β-mercapto ethanol; DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid; *N*-AC: *N*-acetyl cysteine; NAA: nicotinic acid amide; compounds were added from 1000 x concentrated stock solutions to incubation mixtures with standard composition.

Activity of the cell-free assay was strongly affected by the pH of the incubation mixture, which in itself did not change significantly during incubation. Incorporation of radiolabel into NSLs and C4-desmethyl sterols had narrow pH optima between pH 7.4 and 7.6 and pH 7.3 and 7.4, respectively. Incorporation into C4-desmethyl sterols at pH 7.1 and 7.7 was 39 and 45% lower than at pH 7.4, respectively (Fig. 3.4). Formation of C4,4-dimethyl and C4-monomethyl sterols did not show an obvious pH optimum.

Addition of L-methionine to the cofactor solution enhanced production of C4-desmethyl sterols under cell-free conditions. Samples supplied with L-methionine incorporated, on average, comparable amounts of radiolabel into NSLs as those incubated without L-methionine, but the presence of the compound enhanced synthesis of C4-desmethyl sterols (about 30 to 40% of total NSLs). When L-methionine was replaced by the same molar amount of *S*-adenosyl methionine, C4-desmethyl sterols accounted for 52 to 59% of NSLs, but total incorporation of [2-^{14}C]mevalonate into the sterol fraction was very low (only 5 - 6% of the radiolabel added). The addition of glucose-6-phosphate dehydrogenase did not improve cell-free synthesis of C4-desmethyl sterols.

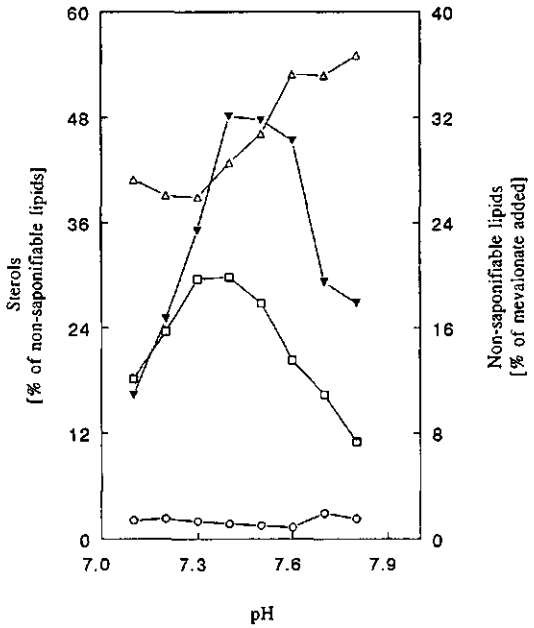

Fig. 3.4 Effect of pH on incorporation of [2-^{14}C]mevalonate into (▾) non-saponifiable lipids, (Δ) C4,4-dimethyl sterols, (o) C4-monomethyl sterols and (□) C4-desmethyl sterols in cell-free preparations from *Botrytis cinerea*.

In preliminary experiments, activity of cell-free extracts using different disruption buffers was investigated. Interestingly, replacement of the standard potassium buffer by a sodium potassium phosphate buffer reduced synthesis of C4-desmethyl sterols and also resulted in an accumulation of C4-monomethyl sterols (Table 3.2). Disruption of germlings in Tris-HCl buffer, commonly used for Δ^{24} sterol:*S*-adenosyl methionine transferase assays,[21-23] reduced synthesis of NSLs by 50% and

resulted in a relatively high amount of eburicol (Table 3.2), indicating negative effects on $P450_{14DM}$ activity. In further experiments the effect of different supplements, often used in $P450_{14DM}$ assays of yeasts,[7,11,24] were tested. MCE, DTT and EDTA reduced incorporation of [2-^{14}C]mevalonate into NSLs by about 50% (Table 3.1). More interestingly, they blocked formation of C4-desmethyl sterols concomitant with an accumulation of lanosterol, which indicates an inhibitory effect of these compounds on side chain alkylation of lanosterol. Addition of *N*-acetyl cysteine and nicotinic acid amide to the disruption buffer severely impaired cell-free sterol synthesis (Table 3.1). Hence, 100 mM potassium phosphate buffer (pH 7.5) was chosen in the standard assay.

Table 3.2 Effect of different disruption buffers (100 mM) on sterol synthesis in cell-free extracts from *Botrytis cinerea*.

		Sterol ratio[2]			
Buffer pH 7.5	NSLs[1]	Eburicol	Lanosterol	C4-monomethyl sterols	C4-desmethyl sterols
K_2HPO_4 KH_2PO_4	21.3	1.0	3.3	n.d.[3]	4.1
Na_2HPO_4 KH_2PO_4	18.3	1.0	1.7	2.5	2.6
Tris-HCl	11.0	1.0	0.1	n.d.	0.3

[1] Percentage of [2-^{14}C]mevalonate added.
[2] Relative to eburicol; based on HPLC analysis.
[3] n.d. - Not detected.

Inhibition studies

The imidazole fungicide imazalil inhibited radial growth of *B. cinerea* by 50% at 1.5×10^{-6} M. Imazalil inhibited cell-free incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols (Fig. 3.1). Inhibition coincided with an increase of radiolabelled C4,4-dimethyl sterols. Effects of imazalil on the formation of C4-monomethyl sterols could not be observed. Figure 3.5 shows a dose-response curve of imazalil for inhibition of C4-desmethyl sterol synthesis obtained from four replicate experiments. The IC_{50} value was calculated to be $9.1 \pm 1.5 \times 10^{-9}$ M. DMSO (1 ml l^{-1}) in control treatments did not have any significant effect on cell-free sterol synthesis.

Identification of radiolabelled sterols

NSLs were analyzed by radio-HPLC. In control samples, six major peaks with retention times of 4.9 (peak 1), 10.0 (peak 2), 12.8 (peak 3), 16.6 (peak 4, consisting of two peaks), 22.8 (peak 5) and 25.1 min (peak 6) were observed (Fig. 3.6A). Peaks 4, 5 and 6 had retention times identical to those of authentic ergosterol, lanosterol and eburicol, respectively, as demonstrated by uv detection at 210 and 280 nm. The identities of other sterols were not studied any further. In imazalil-treated samples (3×10^{-8} M) only two major peaks were observed (Fig. 3.6B): peak 5 (lanosterol) and 6 (eburicol). The size of the latter increased significantly.

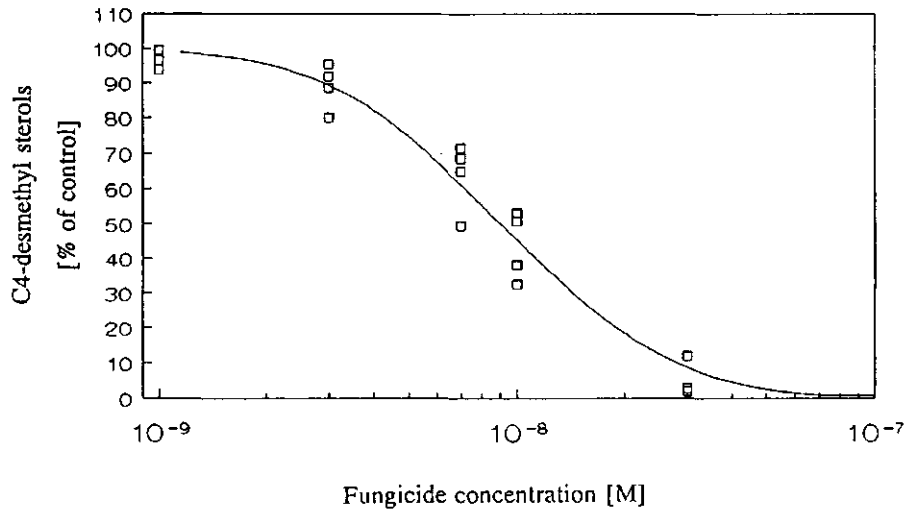

Fig. 3.5 Inhibition of incorporation of (2-^{14}C)mevalonate into C4-desmethyl sterols in cell-free assays (n = 4) from *Botrytis cinerea* at pH 7.4 by imazalil.

Sterols were further analyzed by separation on TLC plates pretreated with silver nitrate. The C4-desmethyl sterol fraction proved to consist of three sterols with R_f values of 0.02, 0.07 and 0.21, which accounted for 13, 63 and 24% of the C4-desmethyl sterol fraction, respectively. The compound with a R_f value of 0.07 co-chromatographed with authentic ergosterol. The other two C4-desmethyl sterols were not identified. C4,4-dimethyl sterols separated into four distinct bands with R_f values of 0.04, 0.31, 0.36 and 0.42. Compounds with R_f values of 0.36 and 0.42 co-chromatographed with authentic eburicol and lanosterol and accounted for 54 and 28% of the C4,4-dimethyl sterol fraction, respectively. No attempt was made to identify the other sterols.

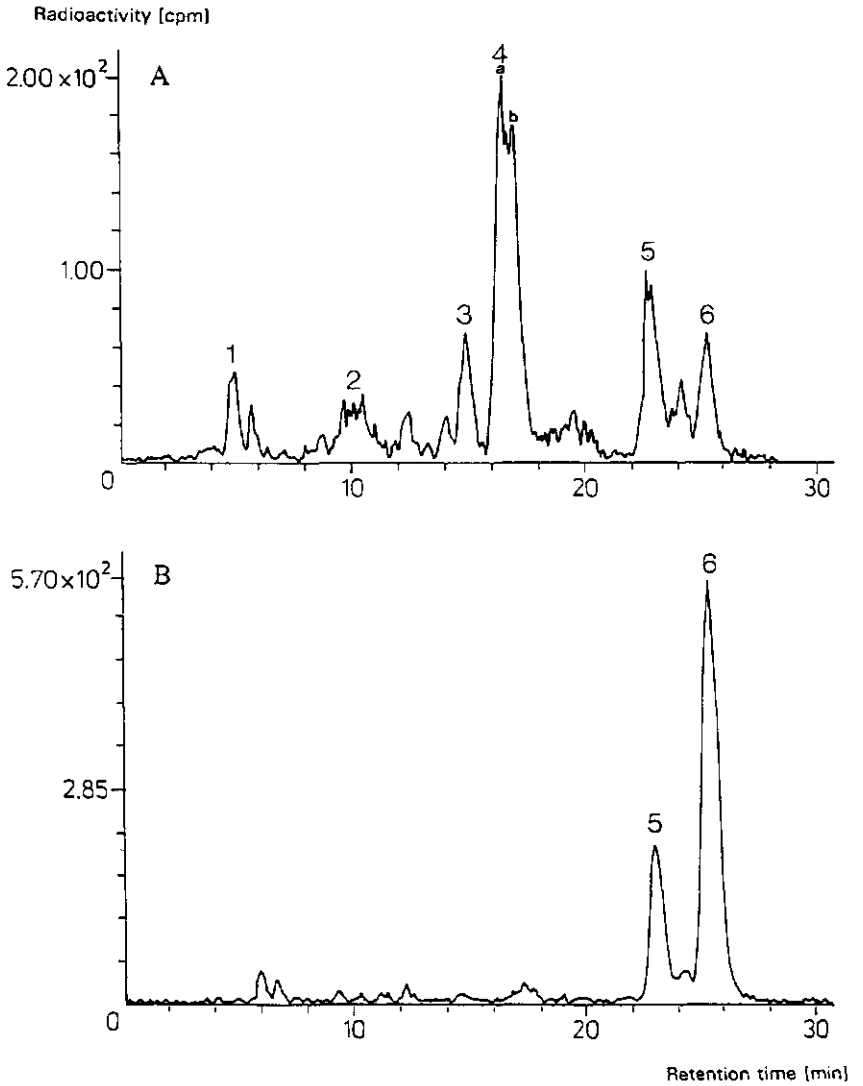

Fig. 3.6 Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from **(A)** control and **(B)** imazalil-treated (3×10^{-8} M) cell-free sterol synthesis assays from *Botrytis cinerea* at pH 7.4. Peak 4 is composed of a mixture of ergosterol (4a) and another non-identified C4-desmethyl sterol (4b). The identities of peak 5 and 6 are lanosterol and eburicol, respectively.

Discussion

Cell-free extracts of *B. cinerea* actively synthesized C4-desmethyl sterols and other sterols from [2-^{14}C]mevalonate. The disruption procedure was relatively vigorous compared with those described for other filamentous pathogens,[15,16] but this proved to be essential. The protein concentration of cell-free extracts in standard assays was 9.0 ± 1.1 mg ml^{-1}, which is comparable to those described for assays developed for yeasts[7,8,11] and ten times higher than reported for cell-free systems of *A. fumigatus*[15] and *P. italicum*.[16] The need for a relatively severe disruption of germlings may be explained by the fact that cells of *B. cinerea* are surrounded by a layer of mucilage. However, it may also be due to differences in quality and quantity of $P450_{14DM}$ in different fungi and $P450_{14DM}$ stability. Additional crucial points for the preparation of active cell-free extracts were the addition of glycerol to filtrates of cell homogenates before centrifugation (Table 3.1) and the pH of the incubation mixture (Fig. 3.4). The cell-free system had a narrow pH optimum for synthesis of C4-desmethyl sterols between pH 7.3 and 7.4. This result is different from that reported for the cell-free assay of *P. italicum*,[16] but resembles data obtained for *A. fumigatus*.[15]

Under optimal conditions the average amount of radioactivity incorporated into C4-desmethyl sterols was about 39% of total NSLs. In intact cells incorporation of radiolabel into C4-desmethyl sterols accounts for more than 90% of the NSLs formed.[25] A possible explanation for the relatively low incorporation of label into C4-desmethyl sterols under cell-free conditions might be a feed-back inhibition of the ergosterol formed, which can not be incorporated into membranes as in intact cells. Increased synthesis of NSLs in extracts treated with imazalil (data not shown) is supportive of this hypothesis. However, the addition of the polyene antibiotic nystatin (10^{-5} M), which forms complexes with C4-desmethyl sterols, to incubation mixtures did not influence cell-free synthesis of C4-desmethyl sterols (data not shown).

Incorporation of [2-^{14}C]mevalonate by cell-free extracts of *B. cinerea* into C4-desmethyl sterols is higher than reported for cell-free preparations derived from other filamentous fungi[15,16] and *C. albicans*.[9] *B. cinerea* assays yielded relatively low amounts of lanosterol as compared with cell-free assays of *A. fumigatus*[15] and *P. italicum*.[16] This is probably due to the addition of L-methionine to incubation mixtures, since its biologically activated form, *S*-adenosyl methionine, is the substrate for the C24 side-chain alkylation of lanosterol.[21,26] Interestingly, the addition of *S*-adenosyl methionine itself resulted in a very high conversion of [2-^{14}C]mevalonate to C4-desmethyl sterols, but in an extremely low formation of total sterols. We have no explanation for this phenomenon.

Analysis of the C4-desmethyl sterol fraction by argentation TLC revealed three distinct C4-desmethyl sterols. The C4-desmethyl sterol, which co-chromatographed with authentic ergosterol, accounted for 63% of the C4-desmethyl sterols synthesized. Radio-HPLC analysis of NSLs revealed three C4-desmethyl sterol peaks (peaks 3, 4a and 4b), which were absent upon imazalil-treatment of cell-free extracts (Fig 3.6). Peak 4a had a retention time identical to that of standard ergosterol. Results indicate that ergosterol is the major C4-desmethyl sterol synthesized in cell-free extracts. The other two C4-desmethyl sterols are not yet identified. According to literature data on sterol composition of intact cells of *B. cinerea*, these sterols might be $\Delta^{5,8,22}$-ergostatrienol and episterol.[27-29] Only a minor amount (3.2 ± 1.1 %) of C4-monomethyl sterols was synthesized under cell-free conditions, which is in agreement with literature data on the sterol content of intact *B. cinerea* cells.[25] The other sterols in the NSLs identified by co-chromatography on radio-HPLC and argentation TLC were lanosterol and eburicol. These data suggest that the cell-free sterol synthesis follows the same pathway as described for intact mycelium.[28,29]

Treatment of cell-free extracts with imazalil led to an accumulation of eburicol and depletion of C4-desmethyl sterols (Fig. 3.1 and 3.6). This suggests that sterol 14α-demethylation is blocked by inhibition of $P450_{14DM}$ activity, and indicates that eburicol is presumably the substrate for the $P450_{14DM}$. The IC_{50} value of imazalil was extremely low (9.1 ± 1.5 x 10^{-9} M) indicating a high affinity for the $P450_{14DM}$ of this fungus. The EC_{50} value of imazalil for radial growth is 1.5 x 10^{-6} M. This difference may be explained by the accumulation, distribution and metabolism of the compound in cells of *B. cinerea*, the intracellular pH and some other unknown factors.

The cell-free assay provides a simple method to screen biochemically for compounds which potentially inhibit sterol biosynthesis at C14-demethylation or, alternatively, at any other site in the sterol biosynthetic pathway between the precursor squalene and the final product ergosterol.

Acknowledgements

The authors are grateful to Prof. Dr. J. Dekker, Prof. Dr. P.J.G.M. De Wit and Dr. R.J. Milling for critical reading of the manuscript. J.C. Kapteyn and C. Stehmann acknowledge the financial support of Schering Agrochemicals Ltd and the "Deutsche Akademische Austauschdienst", respectively.

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Chapter 4

Relationship Between Chemical Structure and Biological Activity of Triazole Fungicides Towards *Botrytis cinerea*

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Pesticide Science (in press)

Abstract

The inhibitory activity of commercial and experimental triazole fungicides on the target enzyme sterol 14α-demethylase ($P450_{14DM}$) was studied in a cell-free sterol synthesis assay from *Botrytis cinerea*. In order to assess structure-activity relationships the inhibitory activity of the compounds on radial growth of the fungus was tested as well. EC_{50}s (concentrations of fungicides inhibiting radial growth of *B. cinerea* on PDA by 50%) of all triazoles tested ranged between 10^{-6} and 10^{-5} M. IC_{50}s (concentrations of fungicides inhibiting incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%) generally ranged between 10^{-9} and 10^{-7} M and correlated with inhibition of mycelial growth. However, differences in IC_{50}s did not quantitatively reflect the observed differences in EC_{50}s, since the ratio between EC_{50} and IC_{50} increased with decreasing fungitoxicity. For a limited number of compounds the correlation between intrinsic inhibitory activity and fungitoxicity was low. Both *in vitro* tests were used to investigate structure-activity relationships for stereoisomers of cyproconazole, SSF-109 and tebuconazole. Fungitoxicity and the potency to inhibit cell-free C4-desmethyl sterol synthesis correlated for all stereoisomers tested. Mixtures of isomers of tebuconazole or cyproconazole were slightly less active than the most potent isomer. The high activity of several commercial triazoles in both experiments implies, that limited field performance of triazole fungicides towards *B. cinerea* is neither due to insensitivity of the $P450_{14DM}$ nor to low *in vitro* sensitivity of the fungus.

Introduction

Botrytis cinerea Pers. ex Fr. is one of the most ubiquitous plant pathogens. The fungus is of high economic importance, as it causes grey mould diseases in various major crops and post-harvest losses of transported and stored flowers, fruits and vegetables.[1]

Methods of disease control include sanitation, cultural methods and chemical control. Chemical control is one of the major disease control strategies for *Botrytis* spp. and is achieved by use of conventional and modern fungicides. Modern fungicides used for control of *Botrytis* spp. are the benzimidazoles, the dicarboximides and a few sterol 14α-demethylation inhibitors (DMIs) such as tebuconazole. In the last two decades development of resistance to benzimidazoles and dicarboximides severely hampered chemical control of *B. cinerea*.[2-4] DMIs are the largest group of modern fungicides. Their primary mode of action is inhibition of sterol synthesis due to binding of the free electron pair in the N-heterocycle to the heme iron of the cytochrome P450-dependent sterol 14α-demethylase ($P450_{14DM}$).[5,6] DMIs have various advantages such as a broad range of antifungal activity, systemic properties, selective action and a relatively low resistance risk.[7-10] A large variety of

plant pathogens is controlled by DMI fungicides, but until now DMIs did not replace the benzimidazoles and dicarboximides in control of *Botrytis* spp.[11-13]

The present study describes the effect of structural variations and stereoisomerism in the N1-moiety of triazole DMIs on inhibition of fungal growth and cell-free C4-desmethyl sterol synthesis. A better insight in the structure-activity relationship may contribute to the development of DMIs for control of grey mould.

Materials and methods

Fungal isolates

The monoascospore isolate SAS56 of *B. cinerea* was generously provided by Dr. F. Faretra (Bari, Italy).[14]

Chemicals

Commercial and experimental triazoles (technical grades) used were generously provided by their respective manufacturers. Triazoles were dissolved as 1000x concentrated solutions in DMSO before use. The racemic mixtures of cyproconazole isomers (diastereomer A: 61.7%, diastereomer B: 38.3%) and tebuconazole enantiomers ((−)enantiomer: 50%, (+)enantiomer: 50%) were used as standards to compare the results of experimental compounds and single isomers. Mevalonic acid DBED-Salt (DL-[2-^{14}C]mevalonate in ethanol, sp. act. 1.9 GBq mmol^{-1}) was purchased from Amersham (Amersham, UK). DMSO, NADPH, $NADP^+$, NAD^+, ATP, glucose-6-phosphate and methionine were supplied from Sigma (St. Louis, MO, USA).

Fungitoxicity assay

Fungitoxicity of compounds was assessed in radial growth tests on PDA-medium. Inoculum was prepared by spreading 200 μl of a spore suspension (10^6 spores ml^{-1}) over the agar surface of a Petri dish. Petri dishes were incubated overnight at 20°C. Agar discs (4 mm diameter) with a thin mycelial mat were transferred upside down on test agar plates amended with DMSO (0.1%) or DMSO-solutions of the fungicides (0.1%). Inhibition of fungal growth was assessed after 36 h of incubation at 20°C in the dark.

Inhibitory activity of commercial triazoles was determined in three experiments and expressed as EC_{50}s (concentrations of compounds inhibiting radial growth of *B. cinerea* on PDA by 50%). Inhibitory potency of structural analogues and stereoisomers of cyproconazole, SSF-109 and tebuconazole was assessed in single experiments. Activity of compounds was expressed as the ratio between the EC_{50} of

the test chemical and the EC_{50} of a reference fungicide tested in the same experiment (R_{EC50}).

Cell-free sterol synthesis assay

Mycelium of *B. cinerea* was grown in an orbital shaker (200 rpm) in liquid medium prepared according to Fritz *et al.* (1977) at 20°C for 14 h.[15] Preparation of cell-free extracts from germlings of *B. cinerea* and assessments of cell-free sterol syntheses were carried out as described previously by Stehmann *et al.*[16] Fungal germlings (14-h-old) were harvested on a sieve, washed thoroughly with tap-water and ice-cold disruption buffer, pH 7.5, after which they were disrupted using a Bead-Beater. Resulting cell-homogenate was filtered through gauze and supplied with glycerol. After centrifugation at low speed the crude cell-extract was used immediately for cell-free sterol synthesis assays. Reaction mixtures - consisting of cell-free extract, cofactor and divalent cation solutions - were adjusted to pH 7.4. DMSO or DMSO-solutions of the fungicides (0.1% of the total reaction volume) were added to the reaction mixtures. Reaction was started by addition of (2-^{14}C)mevalonate (0.5 μCi). Mixtures were incubated at 20°C for 2 h, after which the reaction was stopped by addition of ethanolic-KOH.

Saponification of incubation mixtures, extraction and analysis of non-saponifiable lipids (NSLs) were performed as described by Guan *et al.*[17] After saponification NSLs were extracted with light petroleum ether and separated by thin-layer chromatography (TLC) in a solvent system of cyclohexane + ethyl acetate (4 + 1 by volume). Radioactive areas on the plates were located by autoradiography. Separated NSL fractions were cut out and counted for radioactivity in a liquid scintillation counter (Beckman LS 5800). The amount of radioactivity incorporated into different sterol fractions was expressed as a percentage of radioactivity in NSLs. Subsequently, corresponding incorporation rates of fungicide treatments were calculated as percentages of control treatments (100%). Using the software program Lotus 1-2-3, percentages were plotted against fungicide concentration on a logarithmic scale and regression analysis of these inhibitor-response data was performed. In order to identify components of C4-desmethyl and C4,4-dimethyl sterol fractions, the respective zones were located by autoradiography, cut from TLC plates, extracted with chloroform and rechromatographed on TLC plates pretreated with silver nitrate as described by Kerkenaar *et al.*[18] NSLs were separated by HPLC on a Zorbax C_8 column (4.6 x 250 mm, Chrompack, Middelburg, the Netherlands) at 30°C using 95% methanol (HPLC garde, J.T. Baker B.V., Deventer, the Netherlands) at a flow rate of 1 ml min^{-1} and detected with two UV detectors (Waters 484 and 481, Millipore, Milford, MA) set at 280 and 210 nm, respectively and an on-line radioactivity monitor (Canberra Radiomatic, A-200, Radiomatic Instruments and Chemical Co., Inc., Tampa, IL)

using Pico-Aqua (Packard Instrument Company Inc., Downers Grove, IL) as scintillant at a flow rate of 3 ml min^{-1}. Sterols were identified by comparing retention times of peaks with those of internal standards (ergosterol and lanosterol) and literature data.

Intrinsic inhibitory activity of commercial triazoles was determined in three experiments with cell-free extracts from different disruptions and expressed as IC_{50}s (concentration of compound inhibiting cell-free C-4 desmethyl sterol synthesis by 50%). Inhibitory potency of structural analogues and stereoisomers of cyproconazole, SSF-109 and tebuconazole on cell-free C4-desmethyl sterol synthesis was assessed in single experiments. Activity of compounds was expressed as the ratio between the IC_{50} of the test chemical and the IC_{50} of the reference fungicide tested in the same experiment (R_{IC50}).

Results

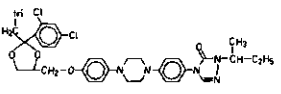
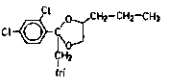
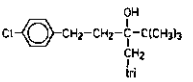
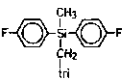
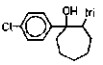
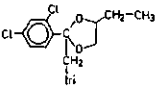
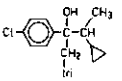
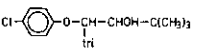
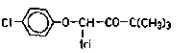
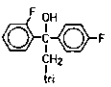
Inhibition of radial mycelial growth

In radial growth experiments the commercial triazoles tested displayed a broad range of biological activity (Table 4.1). The antimycotic compound itraconazole was the strongest inhibitor with an EC_{50} of 1.2 x 10^{-8} M. EC_{50}s of the other commercial triazoles ranged from 7.7 x 10^{-8} (propiconazole) to 8.8 x 10^{-6} M (flutriafol).

EC_{50}s of cyproconazole analogues relative to the EC_{50} of cyproconazole (R_{EC50}s) ranged from 0.03 to 31.4 (Table 4.2). Compounds **1**, **2** and **3** were significantly more active than cyproconazole. Compound **7** was the weakest inhibitor of all cyproconazole analogues tested, being about 30 times less active than cyproconazole. R_{EC50}s of tebuconazole analogues ranged from 0.6 (**8**) to 32.8 (**21**) (Table 4.3). Compounds **8**, **9** and **10** (R_{EC50}s < 1) were more active than the reference compound tebuconazole. Compound **21**, the weakest inhibitor of the tebuconazole analogues tested, was about 30 times less active than tebuconazole.

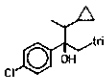
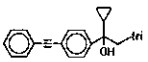
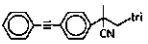
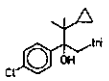
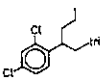
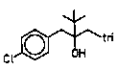
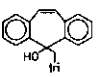
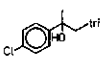
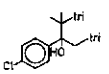
The two (−)enantiomers A_2 and B_2 of cyproconazole displayed EC_{50}s of 2.3 x 10^{-7} and 5.1 x 10^{-8} M, respectively and were more toxic to fungal growth than the (+)enantiomers A_1 and B_1 (Table 4.4). Isomer A_1 was 161 times less toxic to radial mycelial growth than the isomer B_2. The isomer mixture gave an EC_{50} of 2.1 x 10^{-7} M, which is only four times higher than the EC_{50} of the most active isomer B_2. The trans-isomer of SSF-109 was 37 times less toxic to radial mycelial growth than its cis-isomer (Table 4.4). The (+)enantiomer of tebuconazole, assigned to the S-configuration according to the Cahn, Ingold, Prelog principles, was 19 times less active than the (−)enantiomer. The commercial racemic mixture was only slightly less active than the (−)enantiomer (Table 4.4).

Table 4.1 Activity of commercial triazole fungicides to radial growth of *Botrytis cinerea* on PDA (EC_{50}) and inhibitory effects on synthesis of C4-desmethyl sterols in cell-free assays (IC_{50}).

Compound	EC_{50} (M)[1]	IC_{50} (M)[2]	EC_{50}/IC_{50}	Structure
Itraconazole	$1.2 \pm 0.3 \times 10^{-8}$	$8.9 \pm 1.8 \times 10^{-10}$	14	
	(1)[3]	(1)		
Propiconazole	$7.7 \pm 0.6 \times 10^{-8}$	$6.6 \pm 2.6 \times 10^{-9}$	12	
	(6)	(7)		
Tebuconazole	$8.7 \pm 3.8 \times 10^{-8}$	$2.5 \pm 0.9 \times 10^{-9}$	35	
	(7)	(3)		
Flusilazole	$8.7 \pm 4.0 \times 10^{-8}$	$3.0 \pm 0.5 \times 10^{-9}$	29	
	(7)	(3)		
SSF-109	$1.1 \pm 0.3 \times 10^{-7}$	$4.7 \pm 0.7 \times 10^{-9}$	23	
	(9)	(5)		
Etaconazole	$3.6 \pm 1.8 \times 10^{-7}$	$1.3 \pm 0.7 \times 10^{-8}$	28	
	(30)	(15)		
Cyproconazole	$3.8 \pm 0.6 \times 10^{-7}$	$1.4 \pm 0.3 \times 10^{-8}$	27	
	(32)	(16)		
Triadimenol	$1.4 \pm 0.3 \times 10^{-6}$	$1.5 \pm 0.4 \times 10^{-8}$	93	
	(117)	(17)		
Triadimefon	$5.2 \pm 1.6 \times 10^{-6}$	$1.7 \pm 0.5 \times 10^{-7}$	31	
	(433)	(191)		
Flutriafol	$8.8 \pm 1.4 \times 10^{-6}$	$5.1 \pm 2.5 \times 10^{-8}$	173	
	(733)	(57)		

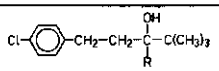
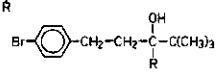
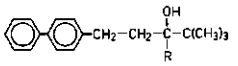
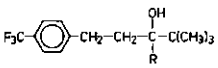
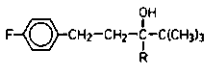
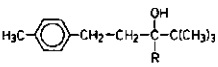
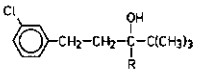
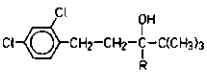
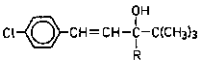
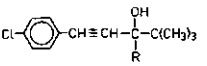
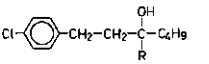
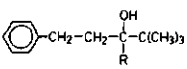
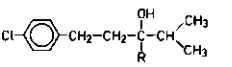
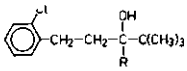
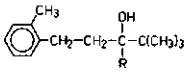
[1] Concentration of compound inhibiting radial growth on PDA by 50% (mean of three experiments).
[2] Concentration of compound inhibiting incorporation of [2-^{14}C] mevalonate into C4-desmethyl sterols by 50% (mean of three experiments).
[3] *Between brackets: ratio between the inhibitory potency of the test compound and itraconazole in radial growth test (R_{EC50}) or cell-free sterol synthesis assay (R_{IC50}).*

Table 4.2 Relative activity of structural analogues of cyproconazole and penconazole on radial growth of *Botrytis cinerea* on PDA and cell-free synthesis of C4-desmethyl sterols.

Compound	R_{EC50} [1]	R_{IC50} [2]	EC_{50}/IC_{50}	Structure
Cyproconazole[3]	1.0	1.0	26	
1	0.03	0.2	4	
2	0.1	0.6	5	
3	0.5	0.4	23	
Penconazole	1.1	2.6	10	
4	1.4	3.3	10	
5	2.5	2.1	19	
6	21.5	347.2	3	
7	31.4	1.3	1199	

[1] Ratio between EC_{50} (concentration of compound inhibiting radial growth by 50%) of test compound and EC_{50} of cyproconazole determined in the same experiment.
[2] Ratio between IC_{50} (concentration of compound inhibiting incorporation of 2-^{14}C-mevalonate into C4-desmethyl sterols by 50%) of test compound and IC_{50} of cyproconazole determined in the same experiment.
[3] EC_{50} 3.4×10^{-7} M (n = 2); IC_{50} $1.4 \pm 0.6 \times 10^{-8}$ M (n = 4).

Table 4.3 Relative activity of structural analogues of tebuconazole on radial growth of *Botrytis cinerea* on PDA and cell-free synthesis of C4-desmethyl sterols.

Compound	R_{EC50} [1]	R_{IC50} [2]	EC_{50}/IC_{50}	Structure
Tebuconazole[3]	1.0	1.0	54	
8	0.6	2.2	24	
9	0.8	0.6	83	
10	0.8	0.8	66	
11	2.4	2.2	95	
12	2.5	2.4	34	
13	3.1	3.1	128	
14	3.6	4.2	57	
15	4.3	2.3	95	
16	4.8	4.6	53	
17	8.0	7.9	80	
18	9.0	5.1	40	
19	13.0	10.0	101	
20	19.9	9.3	120	
21	32.8	31.2	71	

[1] Ratio between EC_{50} (concentration of compound inhibiting radial growth by 50%) of test compound and EC_{50} of tebuconazole determined in the same experiment.
[2] Ratio between IC_{50} (concentration of compound inhibiting incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%) of test compound and IC_{50} of tebuconazole determined in the same experiment.
[3] EC_{50} $2.3 \pm 1.3 \times 10^{-7}$ M (n = 4); IC_{50} $4.3 \pm 2.0 \times 10^{-9}$ M (n = 8).
[4] R = 1-methyl-triazolyl.

Cell-free sterol synthesis assay

Incorporation of [2-^{14}C]mevalonate into NSLs amounted 29.2 ± 3.3% of total radioactivity added (n = 40). Radiolabel present in the C4-desmethyl sterol fraction was 33.3 ± 3.2% (n = 40) of [2-^{14}C]mevalonate incorporated into the NSLs (Table 4.5). C4-desmethyl sterols were characterized by separation on $AgNO_3$-TLC. Ergosterol accounted for 63% of the C4-desmethyl sterols (Fig. 4.1). The two other C4-desmethyl sterols formed were not identified. Triazoles inhibited incorporation of radiolabel into C4-desmethyl sterols at low concentrations concomitant with an accumulation of C4,4-dimethyl sterols (Table 4.5), mainly eburicol (Fig. 4.2).

Table 4.4 Stereoselective toxicity of cyproconazole, SSF-109 and tebuconazole to radial growth of *Botrytis cinerea* on PDA (EC_{50}) and inhibitory effects of these compounds on synthesis of C4-desmethyl sterols in cell-free assays (IC_{50}).

Compound	EC_{50} [M][1]		IC_{50} [M][2]		EC_{50}/IC_{50}
Cyproconazole					
racemic mixture	2.1×10^{-7}	(4)[3]	1.2×10^{-8}	(6)	18
B_2 (−)enantiomer	5.1×10^{-8}	(1)	2.0×10^{-9}	(1)	26
A_2 (−)enantiomer	2.3×10^{-7}	(5)	4.0×10^{-9}	(2)	59
B_1 (+)enantiomer	4.7×10^{-6}	(92)	6.0×10^{-8}	(30)	78
A_1 (+)enantiomer	8.2×10^{-6}	(161)	6.2×10^{-8}	(31)	133
SSF-109					
cis-isomer	8.7×10^{-8}	(1)	5.5×10^{-9}	(1)	16
trans-isomer	3.2×10^{-6}	(37)	5.3×10^{-8}	(10)	60
Tebuconazole					
racemic mixture	6.8×10^{-8}	(2)	3.8×10^{-9}	(1)	18
(−)enantiomer	4.5×10^{-8}	(1)	3.1×10^{-9}	(1)	15
(+)enantiomer	8.7×10^{-7}	(19)	2.7×10^{-8}	(9)	32

[1] Concentration of compound inhibiting radial growth of *B. cinerea* on PDA by 50%.
[2] Concentration of compound inhibiting incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%.
[3] Ratio between activity of test compound and the most potent isomer in radial growth tests (R_{EC50}) and cell-free assays (R_{IC50}).

Inhibition of cell-free C4-desmethyl sterol synthesis

Itraconazole was the most potent inhibitor of cell-free C4-desmethyl sterol synthesis of all compounds tested and had an IC_{50} of 8.9×10^{-10} M (Table 4.1). All other commercial triazoles were less active, displaying IC_{50}s between 6.6×10^{-9} (propiconazole) and 5.1×10^{-8} M (flutriafol). Spearman's ranking coefficient (r_s) for EC_{50}s and IC_{50}s was 0.92 for the ten commercial triazoles tested. There were two exceptions from the ranking correlation: propiconazole and triadimefon. Propiconazole (R_{EC50} 6) was significantly more toxic to radial mycelial growth than the structurally related compound etaconazole (R_{EC50} 30). However, it inhibited sterol C4-desmethyl sterol

synthesis (R_{IC50} 7) only slightly stronger than etaconazole (R_{IC50} 15). Triadimefon (R_{EC50} 433) inhibited radial growth of *B. cinerea* at slightly higher concentrations than triadimenol (R_{EC50} 117), but it was a significantly weaker inhibitor of cell-free C4-desmethyl sterol synthesis (R_{IC50} 191) than triadimenol (R_{IC50} 17).

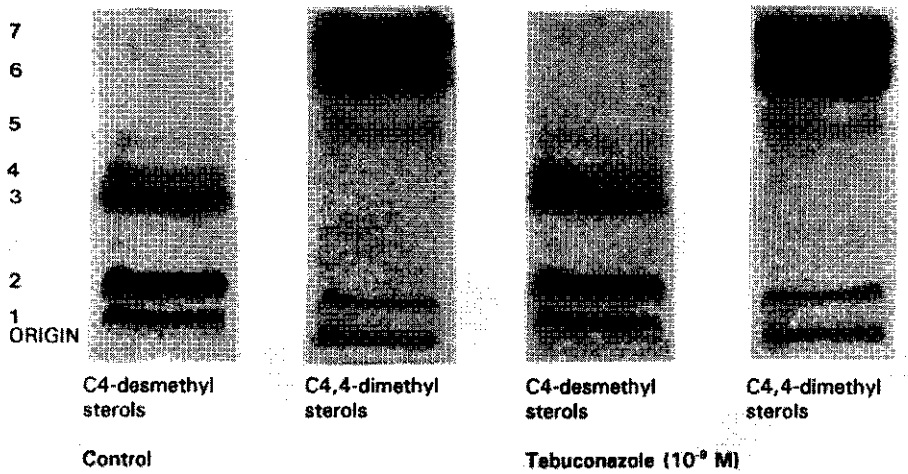


Fig. 4.1 Autoradiogram of an $AgNO_3$-TLC separation of C4-desmethyl sterols and C4,4-dimethyl sterols extracted from cell-free extracts of *Botrytis cinerea* incubated in the absence and presence of tebuconazole. Bands 2, 6 and 7 represent ergosterol, eburicol and lanosterol, respectively. Bands 1, 3, 4 and 5 were not identified.

The IC_{50} of cyproconazole was 1.4×10^{-8} M (Table 4.2). Compounds **1**, **2** and **3** were more potent inhibitors of cell-free C4-desmethyl sterol synthesis than cyproconazole. All other compounds were less active than the reference compound. Spearman's ranking coefficient for EC_{50}s and IC_{50}s of the eight cyproconazole analogues tested was 0.73. Compound **7** (R_{EC50} 31.4) was the least fungitoxic analogue of cyproconazole, but its R_{IC50} (1.3) was about the same as that of cyproconazole.

Tebuconazole inhibited cell-free C4-desmethyl sterol synthesis by 50% at 4.3×10^{-9} M (Table 4.3). The degree of desaturation of sterols accumulated in the presence of tebuconazole was not affected (Fig. 4.1 and 4.2). Compound **8** (R_{EC50} 0.6), which showed a higher inhibitory potency towards radial mycelial growth than tebuconazole, had a R_{IC50} of 2.2. Compounds **9** and **10**, which were also stronger inhibitors of fungal growth than tebuconazole (R_{EC50}s 0.8) had R_{IC50}s below 1. All other analogues were less active than the reference compound. As in radial growth experiments compound **21** was the weakest inhibitor of cell-free C4-desmethyl sterol synthesis displaying a R_{IC50} of 31.2. Spearman's ranking coefficient for EC_{50}s and IC_{50}s of the 15 tebuconazole analogues tested was 0.95.

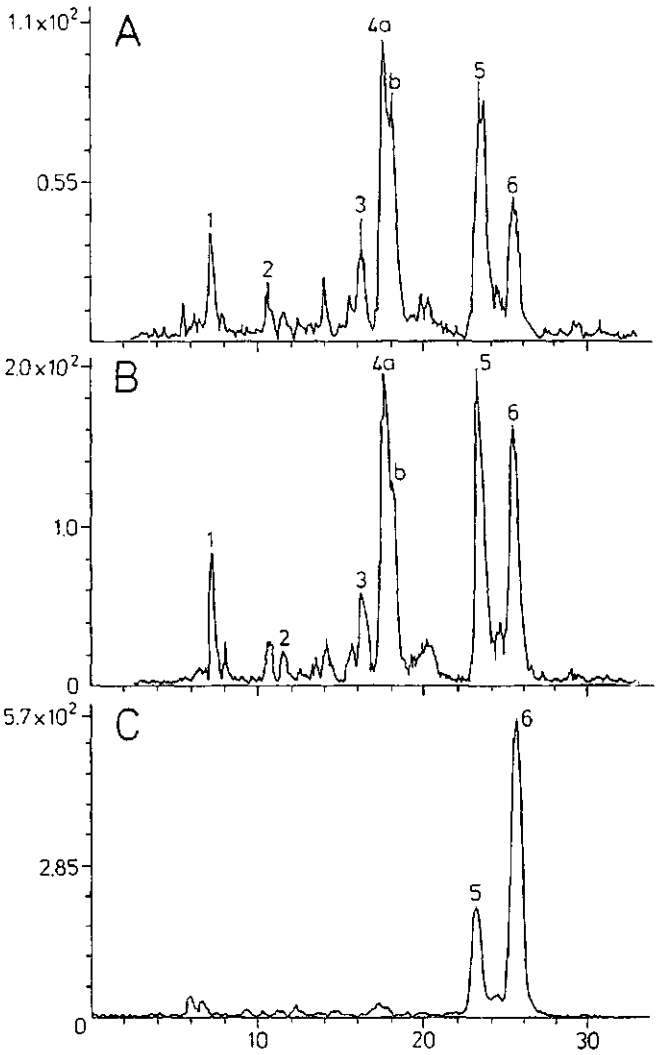


Fig. 4.2 Radio-HPLC separation of NSLs extracted from control (**A**) and tebuconazole-treated (**B**: 10^{-9} M, **C**: 10^{-8} M) cell-free extracts of *Botrytis cinerea*. Peak 4 represents of a mixture of ergosterol (4a) and another C4-desmethyl sterol (4b). Peaks 5 and 6 represent lanosterol and eburicol, respectively. Peaks 1, 2, and 3 were not identified.

Table 4.5 Incorporation of [2-^{14}C]mevalonate into non-saponifiable lipids in cell-free assays from *Botrytis cinerea* upon treatment with cyproconazole.

Cyproconazole (M)	Lipid fraction[1]				
	Squalene + oxidosqualene	C4,4-dimethyl sterols	C4-monomethyl sterols	C4-desmethyl sterols	Origin + unidentified lipids
0	22.1	33.2	2.7	34.6	7.5
10^{-9}	25.4	32.3	2.8	34.0	5.6
7×10^{-9}	24.5	39.3	2.9	26.8	6.5
10^{-8}	27.2	49.3	2.8	15.1	5.6
3×10^{-8}	20.1	56.2	3.6	13.6	6.5

[1] [2-^{14}C]mevalonate incorporated into lipid fractions as percentage of total radiolabel extracted from TLC.

The four stereoisomers of cyproconazole inhibited cell-free C4-desmethyl sterol synthesis to different degrees. The (−)enantiomers A_2 and B_2 exhibited a higher intrinsic inhibitory potency than the (+)enantiomers A_1 and B_1. The least potent isomer A_1 was 31 times less active than the most potent isomer B_2 (Table 4.4). The IC_{50} of the mixture of stereoisomers was only six times lower than the IC_{50} obtained for the most potent isomer B_2. The trans-isomer of SSF-109 was about ten times less potent in inhibiting cell-free C4-desmethyl sterol synthesis than the cis-isomer. The ratio established for fungal growth was higher (37). In cell-free assays the (+)enantiomer of tebuconazole was nine times less active than its (−)enantiomer (Table 4.4). The commercial racemic mixture was slightly less toxic towards fungal growth and cell-free C4-desmethyl sterol synthesis than the most potent (−)enantiomer.

Discussion

Cell-free extracts of *B. cinerea* actively synthesized C4-desmethyl sterols and other sterols from [2-^{14}C]mevalonate. Under optimum conditions the average amount of radioactivity incorporated into NSLs was 29% of radiolabel added and the C4-desmethyl sterol fraction amounted 33% of the NSLs produced. Ergosterol was the main sterol of the C4-desmethyl sterol fraction. C4,4-dimethyl sterols identified were lanosterol and eburicol. The content of the latter sterol increased upon treatment with cyproconazole and tebuconazole (Table 4.5, Fig. 4.2) indicating that C14-demethylation precedes C4-demethylation, as demonstrated earlier.[16,19,20] Therefore, inhibitory potency of DMIs on $P450_{14DM}$ activity can be estimated by assessing inhibition of cell-free C4-desmethyl sterol synthesis. The small deviations in synthesis of total NSLs and C4-desmethyl sterols as well as the low standard deviations in the IC_{50}s obtained from inhibition studies with cell-free extracts confirm that the assay provides a reliable method to screen biochemically for compounds which inhibit $P450_{14DM}$ activity. Therefore, it was decided that structure-activity relations of cyproconazole and tebuconazole analogues can be based on results of single experiments, which always included a reference compound in the same test.

The present study demonstrates that a number of commercial and experimental triazoles inhibit cell-free C4-desmethyl sterol synthesis and fungal growth of *B. cinerea* to different degrees (Tables 4.1, 4.2 and 4.3). The triazole antimycotic itraconazole was the most potent inhibitor of both radial mycelial growth and cell-free C4-desmethyl sterol synthesis. This is of interest, since itraconazole is by far the largest molecule (molecular weight 706) of the compounds tested and has therefore probably more interaction sites with the apoprotein of

$P450_{14DM}$ than the other triazoles tested. Propiconazole, tebuconazole and flusilazole also inhibited radial mycelial growth at relatively low concentrations (EC_{50}s about 8×10^{-9}M). These compounds also inhibited cell-free C4-desmethyl sterol synthesis at extremely low concentrations. Therefore, it can be assumed that limited field performance of triazoles against *B. cinerea*[4,11-13] is not due to a low sensitivity of the target site $P450_{14DM}$. EC_{50}s of all commercial triazoles tested ranged between 10^{-6} and 10^{-5} M. This range of fungitoxicity is comparable to the *in vitro* activity of DMIs against other plant pathogens.[21-25] These results suggest, that limited field performance of DMI-fungicides can also not be ascribed to low sensitivity of the pathogen due to permeability barriers, increased efflux of fungicides from mycelial cells, rapid metabolic breakdown or deposition in cellular compartments.

In general, commercial triazoles most toxic to radial mycelial growth tended to be the most potent inhibitors of cell-free C4-desmethyl sterol synthesis (r_s = 0.92), indicating that intrinsic inhibitory potency and fungitoxicity are correlated (Table 4.1). However, differences in IC_{50}s did not fully reflect the observed differences in fungitoxicity as illustrated by the EC_{50}/IC_{50} ratios. This ratio increases with decreasing fungitoxicity of the compounds tested, which implies that compounds with a low fungitoxicity are still potent inhibitors of cell-free C4-desmethyl sterol synthesis. Thus, toxicity of triazoles to *B. cinerea* is not only determined by their inhibitory potency on $P450_{14DM}$ activity. Other factors such as differential accumulation, metabolism, deposition in cell compartments or binding to fungal cell constituents may be involved. The last possibility is very likely, since compounds with a high affinity for $P450_{14DM}$ will bind preferentially to this enzyme, whereas compounds with a lower affinity for $P450_{14DM}$ may bind relatively stronger to other cell-structures, possibly other P450-dependent enzymes. This hypothesis is supported by papers indicating that DMIs may also bind to various cytochrome P450-dependent mixed-function oxidases.[26,27] The low correlation between complex-formation of P450 isozymes with DMIs and fungitoxicity also indicates that DMIs interact with a pool of fungal cytochrome P450s.[23,24,28-30]

Propiconazole and triadimefon were exceptions from the ranking correlation. Propiconazole and etaconazole are fairly similar analogues, since they only differ in the length of the alkyl substituent of the 1,3-dioxolan-ring. The propyl-substituent of propiconazole may enhance passage of the fungicide through fungal membranes as compared to the ethyl substituent of etaconazole and hence, may explain the relatively low difference in R_{IC50}s as compared to the R_{EC50}s. Triadimefon was slightly less fungitoxic than triadimenol, but was a relatively weak inhibitor of cell-free C4-desmethyl sterol synthesis (Table 4.1). Several papers report that different enzymes are involved in the activation of triadimefon in *Cladosporium cucumerinum*.[31-34] According to Köller (1987) reduction of triadimefon is mediated by cytoplasmic

NADPH-dependent dehydrogenases.[33] Under the test conditions used, cell-free extracts reduce triadimefon only poorly to triadimenol, probably due to a lack of specific enzymes (data not shown). Hence, the high IC_{50} of triadimefon is probably due to a lack of activation.

R_{EC50}s of eight experimental cyproconazole analogues ranged from 0.03 to 31.4 (Table 4.2), exhibiting a difference in fungitoxicity of about 1140 times. This is probably due to a wide variation in chemical structure of the N1-substituent of the cyproconazole analogues tested. IC_{50}s of structural analogues of cyproconazole ranked in the same order of inhibitory potency as in radial growth tests (r_s = 0.73). Compounds **1**, **2** and **3** were more potent inhibitors of cell-free C4-desmethyl sterol synthesis than cyproconazole. All other compounds were less effective in cell-free experiments than the reference compound. Compound **7**, which contains an additional triazole ring in its N1-substituent, was the only exception from this set of data. Although it inhibited radial growth of *B. cinerea* poorly (R_{EC50} 31.4), its intrinsic inhibitory potency was comparable to that of cyproconazole as indicated by the exceptionally high EC_{50}/IC_{50} ratio (1199). This implies that low fungitoxicity of this compound is not due to a low potency to inhibit sterol 14α-demethylation. Mechanisms which influence the concentration of the compound at the target site (accumulation, distribution within the cell, metabolic breakdown) may explain its relatively low fungitoxicity. Compound **6**, the cyproconazole analogue with the smallest N1-substituent, was the least potent inhibitor of cell-free synthesis of C4-desmethyl sterols.

EC_{50}s of 14 experimental tebuconazole analogues relative to the inhibitory potency of tebuconazole ranged from 0.6 to 32.8 (Table 4.3). The least potent compound **21** was about 60 times less toxic to fungal growth than the most potent analogue **8**. The cell-free assay ranked structural analogues of tebuconazole in the same order of intrinsic inhibitory potency as the radial growth test (r_s = 0.95). However, differences in IC_{50}s did not reflect the observed differences in fungitoxicity. Structures of the analogues tested were fairly similar. Minor modifications of the N1-substituent such as the absence of chlorine substituent in the benzyl-ring (**18**) as well as its replacement by a methyl group (**12**) increased both EC_{50} and IC_{50}. Furthermore, the position of chlorine in the benzyl-ring had an impact on activity: a change of the position of chlorine in the benzyl-ring from *para* to *meta* caused a reduction in activity in both tests. Compound **20** with a chlorine substitution of the benzyl ring in *ortho*-position displayed a R_{EC50} and R_{IC50} of 19.9 and 9.3, respectively. Increasing desaturation of the N1-substituent (compounds **15** and **16**) or replacement of the bulky t-butyl group by a linear substituent **17** caused a loss in fungitoxicity and intrinsic activity.

Results obtained indicate that the nature of the N1-substituent strongly affects inhibitory potency of DMIs, presumably by influencing their fit in the apoprotein pocket of the sterol 14α-demethylase, normally occupied by eburicol, the substrate of $P450_{14DM}$. This is consistent with current literature, which suggests that inhibitory potency of DMIs on $P450_{14DM}$ activity is predominantly determined by the interaction of the N1-substituent with the apoprotein of the 14α-demethylase.[35-42] This view is mainly based on differences in affinity of the isomers of triadimenol, paclobutrazol, diniconazol and diclobutrazole to P450 isozymes from yeast and *Ustilago maydis*.[36-42] CO-displacement studies showed that exchange of the N-heterocycles of itraconazole and ketoconazole did not affect the affinity of the compound to microsomal P450 from *Candida albicans*.[41,42] However, spectrophotometric studies with microsomal P450 isozymes from *Aspergillus fumigatus, Penicillium italicum* and *Ustilago maydis* demonstrated that CO-displacement correlated poorly with fungitoxicity of the compounds tested.[28-30] In order to study the impact of the nature of the heterocyclic ring on inhibitory potency of $P450_{14DM}$ activity structural analogues with different N-heterocycles should be tested in the assay described in this paper.

All stereoisomers tested inhibited cell-free C4-desmethyl sterol synthesis with different degrees of potency (Table 4.4). In general, isomers which were more toxic to radial mycelial growth also proved to be more potent inhibitors of cell-free C4-desmethyl sterol synthesis. However, EC_{50}/IC_{50} ratios were higher for less active isomers. This suggests that factors which influence the concentration of these compounds at the target site (accumulation, distribution within the cell, metabolism) are also stereoselective and have the same stereochemical preference as the sterol 14α-demethylase of *B. cinerea*. A more probable explanation is, that isomers with a high affinity for the $P450_{14DM}$ bind preferably to this enzyme, whereas isomers with a lower affinity for the $P450_{14DM}$ bind relatively stronger to other P450-containing cell-structures. The difference established in radial growth tests between the least (A_1 (+)isomer) and the most potent isomer (B_2 (−)isomer) of cyproconazole was 161. This confirms results of Grabski and Gisi[43] who reported that the ratio between fungicidal activity of the most and the least active isomers against perthotrophic fungi was higher than 20. The sequence of decreasing activity in both assays tested was B_2 (−) > A_2 (−) >> B_1 (+) > A_1 (+), which is consistent with the results published by Grabski and Gisi.[43] In their experiments commercial cyproconazole (a 1:1:1:1 mixture of the four isomers) was in most cases more fungitoxic than the most potent isomer B_2. In the present assays, cyproconazole (61.7% A; 38.3% B) was about four to six times less active than the most potent isomer B_2. The significant differences in *in vitro* activity between the cis- and trans-isomer of SSF-109 confirm the results from Masuko *et al.* (1988) and Murabayashi *et al.* (1990).[44,45] They report that the cis-isomer is

significantly more active towards *B. cinerea* than the trans-isomer.[44,45] Therefore, only the cis-isomer was further developed. The (−)enantiomer of tebuconazole was more active than the (+)enantiomer in both radial growth tests and cell-free experiments. These findings support results from Berg *et al.*[46] Interestingly, the IC_{50} and EC_{50} of commercial tebuconazole (a racemic mixture of the two enantiomers) was only slightly higher than the corresponding values of the more potent (−)isomer as already published by Berg *et al.*[46] However, synergy calculations according to the Wadley method[47] only demonstrated an additive interaction, both, on an enzymatic and cellular level (results not shown). Grabski and Gisi[43] and Hermann and Gisi,[48] reported synergistic interactions of cyproconazole isomers with pathogens different from *B. cinerea*. Berg *et al.*[46] indicated a synergistic interaction of isomers of tebuconazole towards *B. cinerea in vitro*. These contradictory results are not easily understood.

Berg *et al.* (1987) showed that *B. cinerea*, *Pyricularia oryzae*, *Fusarium culmorum* and *Pyrenophora teres* accumulated Δ^5-sterols ($\Delta^{5,24}$-lanostadienol, Δ^5-ergostenol, Δ^5-stigmasterol and $\Delta^{5,22}$-stigmadienol) upon treatment of whole cells with low concentrations of tebuconazole.[46] From these findings they stated that $\Delta^8 \rightarrow \Delta^7$ isomerization is a two step process: reduction of the Δ^8-bond succeeded by Δ^7-dehydrogenation, possibly mediated by a cytochrome P450 dependent C7-hydroxylation and subsequent cleavage of water. As another explanation for the exceptional accumulation pattern they discussed a direct interaction of tebuconazole with membranes as described for dithiocarbamates, causing a secondary change in sterol composition by modifying protein/lipid interactions.[49] In our experiments differences in desaturation levels or patterns of the sterols extracted from tebuconazole treated cell-free extracts were never detected (Fig. 4.1 and 4.2). This supports results of Kwok and Loeffler[50], who identified $\Delta^{5,24}$-lanostadienol as obtusifoliol but did not detect any plant sterols.

Acknowledgements

M.A. de Waard acknowledges financial support of Bayer AG (Leverkusen, Germany) and Sandoz AG (Basel, Switzerland). The authors are grateful to Dr. Holmwood and Dr. Lantzsch from Bayer AG and Dr. Schaub from Sandoz AG for providing structural analogues of tebuconazole and cyproconazole, respectively.

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Chapter 5

Factors Influencing Activity of Triazole Fungicides Towards *Botrytis cinerea*

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Crop Protection (submitted)

Abstract

Activity of triazole fungicides towards *Botrytis cinerea* was investigated *in vitro* (radial growth on fungicide-amended agar) and *in vivo* (foliar-sprayed tomato plants and dip-treated grapes). In both tests the benzimidazoles benomyl and thiabendazole and the dicarboximides iprodione and vinclozoline were used as reference fungicides. In all experiments benomyl and tebuconazole proved to be the most active fungicides. The transfer ratio, which is defined as the ratio between the EC_{50} of a particular fungicide determined *in vivo* and *in vitro*, was lowest for benomyl. The transfer ratio of tebuconazole was comparable to or slightly lower than those of dicarboximides. Hence, no obvious correlation between *in vitro* and *in vivo* activity was observed. Field rates of tebuconazole recommended for control of *B. cinerea* are relatively low compared to those of benzimidazoles and dicarboximides. Tomato leaf homogenates and various biological compounds antagonized activity of triazoles and dicarboximides but did not affect inhibitory potency of benzimidazoles. It is suggested that the factors mentioned account only partly for the limited field performance of triazoles towards *B. cinerea*.

Introduction

Since the discovery of antifungal activity of N1-substituted azoles in the late 1960s, a large number of azole derivatives has been developed as fungicides and/or antimycotics. The mode of action of these N1-substituted azoles is based on inhibition of cytochrome P450-dependent 14α-demethylation of lanosterol or eburicol in synthesis of ergosterol, the main sterol of fungal membranes.[1-3] A considerable variation in N1-moieties is compatible with antifungal activity. By now triazoles comprise about 25 commercial agrochemicals and constitute the largest group of modern agricultural fungicides. Most triazole fungicides are used against rusts, powdery mildews and scabs, but are rarely applied in control of grey mould.[4-7]

Grey mould, which is incited by *Botrytis cinerea* Pers. ex Fr., is one of the most ubiquitous diseases of various major crops, and on transported and stored flowers, fruits and vegetables.[8] Benzimidazole and dicarboximide fungicides have been used extensively for grey mould control. Soon after their introduction, serious problems in control of grey mould have been encountered because of rapid development of resistant populations.[9-11] In this context, a replacement of benzimidazoles and dicarboximides by triazole fungicides would be attractive, since they have a number of advantages over other fungicides including a relatively low resistance risk.[12-14]

For several years activity of triazole fungicides towards *B. cinerea* has been discussed in literature[15-19] but no definite data were presented to explain their

limited field performance towards this pathogen. Previous investigations demonstrated that triazole fungicides inhibit effectively activity of the target enzyme sterol 14α-demethylase and *in vitro* mycelial growth of *B. cinerea*.[20,21] Moreover, the degree of *in vitro* activity was comparable to that of triazoles towards other plant pathogens.[22-27] Some of these pathogens are controlled by demethylation inhibitors (DMIs) in practice. In the present study, activity of the triazoles tebuconazole and triadimenol is compared in radial growth and *in vivo* tests with that of selected benzimidazoles and dicarboximides in order to establish whether limited field performance of triazoles towards *B. cinerea* is due to low *in vivo* activity of these fungicides. Furthermore, additional factors such as fungal and plant constituents, which may reduce *in vivo* performance of triazoles towards *B. cinerea*, are studied.

Materials and methods

Fungus and culture conditions

The monoascospore isolate SAS56 of *B. cinerea* was used in all experiments.[28] The isolate was maintained on tomato agar prepared as described by Salinas (1992) and supplied with 1.2% technical agar (Oxoid, Basingstoke, UK) under near-uv light at 20°C.[29] Subcultures were made every three to four days by mass transfers of conidia. Conidia were harvested from 2-3-week-old cultures with 0.1% Tween 20 solution in sterile distilled water. Conidia were separated from mycelium by filtration through sterile glass wool and conidial concentrations were determined with a haemacytometer.

Chemicals

All fungicides were chemically pure active ingredients. They were generously provided by their respective manufacturers. The detergent Agral LN, used in *in vivo* experiments (0.04%, v/v), was purchased from ZENECA (Rotterdam, the Netherlands). Gamborg's B5-medium was purchased from DUCHEFA (Haarlem, the Netherlands). Cuprophan membranes type 150 M (20 μm thickness) were supplied by AKZO (Wuppertal, Germany). Arachidic acid ($C_{20:0}$), behenic acid ($C_{22:0}$), benzimidazole, *cis*-5,8,11,14,17-eicosapentaenoic acid ($C_{20:5}$), elaidic acid ($C_{18:1}$, *trans* 9), ergosterol, erucic acid ($C_{22:1}$), lanosterol, lauric acid ($C_{12:0}$), mannan, nervonic acid ($C_{24:1}$), oleic acid ($C_{18:1}$, *cis* 9), L-α-phosphatidylcholine from soybean, L-α-lysophosphatidylcholine - diarachidoyl, L-α-lysophosphatidylcholine - palmitoyl, L-α-phosphatidylethanolamine ($C_{18:0}$) from soybean, pullulan, stearic acid ($C_{18:0}$), tripalmitin, and tristearin were purchased from Sigma Co. (St. Louis, MO, USA). Arachidonic acid ($C_{20:4}$) was purchased from Fluka AG (Buchs, Switzerland). Progesterol, testosterol and cholesterol were purchased from

BDH Chemicals Ltd. (Poole, England). ß-Sitosterol was supplied by Th. Schmuck GmbH (München, Germany). Pustulan was purchased from Behringer Diagnostics (La Jolla, CA, USA). 22*S*,23*S*-Homobrassinolide was a gift from Prof. Dr. G. Adam (Halle, Germany).

In vitro *activity of test fungicides*

In vitro activity of benzimidazoles (benomyl, thiabendazole), dicarboximides (iprodione, vinclozoline), and triazoles (tebuconazole, triadimenol) was assessed in radial growth experiments on B5-agar inoculating each Petri dish with three drops (5 μl) of conidial suspension in sterile distilled water (10^6 ml^{-1}). Fungicides used were chemically pure active ingredients. Experiments - performed in duplicate - were repeated three times.

Inhibitory activity of fungicides was expressed as EC_{50}s (concentrations of fungicides inhibiting radial growth of *B. cinerea* on B5-agar by 50%). Colony diameters of fungicide treatments were calculated as percentages of control treatments (100%). Using the software-program Lotus 1-2-3, percentages were plotted against fungicide concentration on a logarithmic scale and regression analysis of inhibitor response data was performed.

In vivo *activity of test fungicides*

In vivo activity of test fungicides was determined in foliar spray tests with 6-week-old tomato plants (*Lycopersicon esculentum* Mill. cv. Money Maker). Foliar spray tests were carried out in a spray cabinet, developed at the Department of Phytopathology. The spray cabinet is adapted with a spray gun, which controls the size of the spray droplets and the shape and size of the spray cone. In this experiment a circle-shaped spray cone and a pressure of 0.8 atm were used. Two plants per treatment were accommodated on the turn table and sprayed until run-off with fungicide solu-tion (10 ml) for 2 min. Fungicide solutions were prepared in distilled water containing 0.4% Agral LN as detergent from 1000x concentrated stock solutions of pure active ingredient in DMSO. Spraying was performed at an angle of 45° and from a distance of 75 cm. Fungicide-treated and control plants were allowed to dry for 4 h.

In whole plant experiments the two oldest, fully developed leaves were slightly injured at the upper site with a blunt needle. Each injury site (1-2 mm diameter) was inoculated with a drop (5 μl) of conidial suspension (10^5 ml^{-1}) in potassium phosphate buffer (10 mM, pH 5.0) containing sucrose (10 mM). Inoculated leaves were covered with plastic bags. Plants were incubated in a climate room under fluorescent light (Philips TLMF 40 W/35 RS; 7000 lux; 16-h photoperiod) at 80% RH and 20°C. Radial lesion expansion was assessed after eight days by measuring two diameters per lesion.

In the detached-leaf system the two oldest leaves were - after fungicide treatments and a drying period of 4 h - transferred to water agar amended with benzimidazole (10 μg ml^{-1}). Each leaf was injured at one site as described above and inoculated with standard conidial suspension (5 μl). Petri dishes were sealed with parafilm and incubated in a climate room under fluorescent light (16-h photoperiod) at 20°C. After eight days lesion development was determined by measuring two diameters per lesion.

In an additional experiment, *in vivo* performance of benzimidazoles, dicarboximides and triazoles was determined using detached mature grape berries (*Vitis vinifera* L. cv. Blauwe van Boskoop) as a host. Antifungal activity of test fungicides was evaluated by assessing infection frequency at injury sites of grape berries. Grape berries (20 per treatment) were superficially sterilized with 96% ethanol, injured before or after fungicide treatment with a needle, dipped for 2 min in fungicide solution prepared as described above, allowed to dry for 4 h, and inoculated by spraying bunches of 20 grape berries with conidial suspension (10^5 ml^{-1}) in a mycological peptone (0.1 %, w/v) - maltose (0.4%, w/v) solution, pH 5.5. Grapes were incubated in sealed plastic chambers, lined with moist filter paper, at 20°C. Chambers were opened every second day for 4 h. After eight days, 20 injury sites (10 berries) per treatment were assessed for visual infection.

In all *in vivo* experiments, the pathogen was re-isolated from infection sites and identified microscopically as *B. cinerea*. Additional controls were performed by inoculating injury sites with sterile water. All experiments were performed in duplicate and repeated three times. Inhibitory activity of fungicides was expressed as EC_{50} (concentration of fungicide inhibiting lesion expansion on tomato leaves or colonization of injury sites on grape berries by 50%). Lesion diameters on tomato leaves or infection frequency of injury sites on grape berries were calculated as percentages of control treatments (100%). Using the software-program Lotus 1-2-3, percentages were plotted against fungicide concentration on a logarithmic scale and regression analysis of inhibitor response data was performed.

Effects of tomato leaf homogenate (TLH) on activity of fungicides

Effects of TLH on inhibitory activity of test fungicides on conidial germination and germ tube elongation were investigated with B5-agar, pH 6.0, amended with homogenate of fresh tomato leaves (TLH; 10 g 100 ml^{-1} B5-agar). Test media (15 ml) with and without TLH were mixed with fungicides from 1000x concentrated stock solutions in DMSO and poured in Petri-dishes (9 cm diameter). Sterilized Cuprophan membranes were transferred to dried agar surfaces. Membranes were inoculated with 100 μl of conidial suspension (3 - 5 x 10^3 conidia ml^{-1}) evenly spread over the membrane surface. After 16 h of incubation at 20°C in the dark, germination of 100

conidia and germ tube length of 50 conidia per treatment were assessed microscopically. Inhibition of mycelial growth was tested on the same media without membranes. Petri dishes were inoculated with one drop (5 μl) of conidial suspension (10^6 ml^{-1}) in sterile water. Mycelial growth was determined after incubation at 20°C in the dark for two days. All experiments were performed in duplicate and repeated three times. Antagonistic activity of TLH was expressed as antagonism factor (AF), which is the ratio between the EC_{50} of a fungicide tested on B5+TLH and the EC_{50} of the same fungicide tested on B5-agar.

Preparation of test material

Fungal mycelium was harvested from overnight cultures incubated in liquid medium[30] at 20°C for 14 h. Fresh mycelium (10 and 1 g wwt) and equivalent amounts of freeze-dried mycelium (1.4 and 0.14 g dwt) were homogenized in a Braun homogenizer. The same procedure was followed for fresh tomato leaves (10 and 1 g fwt) and freeze-dried tomato leaves (1 and 0.1 g dwt).

Lipids and non-saponifiable lipids (NSLs) were extracted from freeze-dried mycelium (1.4 g dwt) or plant tissue (1 g dwt) with chloroform + methanol (2 + 1 by volume) or petroleumether (b.p. 40 - 60°C), respectively, dried under a stream of nitrogen, and resuspended in chloroform (200 μl). Water extracts of plant tissue were prepared by homogenizing fresh tomato leaves (10 g) with distilled water (10 ml). Cell debris were removed by filtration and subsequent centrifugation (10 min) at 2000 g. The supernatant was dried under a stream of nitrogen and dissolved in distilled sterile water (200 μl).

Mannans from *B. cinerea* were isolated from liquid cultures (3 l) according to Kamoen, Geeraert and Van der Cruyssen (1992).[31]

Paper disc assay

Fungal and plant material were tested for agonistic effects on activity of benzimidazoles, dicarboximides and triazoles towards *B. cinerea* in a paper disc assay. Flasks with molten B5-agar, pH 6.0, (100 ml, 40 - 45°C) amended with agonists were seeded with 1 ml of conidial suspension (10^7 conidia ml^{-1}). Paper discs (9 mm diameter) were dipped in chloroform solutions of fungicides (30 μg ml^{-1} benomyl; 100 μg ml^{-1} iprodione; 40 μg ml^{-1} tebuconazole; 150 μg ml^{-1} thiabendazole; 500 μg ml^{-1} triadimenol; or 400 μg ml^{-1} vinclozoline) for 10 sec and dried for 2 min. Paper discs (3) were transferred to solidified B5-agar (10 ml) in Petri dishes (9 cm diameter). After incubation at 20°C in the dark for five days, diameters of inhibition zones were measured. Experiments were performed in duplicate.

Table 5.1 Activity of fungicides on radial growth of *Botrytis cinerea* on B5-agar, lesion expansion on tomato leaves and infection frequency on grape berries.

	EC_{50} ($\mu g\ ml^{-1}$)[1]					
Fungicide	B5-agar[2]	Intact tom.plant[3]	Det.tom.leaves[3]	Grapes (i.b.t.)[4]	Grapes (i.a.t.)[4]	Field rate[5]
Benomyl	0.08 ± 0.01	1.5 ± 0.2 [18][6]	1.5 ± 0.2 [19]	0.8 ± 0.3 [10]	7.4 ± 2.8 [92]	1250 - 2500 500 - 1000
Tebuconazole[7]	0.11 ± 0.01	5.0 ± 1.2 [53]	5.3 ± 2.8 [48]	1.9 ± 0.6 [17]	13.3 ± 7.1 [121]	250 300 - 400
Vinclozoline	0.22 ± 0.02	8.8 ± 1.3 [40]	9.5 ± 2.0 [43]	21.5 ± 1.7 [98]	24.0 ± 8.2 [109]	1250 - 2500 500 - 1000
Iprodione	0.31 ± 0.04	42.1 ± 4.2 [136]	30.9 ± 3.8 [100]	51.8 ± 9.6 [167]	98.9 ± 11.2 [319]	1250 500
Thiabendazole	0.43 ± 0.07	10.4 ± 1.9 [24]	10.8 ± 1.8 [25]	12.3 ± 3.7 [29]	31.0 ± 7.2 [72]	1000 300
Triadimenol[8]	1.9 ± 0.4	35.0 ± 8.5 [19]	35.0 ± 3.9 [21]	33.7 ± 9.8 [18]	69.4 ± 12.5 [37]	- -

[1] Average EC_{50} from four experiments.
[2] Concentration of fungicide inhibiting radial growth on B5 agar by 50%.
[3] Concentration of fungicide inhibiting lesion expansion on intact tomato plants or detached tomato leaves by 50%.
[4] Concentration of fungicide inhibiting colonization of infection sites on grape berries by 50%; berries injured before (i.b.t.) or after (i.a.t.) fungicide treatment.
[5] Rates advised in Crop Protection Manual of the Netherlands (1993) and Product Information Manual, Bayer AG, Germany (1993) in mg l^{-1} (up) and g ha^{-1} (down) for control of *B. cinerea* by benomyl (beans and cucumber), 10% tebuconazole as mixture with 40% dichlofluanid (grapes), vinclozoline (beans, cucumber, peas and lettuce), iprodione (beans, cucumber, peas and lettuce), and thiabendazole (ornamentals).
[6] Transfer ratio: EC_{50} of fungicide in *in-vivo* experiment relative to EC_{50} of the same fungicide in radial growth test on B5 agar.
[7] Tebuconazole is advised as a single product for control of powdery mildews, rusts and leaf blight in cereals and *Phoma* and *Sclerotinia* in rape seed at 830 - 1500 mg l^{-1} or 250 - 375 g ha^{-1}.
[8] Triadimenol is advised for control of powdery mildews, rusts and leaf blight in cereals at 1000 mg l^{-1} or 500 g ha^{-1}.

Results

In vitro *activity*

Radial growth tests demonstrated that tebuconazole and benomyl were the most potent fungicides, giving EC_{50}s of 0.08 and 0.11 μg ml^{-1}, respectively (Table 5.1). All other fungicides were significantly less potent and displayed EC_{50}s ranging between 0.22 (vinclozoline) and 1.9 μg ml^{-1} (triadimenol).

In vivo *performance on tomato*

In vivo performance of the selected fungicides was tested on foliar sprayed tomato plants and detached tomato leaves. In control treatments, 89 $\pm$ 7% (n = 36) of inoculation sites on leaves of intact plants and 91 $\pm$ 8% (n = 36) of inoculation sites on detached tomato leaves developed lesions. Average lesion diameter determined from 36 control treatments on intact plants and detached leaves was 27 $\pm$ 5 and 20 $\pm$ 6 mm, respectively. Lesions did not develop on leaves inoculated with sterile water.

In both experiments, benomyl was the strongest inhibitor of lesion expansion, giving an EC_{50} of 1.5 μg ml^{-1} (Table 5.1). Activity of tebuconazole was slightly lower (EC_{50}s 5.0 and 5.3 μg ml^{-1}). All other fungicides tested were less potent, displaying EC_{50}s between 8.8 and 42.1 μg ml^{-1}. Benomyl had the lowest transfer ratios (18 and 19) from *in vitro* to *in vivo* test situations of all fungicides tested (Table 5.1). The transfer ratios of tebuconazole (53 and 48) were comparable with those of vinclozoline (40 and 43). Iprodione showed the highest transfer ratios of all fungicides tested (136 and 100).

In vivo *performance on grapes*

In a further experiment, *in vivo* performance of fungicides was assessed in dip treatments with grape berries injured before or after application of fungicides. In control treatments, all injury sites became colonized with *B. cinerea*. Grape bunches inoculated with sterile water remained healthy. In general, performance of all fungicides was significantly reduced, if berries were injured after fungicide treatment (Table 5.1). In both experiments benomyl was the most active fungicide tested (EC_{50}s 0.8 and 7.4 μg ml^{-1}, respectively). Tebuconazole was less active than benomyl (EC_{50}s 1.9 and 13.3 μg ml^{-1}, respectively) but controlled *B. cinerea* more effectively than the other fungicides tested. The highest transfer ratios between *in vitro* activity and *in vivo* performance on grape berries were determined for iprodione (167 and 319).

Effect of tomato leaf homogenate (TLH) on fungicide activity

The influence of TLH on the potency of test fungicides to inhibit germination of conidia, germ tube elongation and radial mycelial growth was tested with B5-agar amended with TLH. After 16 h incubation, germination of conidia in control treatments ranged around 90%. At the concentrations tested, all fungicides hardly reduced germination of conidia (data not shown). TLH did not affect germination, both in the absence and presence of fungicides.

Table 5.2 Activity of fungicides towards germ tube elongation and radial mycelial growth of *Botrytis cinerea* on B5-agar, pH 6.0, with and without tomato leaf homogenate (TLH).

	EC_{50} (μg ml^{-1})			
	Germ tube elongation[1]		Mycelial growth[2]	
Fungicide	B5	B5+TLH	B5	B5+TLH
Benomyl	0.09 ± 0.01 a[3]	0.09 ± 0.01 a [1][4]	0.08 ± 0.01	0.1 [1]
Tebuconazole	0.06 ± 0.01 a	1.3 ± 0.6 b [22]	0.11 ± 0.01	0.8 [8]
Vinclozoline	0.32 ± 0.01 a	0.9 ± 0.2 b [3]	0.22 ± 0.03	0.5 [2]
Iprodione	0.37 ± 0.02 a	0.9 ± 0.2 b [2]	0.31 ± 0.05	0.6 [2]
Thiabendazole	0.23 ± 0.03 a	0.3 ± 0.1 a [1]	0.4 ± 0.1	0.2 [0.5]
Triadimenol	1.0 ± 0.2 a	3.9 ± 0.5 b [4]	1.9 ± 0.5	2.8 [2]

[1] Concentration of fungicide inhibiting germ tube elongation by 50% (n = 3), germtube length in control: 377 ± 102 μm.
[2] Concentration of fungicide inhibiting radial growth by 50% (n = 3), colony diameter in controls: 26 ± 1 mm.
[3] Figures in the same row followed by the same letter do not differ significantly (P = 0.05).
[4] Antagonism factor (AF): EC_{50} B5+TLH / EC_{50} B5.

On B5-agar benomyl and tebuconazole were the strongest inhibitors of germ tube elongation and radial mycelial growth of all fungicides tested with EC_{50}s between 0.06 and 0.11 μg ml^{-1} (Table 5.2). All other fungicides tested were 3 to 19 times less active. Triadimenol was the least active fungicide tested with EC_{50}s of 1.0 and 1.9 μg ml^{-1} for inhibition of germ tube elongation and radial mycelial growth, respectively (Table 5.2). TLH significantly (P = 0.05) reduced activity of tebuconazole towards germ tube (AF: 22) and mycelial (AF: 8) growth (Table 5.2). TLH also

Table 5.3 Interaction of fungal and plant material and biological compounds with activity of fungicides towards *Botrytis cinerea* in paper disc assays.

Test material	Conc.	Growth inhibition zone around paper disc impregnated with fungicide (mm)[1]					
		Benomyl	Thiabendazole	Iprodione	Vinclozoline	Tebuconazole	Triadimenol
Control		27.5 ± 4.9	27.5 ± 5.2	26.5 ± 1.0	24.6 ± 0.6	25.9 ± 1.1	27.0 ± 0.6
Mycelium of *B. cinerea*[2]							
Fresh	1	40	43	23	20	22	24
	10	43	43	18	16	17	21
Freeze-dried	1	39	38	16	16	17	21
	10	50	50	19	20	16	22
Lipids	10	25	26	22	22	16	22
NSLs	10	26	27	22	21	19	24
Tomato leaf homogenates[3]							
Fresh	1	33	32	24	20	20	25
	10	44	46	16	19	14	18
Freeze-dried	1	35	35	19	17	18	20
	10	38	40	16	15	18	20
Water extract	10	40	46	20	21	26	25
Lipids	10	31	31	21	16	17	22
NSLs	10	27	25	21	20	21	25
Sterols and related compounds[4]							
Cholesterol	10	27	27	27	27	26	27
Ergosterol	10	25	27	25	25	22	25
S,S-Homobrassinolide	10	32	36	27	27	24	26
Lanosterol	10	26	29	27	26	25	24
Progesterol	10	31	33	17	18	17	17
ß-Sitosterol	10	29	30	26	27	25	25
Testosterol	10	41	45	21	23	24	24

Table 5.3 continued

Saturated fatty acids[4]							
Arachidic acid	100	26	28	27	23	25	27
Behenic acid	100	43	47	26	30	19	17
Lauric acid	100	40	42	26	25	26	30
Stearic acid	100	26	29	27	22	25	26
Unsaturated fatty acids[4]							
Arachidonic acid	100	30	33	22	22	14	17
Eicosapentaenoic acid	100	33	35	26	25	24	24
Elaidic acid	100	42	47	30	30	26	30
Erucic acid	100	26	27	24	24	21	25
Nervonic acid	100	29	31	25	25	22	25
Oleic acid	100	23	25	24	22	22	26
Phospholipids[4]							
Phosphatidylcholine							
- Diarachidonic acid	100	25	22	26	24	24	28
- Palmitoyl	100	28	30	27	23	25	28
- Soybean	100	30	31	24	23	23	26
Phosphatidylethanol-amine	100	43	43	32	30	31	31
Acylglycerides[4]							
Tripalmitin	100	28	31	27	23	24	26
Tristearin	100	31	32	28	23	24	26
Polysaccharides[4]							
Pustulan	100	25	23	26	24	25	28
Pullulan	100	29	26	26	23	24	28
Mannan	100	30	28	27	25	24	27
B. cinerea mannans[5]	3	34	32	25	26	24	27

[1] Controls: average of four experiments; tests: average of two experiments.
[2] g wet weight or amounts equivalent to wet weight per 100 ml B5-agar.
[3] g fresh weight or amounts equivalent to fresh weight per 100 ml B5-agar.
[4] μg ml^{-1} B5-agar.
[5] Extracts from 3 l liquid culture 100 ml^{-1} B5-agar.

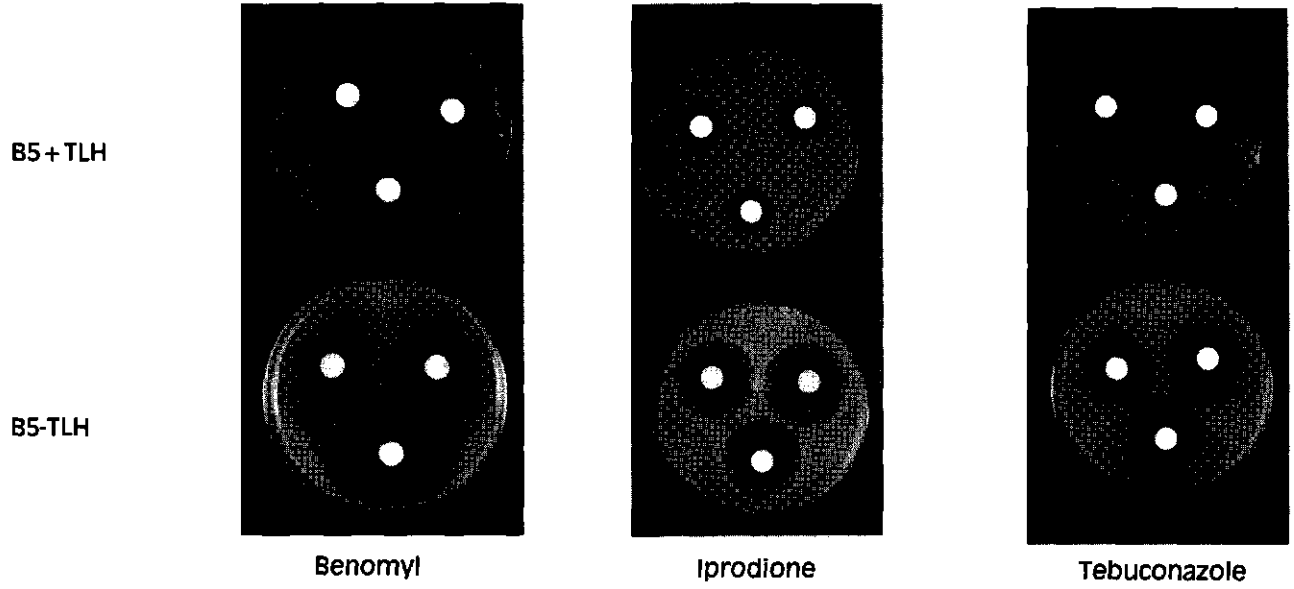

Figure 5.1 Effect of homogenized fresh tomato leaves (TLH) on activity of fungicides towards *Botrytis cinerea* in paper disc assays.

significantly (P = 0.05) reduced activity of triadimenol and the dicarboximides tested on germ tube elongation. However, it did not affect activity of benomyl and thiabendazole.

Interaction of biological materials with fungicide activity

Since TLH influenced activity of fungicides on germ tube and mycelial growth, the effect of a broader spectrum of biological material was tested in a paper disc assay. Fungal and plant biomass effectively counteracted activity of dicarboximides and triazoles tested, but enhanced inhibitory potency of benomyl and thiabendazole (Table 5.3, Fig. 5.1). Lipids and non-saponifiable lipids extracted from fungal mycelium and tomato leaf tissue also reversed toxicity of dicarboximides and triazoles but showed no effect or slight synergism with benzimidazoles. Water extracts of plant material alleviated activity of dicarboximides, but had no effect on activity of triazoles and enhanced inhibitory potency of benomyl and thiabendazole. Sterols and related steroids tested either reduced or enhanced toxicity of the particular fungicides. Progesterol most effectively antagonized activity of dicarboximides and triazoles, but seemed to enhance activity of the benzimidazoles tested. Of the saturated and unsaturated fatty acids tested, arachidonic acid showed the most prominent result: it alleviated activity of dicarboximides and triazoles and potentiated activity of benzimidazoles. Some saturated fatty acids increased activity of benzimidazoles. Among the phospholipids and acylglycerides phosphatidylethanolamine synergized activity of all fungicides tested, whereas all other test compounds were less active. Polysaccharides did not show any obvious effect on activity of test fungicides.

Discussion

In all *in vivo* experiments tebuconazole was slightly less active than benomyl. However, tebuconazole performed better than the commercial botryticides iprodione, thiabendazole and vinclozoline (Table 5.1). The same applies to triadimenol, when compared with iprodione. Therefore, these results do not explain why triazoles only have a limited field performance towards *B. cinerea*. The good field performance of benomyl may in part be explained by its relatively low transfer ratios and the relatively high application rates recommended (Table 5.1). The transfer ratios determined for tebuconazole were comparable to or lower than those of the dicarboximides tested. Hence, the magnitude of the transfer ratio of triazole fungicides can not explain their limited field performance. Field rates of triazoles recommended for control of target pathogens such as powdery mildews and rusts in

cereals are considerably lower than those advised for control of grey mould diseases on fruits by benzimidazoles and dicarboximides.[32,33] Low efficacy of triazoles may be related to the fact that the field rates recommended are relatively low for *Botrytis* control. However, application of higher rates may be limited by phytotoxic (plant growth regulatory) effects of triazoles.[34-37]

In general, performance of all fungicides was significantly reduced, if grape berries were injured after fungicide treatment. This can be explained by a lack of fungicide deposit on/in the wound, which enables the fungus to establish in the host. In general, data presented in Table 5.1 indicate that the correlation between *in vivo* performance and the potency to inhibit radial mycelial growth on B5-medium is only weak. This is probably due to factors as uptake, transport and persistence of fungicides in the plant and interaction of plant constituents with antifungal activity.

All fungicides tested barely affected germination of conidia. This corroborates earlier reports that triazoles and benzimidazoles are poor inhibitors of spore germination.[38-41] With both groups of fungicides germ tubes of treated conidia are relatively shorter, swollen and deformed. Dicarboximides are known to inhibit both germination and mycelial growth, but inhibit the latter much more efficiently.[30,42] Hence, germinating conidia may rapidly invade underlying plant tissue before being affected by fungicide residues. Once inside the plant tissue antagonistic effects of plant constituents on fungicide activity may occur. Therefore, effects of plant constituents on inhibition of conidial germination, germ tube elongation and mycelial growth of *B. cinerea* by the fungicides selected were studied on agar amended with TLH. TLH hardly influenced spore germination, but enhanced germ tube elongation and radial mycelial growth. TLH barely influenced inhibitory potency of benomyl and thiabendazole towards germ tube elongation and mycelial growth, whereas it reduced activity of dicarboximides and triazoles significantly (Table 5.2). The effect of TLH on activity of tebuconazole was most prominent. However, the transfer ratio between *in vitro* and *in vivo* activity of tebuconazole was still comparable or even lower than that of the dicarboximides tested (Table 5.1). Since TLH antagonized activity of both, dicarboximides and triazoles, its effect is probably non-specific. A possible explanation may be that these fungicides partition into lipophilic components present in TLH.

It is well known that activity of sterol demethylation inhibitors (DMIs) can be antagonized by various compounds, including sterols and related compounds, phospholipids, acylglycerides, fatty acids and surfactants.[38,43-50] These observations might in part explain the antagonism of triazole activity by TLH as described above. Little is known about the specificity of these effects since these experiments were not performed with benzimidazoles and dicarboximides. Results from paper disc assays showed that fungal mycelium or TLH counteract activity of dicarboximides and

triazoles, but enhanced activity of benzimidazoles (Table 5.3, Fig. 5.1). These results corroborate results from radial growth experiments presented in Table 5.2. Synergistic effects of TLH on activity of the benzimidazoles could not be detected in radial growth experiments. This apparent discrepancy is not understood.

Antagonistic effects of TLH on activity of the triazoles resided in the lipid-fraction of TLH, as diameters of inhibition zones of triazoles were not affected by addition of water extracts to test medium. Antagonistic effects of protoplast membranes and lipid fractions isolated from *Candida albicans* on activity of clotrimazole and miconazole were already reported by Yamaguchi (1977).[46]

Progesterone and testosterone alleviated activity of triazoles and dicarboximides. Such effects have been described earlier for triazoles.[38,39,47] Other steroids tested were not active. These findings support results of Yamaguchi (1977) reporting an effective antagonism on activity of clotrimazole and miconazole only by uv-activated steroids, which lack a 3β-OH group and a saturated or unsaturated hydrocarbon side chain.[46] This may be due to steric requirements for uptake of the antagonist into the fungal cell.

The saturated fatty acid behenic acid ($C_{20:0}$) and the unsaturated fatty acid arachidonic acid ($C_{20:4}$) strongly antagonized activity of tebuconazole and triadimenol. This observation is in agreement with several reports on alleviation of the activity of various antifungal compounds by phospholipids and free fatty acids[46,47,49-53] and supports the assumption that unsaturated fatty acids and phospholipids or acylglycerides with an unsaturated acyl group have the highest antagonistic activity.[46,51] Further studies demonstrated that various antifungals form hydrophobic complexes with egg lecithin and purified unsaturated fatty acids, which results in a reduced concentration of the antimicrobial agent in the medium.[49,54,55] Another indication for interaction of triazoles with membrane lipids are rapid direct membrane-damaging effects of some DMIs when applied at fungicidal concentrations.[36,56-59]

The antagonists found alleviated toxicity of triazoles and dicarboximides to comparable extends, suggesting a non-specific interaction with those fungicides. However, the antagonistic effect on activity of the benzimidazoles tested was limited. This may be due to differences in lipophilicity of the fungicides tested. Tebuconazole has a log P of 3.7. Log P values of triadimenol, iprodione and vinclozoline are around 3.1. Those of benomyl and thiabendazole are 2.1 and 1.6, respectively. It is likely that the reduction of toxicity may be a consequence of partitioning of the fungicides into undissolved residues of the antagonist. This would suggest a lack of specificity in *in vitro* antagonism towards different fungi, which is not always true.[38,43,44,46,49] Hence, additional mechanisms may be involved as well. Since

wounded plant tissue provides infection sites for *B. cinerea*,[60-62] antagonistic effects of plant constituents may become relevant during pathogenesis.

In summary, it is concluded that limited field performance of triazole fungicides towards grey mould is not due to low *in vitro* and *in vivo* sensitivity of the causal agent *B. cinerea*. It can also not be ascribed to a short half-life time of triazoles under field conditions, as these fungicides generally perform extremely well against various other foliar pathogens. Antagonizing effects of plant and fungal constituents can only partly explain the relatively low field performance of triazoles, since these effects were also observed with dicarboximide botryticides. Possibly, the relative low field rates recommended for DMIs as compared to those for benzimidazoles and dicarboximides for *Botrytis* control also account in part for limited field performance of DMI fungicides. An additional factor may be a high genetic diversity in sensitivity of the pathogen population to triazoles. This topic will be subject of further research.

Acknowledgements

This work was supported by Bayer AG (Leverkusen, Germany) and Sandoz AG (Basel, Switzerland). The authors are grateful to Prof. Dr. G. Adam (Halle, Germany) for providing *S,S*-Homobrassinolide and Dr. J. Hermens (Utrecht, the Netherlands) for calculation of log P values of test fungicides. Also, skilful assistance of Mr. A.J. Cozijnsen in *in vivo* experiments is gratefully acknowledged.

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Chapter 6

Sensitivity of Populations of *Botrytis cinerea* to Triazoles, Benomyl and Vinclozoline

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European Journal of Plant Pathology (submitted)

Abstract

Sensitivity of field isolates (121) of *Botrytis cinerea* from France (1992), Germany (1979 - 1992), Israel (1990) and the Netherlands (1970 - 1989) to the triazoles tebuconazole and triadimenol, the benzimidazole benomyl and the dicarboximide vinclozoline were tested in radial growth experiments. Resistance to benomyl (in 21 to 100% of isolates tested) and vinclozoline (in 25 to 71% of isolates tested) was common in most countries. EC_{50}s (concentrations of fungicides inhibiting radial mycelial growth of *B. cinerea* on B5-agar by 50%) for tebuconazole and triadimenol ranged between 0.01 - 1.64 and 0.4 - 32.6 μg ml^{-1}, respectively, and were log-normally distributed. The variation factor (ratio between EC_{50} of the least and most sensitive isolate tested) amounted 164 and 82 for tebuconazole and triadimenol, respectively. These values are comparable to those for azole fungicides applied in control of other pathogens. Hence, variation in sensitivity to triazoles can probably not explain limited field performance of triazoles towards *B. cinerea*. Isolates from south west Germany (1992) were significantly less sensitive to tebuconazole than isolates collected earlier in Germany, Israel and the Netherlands. This can be due to rapid development of resistance or to a variation in natural sensitivity within local populations of *B. cinerea* to DMI fungicides. Less sensitive populations may contribute to the limited field performance of DMI fungicides towards *B. cinerea*. The sensitivity of isolates from south west Germany to tebuconazole was similar to that of DMI-resistant mutants generated in the laboratory. These mutants displayed stable resistance with Q-values (ratio between EC_{50} of resistant mutant and wild type isolate) between 5 and 20. Sensitivity of field isolates and laboratory mutants to tebuconazole and triadimenol was correlated.

Introduction

Botrytis cinerea Pers. ex Fr., the causal agent of grey mould, is a plant-pathogenic fungus with an extensive geographic distribution and host range.[1] Chemical control of the fungus is achieved by treatments with protectant, benzimidazole and dicarboximide fungicides. Protectants are not affected by development of resistance because of their multisite effects, whereas benzimidazoles selected rapidly for highly resistant pathogen populations.[2,3] Dicarboximides, which generally replaced the benzimidazoles during the late 1970s and early 1980s, also severely suffer from resistance development.[4-6]

Triazoles, which have been introduced from 1973 onwards, are widely used for disease control in a variety of crops but rarely for control of grey mould.[7,8] These fungicides specifically inhibit sterol 14α-demethylation during synthesis of ergosterol, the main sterol of fungal membranes.[9-11] This implies a risk of resistance development. Strains resistant to sterol demethylation inhibitors (DMIs) can be readily

isolated under laboratory conditions. However, the risk of resistance development towards DMIs in the field is considered to be lower than that of benzimidazoles and dicarboximides,[12,13] although eroding performance due to resistance development has been reported for a number of pathogens.[14]

Previous studies demonstrated that triazole fungicides inhibit effectively sterol 14α-demethylase activity, *in vitro* mycelial growth, and development of grey mould on tomato leaves and grape berries under growth chamber conditions.[15-17] In the present paper, triazole sensitivity of field isolates of *B. cinerea* from different sites in Europe and Israel is studied in order to determine whether limited field performance of triazoles towards *B. cinerea* can be attributed to a large variation in sensitivity of the pathogen populations to these fungicides. The experiments were carried out with tebuconazole, a DMI fungicide with high *in vitro* and *in vivo* activity towards *B. cinerea* and registered in combination with dichlofluanid for control of grey mould. Sensitivity of *B. cinerea* isolates towards triadimenol was tested as well, since this fungicide is often applied for control of other pathogens (*e.g.* powdery mildews) in crops which can also be infected by *B. cinerea*. Triadimenol is only moderately active against *B. cinerea* and therefore not registered for control of grey mould. For comparison, a limited number of DMI-resistant laboratory mutants were included in radial growth experiments. Isolates were also tested for resistance to the benzimidazole benomyl and the dicarboximide vinclozoline.

Materials and methods

Chemicals

Benomyl (Du Pont de Nemours & Co., Wilmington, Del., USA), tebuconazole and triadimenol (Bayer AG, Leverkusen, Germany) and vinclozoline (BASF AG, Ludwigshafen, Germany) were kindly supplied by their respective manufacturers. They were used as pure active ingredients.

Fungal isolates

Isolate SAS56, obtained from the ascospore progeny of a cross from WS55 (a field isolate from roses) and another undefined parent was used as a reference in all experiments.[18,19] It was kindly supplied by Dr. F. Faretra (University of Bari, Italy). Isolates NF1 to NF10 and SF1 to SF20 were isolated in 1992 from untreated grapes (control plots) in experimental vineyards in northern and southern France, respectively. They were generously provided by Dr. R. Pontzen (Bayer AG, Leverkusen, Germany). Isolates SD1 to SD38 were collected from control plots in experimental vineyards in south west Germany in 1992. Isolates D1 to D15 were isolated from grape

Sensitivity of populations

vine (berries, leaves and stems), lettuce and strawberry in Germany during 1979 - 1992. Isolates SD1 - SD38 and D3 - D6, D8, D9 and D11 - D13 were kindly provided by Dr. R. Pontzen (Bayer AG, Leverkusen, Germany) and isolates D1, D2, D7, D10, D14 and D15 by Dr. G. Lorenz (BASF AG, Ludwigshafen, Germany). Isolates I1 to I30 were isolated during January - March 1990 from tomato and cucumber in commercial plastic greenhouses in Israel.[20] They were generously provided by Dr. Y. Elad (Agricultural Research Organization, Bet Dagan, Israel). Isolates N1 to N7 were isolated during 1970 - 1989 from gerbera, tomato and roses in commercial greenhouses in the Netherlands.[21] They were kindly supplied by Dr. J. Salinas (LMA-IPO, Wageningen, the Netherlands). All isolates are bulk-isolates derived from crops not treated with tebuconazole. However, other DMIs (*e.g.* triadimenol) were used frequently for control of other diseases in the same or other crops in the sampling region.

Isolate B3 is a DMI-sensitive strain isolated from tomato in Greece. The monospore isolates G25, G39, G66 and G68 are laboratory-generated mutants with reduced sensitivity to DMIs, obtained by selection with triadimefon (100 μg ml^{-1}) after a 4-h MNNG (10 μg ml^{-1}) treatment (Ziogas, pers. comm.). They were kindly provided by Dr. B.N. Ziogas (University of Athens, Greece).

Preservation and culture conditions

Isolates were cultured on tomato agar prepared as described by Salinas (1992) and supplied with 1.2% technical agar (Oxoid, Basingstoke, UK) under near-uv light at 20°C. Subcultures were made every three to four days by mass transfers of conidia. Conidia were harvested from 2-3-week-old cultures with 0.1% Tween 20 solution in sterile distilled water. Conidia were separated from mycelium by filtration through sterile glass wool. Conidial concentrations were determined with a haemacytometer.

Mycelium and conidia were harvested from tomato agar cultures with 10% glycerol in sterile distilled water and preserved at -80°C in Eppendorf vials.

Sensitivity tests

Dose response relationships for inhibition of mycelial growth by the triazoles tebuconazole and triadimenol were assessed in radial growth experiments on fungicide-amended B5-agar using drops (5 μl) of conidial suspensions in sterile water (10^6 ml^{-1}) as inoculum. After incubation in the dark at 20°C for three days colony diameters were measured. Average diameters of colonies on fungicide-amended agar were calculated as percentages of colony diameters in control treatments. Using the software-program Lotus 1-2-3, percentages were plotted against fungicide concentrations on a logarithmic scale and EC_{50}s (concentrations of fungicides inhibiting radial mycelial growth by 50%) were determined by regression analysis of inhibitor response data. Experiments were performed in duplicate. Average EC_{50}s of

isolates from different regions were tested for significant differences after log transformation according to the ANOVA method.

Resistance of isolates to benomyl and vinclozoline was determined in radial growth experiments on B5-agar amended with discriminating concentrations (1 or 5 μg active ingredient (AI) ml^{-1}) allowing growth of resistant but fully inhibiting growth of sensitive isolates. Petri dishes were inoculated with drops (5 μl) of conidial suspension in sterile water (10^6 ml^{-1}). After incubation in the dark at 20°C for three days, colony diameters were assessed. Isolates barely affected in growth by 5 μg AI ml^{-1} were defined as highly resistant. Isolates affected in growth by 5 μg AI ml^{-1} but not by 1 μg AI ml^{-1} were defined as moderately resistant. Isolates slightly affected in growth by 1 μg AI ml^{-1} were defined as low-resistant.

Results

Sensitivity of field isolates to tebuconazole

The EC_{50} of the reference isolate SAS56 for tebuconazole was 0.16 $\pm$ 0.02 μg ml^{-1} (n = 18). Sensitivity distribution of all isolates tested (n = 121) was log-normal in character with an average EC_{50} of 0.38 $\pm$ 0.30 μg ml^{-1}. As an example dose-response curves of the reference isolate SAS56 and isolates collected in Germany during 1978 - 1992 are presented in Fig. 6.1A. Isolates were arbitrarily classified in categories with EC_{50}s < 0.1 (I), 0.1 - 0.25 (II), 0.25 - 0.5 (III), 0.5 - 1.0 (IV) and > 1.0 μg tebuconazole ml^{-1} (V). The distribution of isolates from different countries over the categories is given in Table 6.1. Highest sensitivity levels of isolates range from 0.01 (Israel, class I) to 0.16 μg ml^{-1} (south west Germany, class II). Similarly, lowest sensitivity levels of isolates range from 0.3 (the Netherlands, class III) to 1.64 μg ml^{-1} (Germany, class V). Hence, the variation factor (ratio between the highest and lowest EC_{50} found) of the populations tested varies between 2 (the Netherlands) and 62 (Israel) and amounts 164 for all isolates tested. The average EC_{50} determined for the 39 isolates collected in south west Germany during 1992 differed significantly from the average EC_{50}s of the isolates collected in Germany (1979 - 1981), Israel and the Netherlands. Isolates NF7 from northern France (EC_{50} 1.3 μg ml^{-1}), D12 from Germany (EC_{50} 1.6 μg ml^{-1}) and SD29 from south west Germany (EC_{50} 1.5 μg ml^{-1}) showed the lowest sensitivity to tebuconazole.

Sensitivity of field isolates to triadimenol

The EC_{50} of the reference isolate SAS56 for triadimenol was 2.2 $\pm$ 0.4 μg ml^{-1} (n = 18). The sensitivity distribution of all isolates tested (n = 121) was log-normal in character with an average EC_{50} of 4.1 $\pm$ 3.7 μg ml^{-1}. As an example dose-response curves of the

Table 6.1 Sensitivity of *Botrytis cinerea* isolates to tebuconazole and triadimenol.

Country	Year of isolation	Number of isolates	Fungicide Teb/Tri	EC_{50} range I[1]	II	III	IV	V	Average EC_{50} ± SD ($\mu g\ ml^{-1}$)	Lowest and highest EC_{50}	VF[3]
France (north)	1992	10	Teb	0[2]	5	3	1	1	0.4 ± 0.3 ab[4]	0.13 - 1.28	10
			Tri	0	3	4	2	1	5.1 ± 5.1 B	1.7 - 18.7	11
France (south)	1992	20	Teb	0	7	10	3	0	0.3 ± 0.2 ab	0.12 - 0.65	5
			Tri	0	7	11	1	1	3.6 ± 2.6 AB	1.6 - 13.7	9
Germany (south west)	1992	39	Teb	0	6	18	9	6	0.6 ± 0.4 b	0.16 - 1.51	9
			Tri	0	5	21	12	1	4.6 ± 2.5 B	1.8 - 13.7	8
Germany	1979 - 1991	14	Teb	1	6	6	0	1	0.3 ± 0.4 a	0.07 - 1.64	23
			Tri	0	7	6	0	1	5.0 ± 8.0 AB	1.1 - 32.6	30
Israel	1990	31	Teb	2	13	15	1	0	0.3 ± 0.1 a	0.01 - 0.62	62
			Tri	1	10	13	6	1	3.7 ± 2.3 AB	0.4 - 12.4	31
The Netherlands	1970 - 1989	7	Teb	0	5	2	0	0	0.2 ± 0.1 a	0.13 - 0.3	2
			Tri	0	5	2	0	0	2.2 ± 0.7 A	1.5 - 3.3	2
Total	1970 - 1992	121	Teb	3	42	54	14	8	0.4 ± 0.3	0.01 - 1.64	164
			Tri	1	37	57	21	5	4.1 ± 3.7	0.4 - 32.6	82

[1] EC_{50} of tebuconazole (Teb): I < 0.1, II = 0.1 - 0.25, III = 0.25 - 0.5, IV = 0.5 - 1.0 and V > 1.0 $\mu g\ ml^{-1}$.
EC_{50} of triadimenol (Tri): I < 1.0, II = 1.0 - 2.5, III = 2.5 - 5.0, IV = 5.0 - 10.0 and V > 10.0 $\mu g\ ml^{-1}$.
[2] Number of isolates.
[3] Variation factor: ratio between highest and lowest EC_{50} determined.
[4] Average EC_{50}s for the same fungicide followed by the same letter(s) do not differ significantly (P = 0.05).

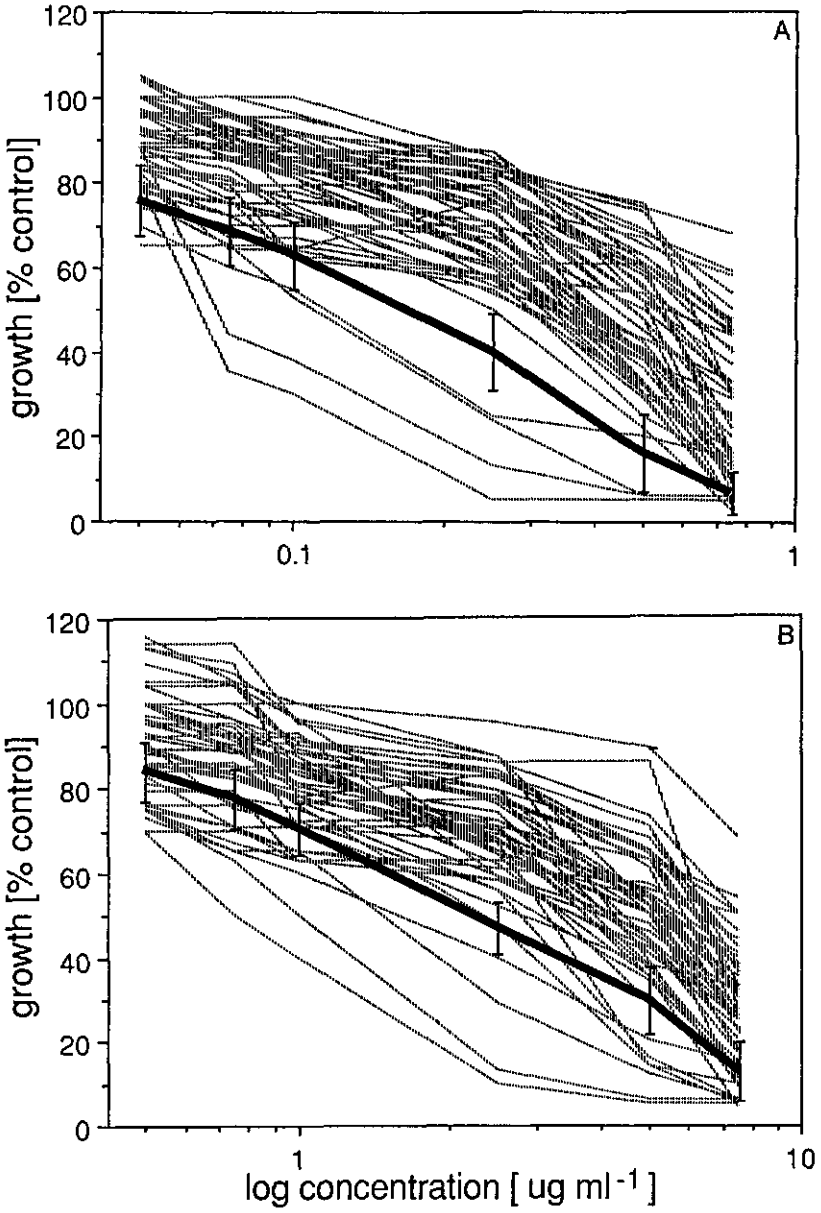

Fig. 6.1 Effect of tebuconazole **(A)** and triadimenol **(B)** on radial mycelial growth of 53 field isolates of *Botrytis cinerea* collected in Germany during 1979 - 1992 and reference isolate SAS56.

Sensitivity of populations

reference isolate SAS56 and isolates collected in Germany during 1978 - 1992 are presented in Fig. 6.1B. Isolates were arbitrarily classified in categories with EC_{50}s < 1.0 (I), 1.0 - 2.5 (II), 2.5 - 5.0 (III), 5.0 - 10.0 (IV) and > 10.0 μg triadimenol ml^{-1} (V). The distribution of isolates from different countries over the categories is given in Table 6.1. Highest sensitivity levels of isolates range from 0.4 (Israel, class I) to 1.8 μg ml^{-1} (south west Germany, class II). Similarly, lowest sensitivity levels of isolates range from 3.3 (the Netherlands, class III) to 32.6 μg ml^{-1} (Germany, class V). Hence, the variation factor of populations tested varies between 2 (the Netherlands) and 31 (Germany) and amounts 82 for all isolates tested. Average EC_{50}s of the isolates collected in south west Germany and northern France in 1992 differed significantly from the average EC_{50} of isolates collected in the Netherlands during 1970 - 1989. Isolates NF7 (EC_{50} 18.7 μg ml^{-1}) and D12 (EC_{50} 32.6 μg ml^{-1}) showed the lowest sensitivity to triadimenol.

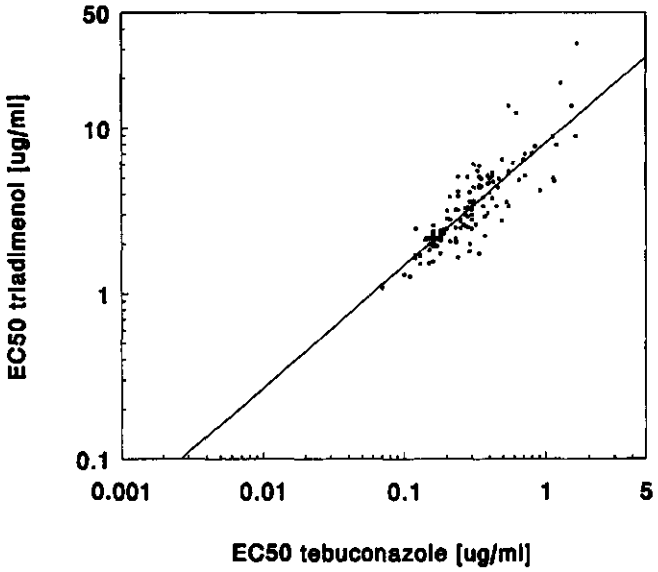

Fig. 6.1 Correlation between sensitivity of the reference isolate SAS56 (+) and 121 field isolates (□) of *Botrytis cinerea* towards tebuconazole and triadimenol.

Cross-sensitivity to tebuconazole and triadimenol

Regression analysis of EC_{50}s of tebuconazole and triadimenol for all field isolates tested (y = 0.91304 + 0.741244 x) indicates that sensitivity to tebuconazole and triadimenol are correlated (R^2 = 0.86; Fig. 6.2). Cross-sensitivity is not absolute, since

resistance factors of particular isolates for tebuconazole and triadimenol vary considerably (data not shown).

Table 6.2 Sensitivity of *Botrytis cinerea* isolates to benomyl and vinclozoline.

Country	Year of isolation	Number of isolates	Fungicide Ben/Vin	Sensitivity[1,2] S	LR	MR	HR
France (north)	1992	10	Ben	0	0	0	10
			Vin	3	6	1	0
France (south)	1992	20	Ben	14	0	0	6
			Vin	12	6	2	0
Germany (south west)	1992	39	Ben	30	1	0	8
			Vin	19	5	15	0
Germany	1979 - 1991	14	Ben	5	1	0	8
			Vin	3	4	6	1
Israel	1990	31	Ben	15	2	0	14
			Vin	14	8	9	0
The Netherlands	1970 - 1989	7	Ben	2	0	0	5
			Vin	2	4	1	0
Total	1970 - 1992	121	Ben	66	4	0	51
			Vin	53	33	34	1

[1] Sensitive (S): radial growth fully inhibited at 1 μg AI ml^{-1} B5-agar; low resistance (LR): radial growth slightly inhibited by 1 μg AI ml^{-1} B5-agar; Moderate resistance (MR): radial growth inhibited by 5 μg AI ml^{-1} B5-agar; high resistance (HR): radial growth not inhibited by 5 μg AI ml^{-1} B5-agar.
[2] Number of isolates.

Resistance of field isolates to benomyl and vinclozoline

In all experiments, mycelial growth of the sensitive reference isolate SAS56 was completely inhibited on agar amended with benomyl (1 μg ml^{-1}) or vinclozoline (1 μg ml^{-1}). Of all 121 isolates tested, 17 were benomyl resistant and vinclozoline sensitive, and 30 isolates were benomyl sensitive and vinclozoline resistant. The highest frequency of benzimidazole (HR) or dicarboximide (LR, MR) resistant isolates was detected in northern France and Germany, respectively (Table 6.2). Of all isolates tested, 38 were resistant to both benomyl and vinclozoline. Highest frequencies of isolates with double resistance were detected in northern France (ca. 70%), Germany (ca. 60%) and the Netherlands (ca. 60%). In the other regions, between ca. 10 (southern France, south west Germany) and ca. 40% (Israel) of the isolates tested

were resistant to both fungicides. Isolates D12 and NF7 were resistant to benomyl and vinclozoline and had a relatively low sensitivity to triazoles.

Characterization of laboratory mutants

Isolate B3 with wild-type sensitivity to triazoles was also sensitive to benomyl but had a low degree of resistance to vinclozoline. EC_{50}s of tebuconazole and triadimenol for inhibition of radial mycelial growth were 0.07 and 1.1 μg ml^{-1}, respectively (Table 6.3). Of the laboratory mutants, G25 had the lowest degree of sensitivity to tebuconazole with a Q-value (ratio between EC_{50} of resistant mutant and sensitive wild-type) of 13. Isolate G66 had the lowest sensitivity to triadimenol (Q value 19). Sensitivity of B3 and DMI-resistant mutants to benomyl and vinclozoline did not differ significantly.

Table 6.3 Sensitivity of the wild-type isolate B3 and DMI-resistant laboratory mutants of *Botrytis cinerea* to tebuconazole and triadimenol.

	EC_{50} (μg ml^{-1})[1,2]			
Isolate	Tebuconazole		Triadimenol	
B3	0.07		1.1	
G25	0.9	(13)	10.0	(9)
G39	0.4	(6)	5.1	(5)
G66	0.4	(6)	20.5	(19)
G68	0.5	(7)	5.5	(5)

[1] Concentration of fungicide inhibiting radial mycelial growth on BS-agar by 50%.
[2] Between brackets: Q-value (ratio between EC_{50} of resistant mutant and wild-type isolate B3).

Discussion

The sensitivity distribution of 121 field isolates of *B. cinerea* from different regions in Europe and Israel was based on EC_{50}s for inhibition of radial mycelial growth by the DMI fungicide tebuconazole. EC_{50}s were log-normally distributed and ranged from 0.01 to 1.64 μg ml^{-1}. The variation factor for all isolates tested was 164, indicating a considerable variation in sensitivity of *B. cinerea* populations towards tebuconazole. Similar variations in sensitivity to the same and other classes of fungicides were demonstrated earlier.[20,23,24] Experiments performed at BASF AG indicated that sensitivity to DMIs in populations of *B. cinerea* varied regardless whether populations were treated with DMI fungicides or not (Lorenz, pers. comm.). A similar phenomenon was described by Schulz *et al.* (1986) for *Erysiphe graminis* f.sp. *tritici*,

Table 6.4 Literature data on the sensitivity distribution to DMIs in populations of plant pathogens.

Pathogen	Fungicide	Sensitivity distribution			VF[1]	Reference	
Populations not treated with DMIs							
Venturia inaequalis	Flusilazole	0.0006	-	0.17[2]	283	Smith *et al.*, 1991	(31)
Puccinia recondita f.sp. *tritici*	Cyproconazole	0.07	-	2.0[3]	29	Ohl & Gisi, 1994	(32)
Populations treated with DMIs							
Botrytis cinerea	Tebuconazole	0.1	-	4.0[2]	40	Elad, 1992	(20)
Botrytis cinerea	Tebuconazole	0.01	-	1.6[2]	164	Stehmann & De Waard, this paper	
Botrytis cinerea	Triadimenol	0.4	-	33.0[2]	82	Stehmann & De Waard, this paper	
Erysiphe graminis f.sp. *hordei*	Triadimenol	< 0.025	-	> 0.625[4]	> 25	Fletcher & Wolfe, 1981	(33)
Puccinia recondita f.sp. *tritici*	Cyproconazole	0.05	-	9.0[3]	180	Ohl & Gisi, 1994	(32)
Rhizoctonia spp.	Cyproconazole	0.2	-	14.0[5]	70	Kataria *et al.*, 1991	(34)
Rhizoctonia spp.	Triadimenol	1.0	-	23.0[5]	23	Kataria *et al.*. 1991	(34)
Rhynchosporium secalis	Triadimenol	1.0	-	> 30.0[6]	> 30	Hunter *et al.*, 1986	(35)
Pseudocercosporella	Prochloraz	0.03	-	0.9[2]	30	Leroux & Marchegay, 1991	(36)
herpotrichoides	Prochloraz	0.002	-	0.08[7]	40	Leroux & Marchegay, 1991	(36)
Septoria tritici	Flutriafol	0.04	-	40.0[2]	1000	Hollomon, pers. comm.	
Septoria tritici	Cyproconazole	0.01	-	0.9[2]	90	Gisi & Hermann, 1994	(37)
Septoria tritici	Flutriafol	0.04	-	6.3[2]	158	Gisi & Hermann, 1994	(37)
Sphaerotheca fuliginea	Fenarimol	< 0.007	-	> 0.2[3]	> 32	Schepers, 1985	(38)
Uncinula necator	Triadimenol	0.1	-	10.0[8]	100	Steva *et al.*, 1990	(39)
Venturia inaequalis	Flusilazole	0.0007	-	0.14[2]	200	Smith *et al.*, 1991	(31)
Venturia inaequalis, V. piri	Bitertanol	< 0.1	-	> 7.5[2]	> 75	Creemers *et al.*, 1988	(40)
Venturia inaequalis, V. piri	Fenarimol	< 0.25	-	> 2.5[2]	> 10	Creemers *et al.*, 1988	(40)
Venturia nashicola	Triflumizole	< 0.2	-	> 1.0[2]	> 5	Ishii *et al.*, 1990	(41)
Venturia nashicola	Triflumizole	1.56	-	25.0[6]	16	Ishii *et al.*, 1990	(41)
Venturia nashicola	Bitertanol	0.78	-	25.0[6]	32	Ishii *et al.*, 1990	(41)

[1] VF: variation factor (ratio between lowest and highest sensitivity detected).
[2] EC_{50} mycelial growth on agar ($\mu g\ ml^{-1}$).
[3] EC_{90} leaf segment test ($\mu g\ ml^{-1}$).
[4] ED_{50} seed treatment (g AI kg^{-1} seed).
[5] EC_{90} mycelial growth on agar ($\mu g\ ml^{-1}$).
[6] MIC mycelial growth on agar ($\mu g\ ml^{-1}$).
[7] EC_{50} germtube elongation on agar ($\mu g\ ml^{-1}$).
[8] MIC *in vivo* ($\mu g\ ml^{-1}$).

who discussed the influence of environmental factors on sensitivity of the pathogen to triazoles.[25] Heterokaryosis is widely accepted as an important source of genetic variation in *B. cinerea*,[26-28] although its role in natural variability is not well understood and evidence for vegetative incompatibility, which would limit formation of heterokaryons, has been published for *B. cinerea*[29] and other members of the *Sclerotiniaceae*.[30]

In order to judge whether variation in sensitivity to triazoles may account for the limited field performance towards *B. cinerea*, literature data on variations in sensitivity of other pathogens to DMI fungicides were collected (Table 6.4). These data indicate, that the variation in sensitivity of *B. cinerea* populations to tebuconazole and triadimenol is in the same order of magnitude as that of other pathogens for the same or related DMI fungicides (Table 6.4). For most pathogens listed, disease control by DMIs was achieved in the period investigated. Hence, it is concluded that variation in sensitivity of *B. cinerea* populations to triazoles can probably not explain the limited field performance of triazoles in grey mould control.

The average EC_{50} of tebuconazole for isolates collected in south west Germany during 1992 was significantly higher than average EC_{50}s of populations from Germany (1979 - 1991), Israel (1990) and the Netherlands (1970 - 1989). This may be due to selection for resistance to DMI fungicides by treatments with tebuconazole or other DMIs. In Germany, tebuconazole (10%) is registered in combination with dichlofluanid (40%) for control of *B. cinerea*, *Uncinula necator* and *Plasmopara viticola* in grape vine. Tebuconazole and other DMIs (*e.g.* triadimenol) have also been used for control of other pathogens (*e.g.* powdery mildews) in other crops, which can also be infected by *B. cinerea*. These treatments could have exerted a selection pressure on *B. cinerea*, which may have resulted in reduced sensitivity of the pathogen population to DMI fungicides. Similar conditions led Elad (1992) assume that resistance to DMI fungicides developed in Israel.[20] Such a resistance development may be caused by quantitative shifts in sensitivity of *B. cinerea* populations as described for other pathogens.[25,42] The rapid selection of DMI-resistant isolates of *B. cinerea* under laboratory conditions may corroborate the resistance development in the field. However, it remains unclear why selection for reduced sensitivity would have occurred exclusively in south west Germany. Data on the selection pressure exerted by tebuconazole and other DMIs in the sampling regions are not available, but it seems unlikely that it has been highest in south west Germany. Another explanation for the reduced sensitivity of the *B. cinerea* population in south west Germany to tebuconazole may be a high local variation in natural sensitivity. Conclusive evidence for both hypotheses is lacking since data on the baseline sensitivity of *B. cinerea* populations in south west Germany and other sampling regions are not available.

These facts underline the necessity to establish baseline sensitivities of pathogen populations before introduction of a new fungicide.

It is suggested, that for isolates with a relatively low *in vitro* sensitivity to DMIs *in vivo* sensitivity may be considerably lower than described for the reference isolate SAS56.[17] This is especially relevant, since field rates of DMIs recommended were assessed to be relatively low for control of grey mould.[17] Hence, the existence of populations with relatively low sensitive isolates may contribute to the limited field performance of DMI fungicides against *B. cinerea*. Results emphasize the need to use reference isolates in screening programmes for candidate fungicides which represent the mean or even the lowest sensitivity of isolates in a pathogen population.

Distribution of isolates tested over the EC_{50} categories designed for tebuconazole and triadimenol was similar, suggesting a positive correlation between sensitivity to tebuconazole and triadimenol. Regression analysis confirms that isolates with reduced sensitivity to tebuconazole are cross-resistant to triadimenol (Fig. 6.2). However, cross-resistance is not absolute, since resistance factors for tebuconazole and triadimenol may vary considerably. Similar results were published for other pathogens such as *Septoria tritici* and *Erysiphe graminis* f.sp. *tritici* treated with DMIs.[37,43] Until the mechanism of field resistance to DMIs is not understood, the basis of cross-resistance and its variation will remain unclear. Results indicate that studies on cross-resistance between DMI fungicides should be performed at a population level and not with single isolates.

Since resistance to benzimidazoles and dicarboximides has a qualitative character,[44] resistance in *B. cinerea* populations to benomyl and vinclozoline was studied with discriminating concentrations (1 or 5 μg AI ml^{-1}). Frequency of benzimidazole-resistant isolates varied from 21 to 100% in the different sampling regions. The degree of resistance was generally high, although a few low resistant phenotypes were identified. This is ascribed to the presence of distinct alleles (*Mbc1HR* and *Mbc1LR*) at the *Mbc1* locus.[19,45] Between 25 and 60% of the isolates collected at different sites were moderately resistant to vinclozoline. Part of the isolates (32%) was multiple resistant to benomyl and vinclozoline. Resistance to benomyl, triazoles and vinclozoline was not correlated (data not shown). Data confirm that a major part of the *B. cinerea* population in Europe and Israel has developed resistance to both, benzimidazoles and dicarboximides, which will seriously hamper grey mould control. Results also emphasize the need for registration of new fungicides for grey mould control.

Acknowledgements

This research was supported by Bayer AG, Leverkusen, Germany. The authors acknowledge skilful assistance of Ms. I. Candilidis and Ms. H. Wassenaar. We are grateful to Dr. Y. Elad, Dr. F. Faretra, Dr. G. Lorenz, Dr. R. Pontzen, Dr. J. Salinas and Dr. B.N. Ziogas for generously providing isolates of *Botrytis cinerea*.

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Chapter 7

Accumulation of Tebuconazole by Isolates of *Botrytis cinerea* Differing in Sensitivity to DMI Fungicides

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Pesticide Science (submitted)

Abstract

Accumulation of tebuconazole by germlings of wild-type isolates of *Botrytis cinerea* was characterized by a high initial level of intracellular tebuconazole during the first 10 min of incubation and a subsequent gradual release of the fungicide into the surrounding medium. This transient accumulation indicates that the efflux of the fungicide has an inducible character. Accumulation of tebuconazole by laboratory-generated mutants resistant to sterol demethylation inhibitors (DMIs) was significantly lower, suggesting that efflux capacity in these mutants is correlated with resistance. Accumulation of tebuconazole by field isolates with a relatively low sensitivity to DMI fungicides was transient in time and accumulation levels did not differ significantly from those of the wild-type isolates tested. These results suggest, that reduced accumulation of tebuconazole in fungal mycelium may account for resistance in laboratory-generated mutants but not in field isolates of *B. cinerea* with a relatively low sensitivity to the fungicide. Various chemicals such as BAS 490F, carbonyl cyanide 3-chlorophenylhydrazone, copper sulphate and fluazinam enhanced accumulation of tebuconazole by a DMI-sensitive and -resistant isolate. This enhanced accumulation is ascribed to inhibition of the energy-dependent efflux of the fungicide. It is suggested that chemicals, which inhibit the energy-dependent efflux, may synergize activity of tebuconazole or other DMIs. Such chemicals are candidate companion compounds in mixtures with DMI fungicides.

Introduction

Tebuconazole is a triazole fungicide with a broad spectrum of antifungal activity including powdery mildews, rusts, *Sclerotinia* spp. and *Botrytis cinerea*.[1,2] For control of *B. cinerea*, tebuconazole (10%) is marketed as Folicur® E in mixture with dichlofluanid (40%).[3,4] The antifungal action of triazoles is based on inhibition of cytochrome P450-dependent 14α-demethylation of lanosterol or eburicol in the synthesis of ergosterol, the main sterol of fungal membranes.[5-7] Development of resistance to fungicides with a specific mode of action is a major threat for effective chemical control. This also accounts for sterol demethylation inhibitors (DMIs). Field resistance to these fungicides developed relatively slowly as compared with other site-specific inhibitors.[8] However, resistant mutants of various pathogens can be readily selected in the laboratory.[9-11] In a previous study, both laboratory-generated mutants with reduced sensitivity to tebuconazole and field isolates of *B. cinerea* with a relatively low degree of sensitivity to tebuconazole were characterized.[12]

In the present study, a putative mechanism of resistance which may operate in these isolates is investigated. This mechanism is based on increased energy-dependent efflux of the fungicide from mycelial cells into the surrounding medium

by which the intracellular concentration of the fungicide decreases and saturation of the target site, sterol 14α-demethylase, is not achieved.[13-16] Such an increased energy-dependent efflux has been described as a mechanism of resistance to DMIs in laboratory-generated mutants of *Aspergillus nidulans*,[13,14] *Candida albicans*,[17] *Monilia fructicola*,[18] *Nectria haematococca* var. *cucurbitae*[10] and *Penicillium italicum*.[15,19] Information on the mechanism of resistance to DMI fungicides operating in field isolates is not available.

Accumulation of tebuconazole in *B. cinerea* was investigated in the presence of inhibitors of fungal respiration and other fungicides active against *B. cinerea* which interfere with energy generation. Fungicides enhancing intracellular accumulation of tebuconazole can be regarded as potential companion compounds in mixtures with tebuconazole or other DMI fungicides.

Materials and methods

Chemicals

[Phenyl-UL-^{14}C]tebuconazole (sp. act. 3.47 MBq mg^{-1}) and tebuconazole were generously provided by Bayer AG (Leverkusen, Germany). Captan and chlorothalonil were generously provided by Ciba Geigy Agro B.V. (Roosendaal, the Netherlands), and maneb and thiram by Luxan B.V. (Elst, the Netherlands). Fenpiclonil (Ciba Geigy AG, Basel, Switzerland), fluazinam (ISK Biosciences Co., Mentor, Ohio, USA), pyrimethanil (AgrEvo, Berlin, Germany) and BAS 490F (BASF AG, Ludwigshafen, Germany) were generously provided by their respective manufacturers. Dichloran was a gift from Dr. J.W. Eckert (University of California, USA). All fungicides were used as pure active ingredients. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from Sigma (St. Louis, Mo., USA).

Fungal isolates, culture conditions and preparation of mycelial suspensions

The monoascospore isolate SAS56 was provided by Dr. F. Faretra (University of Bari, Italy).[20] Wild-strain isolate B3 and laboratory mutants G25 and G66 were obtained from Dr. B.N. Ziogas (University of Athens, Greece). Field isolates D12 and SD29 were provided by Dr. R. Pontzen (Bayer AG, Leverkusen, Germany). D12 was isolated in 1990 from untreated lettuce (control plots) of experimental stations in Germany. SD29 was isolated in 1992 from untreated grapes (control plots) of experimental vineyards in south west Germany.[12]

Isolates were cultured on tomato agar prepared as described by Salinas (1992) and supplied with 1.2% technical agar (Oxoid, Basingstoke, UK) under near-uv light at 20°C.[21] Subcultures were made every three to four days by mass transfers of conidia.

Accumulation of tebuconazole

Conidia were harvested from 2-3-week-old cultures with 0.1% Tween 20 solution in sterile distilled water. Conidia were separated from mycelium by filtration through sterile glass wool and concentrations of conidia in suspensions were determined with a haemacytometer.

Flasks (2 l) with liquid synthetic media (1 l) prepared according to Fritz *et al.* (1987) were inoculated at an initial density of 2 x 10^6 conidia ml^{-1}.[22] The cultures were incubated in a rotary shaker (200 rev min^{-1}) in the dark at 20°C for 12 h. Standard germling suspensions were made by passing cultures through a 0.5-mm pore sieve to remove clusters of mycelium and collecting germlings on a 0.05-mm pore stainless steel sieve. Germlings collected were washed three times by resuspending wet mycelium (1 g) in 23.4 mM potassium phosphate buffer (pH 6.0, 50 ml) containing 0.1 mM calcium chloride and 10 g l^{-1} D-glucose. Standard mycelial suspensions were made by resuspending the washed mycelium (1 g wwt) in 100 ml of the same buffer.

Radial growth experiments

Sensitivity of isolates to tebuconazole was assessed in radial growth experiments performed as described earlier.[12,23] Petri dishes (9 cm diameter) containing B5-agar (25 ml) amended with fungicide were inoculated with drops of conidial suspension[12] or mycelial plugs from overnight cultures on B5-agar.[23]

Accumulation of tebuconazole

Experiments were carried out according to methods described previously.[13] Standard germling suspensions (70 ml in 300 ml Erlenmeyer flasks) were shaken on a reciprocal shaker at 20°C for 20 min. Experiments were initiated by adding tebuconazole (10, 30, 100, 300 or 500 μM initial external concentration) supplied with [^{14}C]tebuconazole (initial external concentration 0.1 KBq ml^{-1}) from a 100x concentrated stock solution in methanol to standard germling suspensions. Accumulation of tebuconazole was determined in germlings collected from 5-ml samples at several incubation times. Collected germlings were washed 4 times in 30 s with buffer (5 ml) containing the same concentration of tebuconazole as present in the incubation medium. Radioactivity in mycelium was extracted with scintillation liquid (Pico Aqua, Packard Instruments Company Inc., Downers Grove, IL, USA) for 1 day and counted in a liquid scintillation spectrometer.

Effects of chemicals on uptake of tebuconazole were determined by addition from 100x concentrated stock solutions in methanol or distilled water 60 min after addition of [^{14}C]tebuconazole (initial external concentration 100 or 300 μM) to standard mycelial suspensions (30 ml in 100 ml Erlenmeyer flasks). Samples (5 ml) were collected every 15 min and assessed for accumulation of tebuconazole.

Results

Sensitivity

In radial growth experiments, in which drops of conidial suspension were used as inoculum, EC_{50}s (concentrations of fungicide inhibiting radial mycelial growth by 50%) of the reference isolates SAS56 and B3 for tebuconazole were 0.16 and 0.07 μg ml^{-1}, respectively (Table 7.1). EC_{50}s of field isolates ranged around 1.6 μg ml^{-1}. Laboratory mutants displayed Q-values (ratio between EC_{50} of resistant mutant and sensitive wild-type isolate) between 6 and 13. Experiments were also carried out using mycelial plugs as inoculum. Under these conditions, EC_{50}s of tebuconazole for the reference isolates SAS56 and B3 were 0.04 and 0.03 μg ml^{-1}, respectively, and Q-values of the mutants G66 and G25 were 32 and 36, respectively.

Table 7.1 Inhibition of radial mycelial growth of *Botrytis cinerea* isolates by tebuconazole.

	EC_{50} (μg ml^{-1})[1]			
Isolate	Spore drops[2]	Q-value[3]	Mycelial plugs[4]	Q-value
B3	0.07[5]		0.03	
G25	0.89	13	1.05	36
G66	0.40	6	0.94	32
SAS56	0.16 ± 0.04[6]		0.04 ± 0.01[7]	
D12	1.64		1.00	
SD29	1.56		0.86	

[1] Concentration of fungicide inhibiting radial mycelial growth on B5-agar by 50%.
[2] Inoculation of Petri dishes with drops (5 μl) of conidial suspension (10^6 ml^{-1}).
[3] Ratio between EC50 of resistant mutant and sensitive wild-type isolate.
[4] Inoculation of Petri dishes with agar plugs from overnight cultures.
[5] n = 1
[6] n = 18
[7] n = 3

Accumulation by SAS56

At low external concentrations of tebuconazole (10 and 30 μM) accumulation of the fungicide by the DMI-sensitive reference isolate SAS56 was constant in time and ranged around 0.2 and 0.6 nmol mg^{-1} dry weight mycelium, respectively (Table 7.2). At 100 μM or higher, accumulation of tebuconazole became transient in time with a maximum after about 10 min of incubation (Fig. 7.1). Increasing external

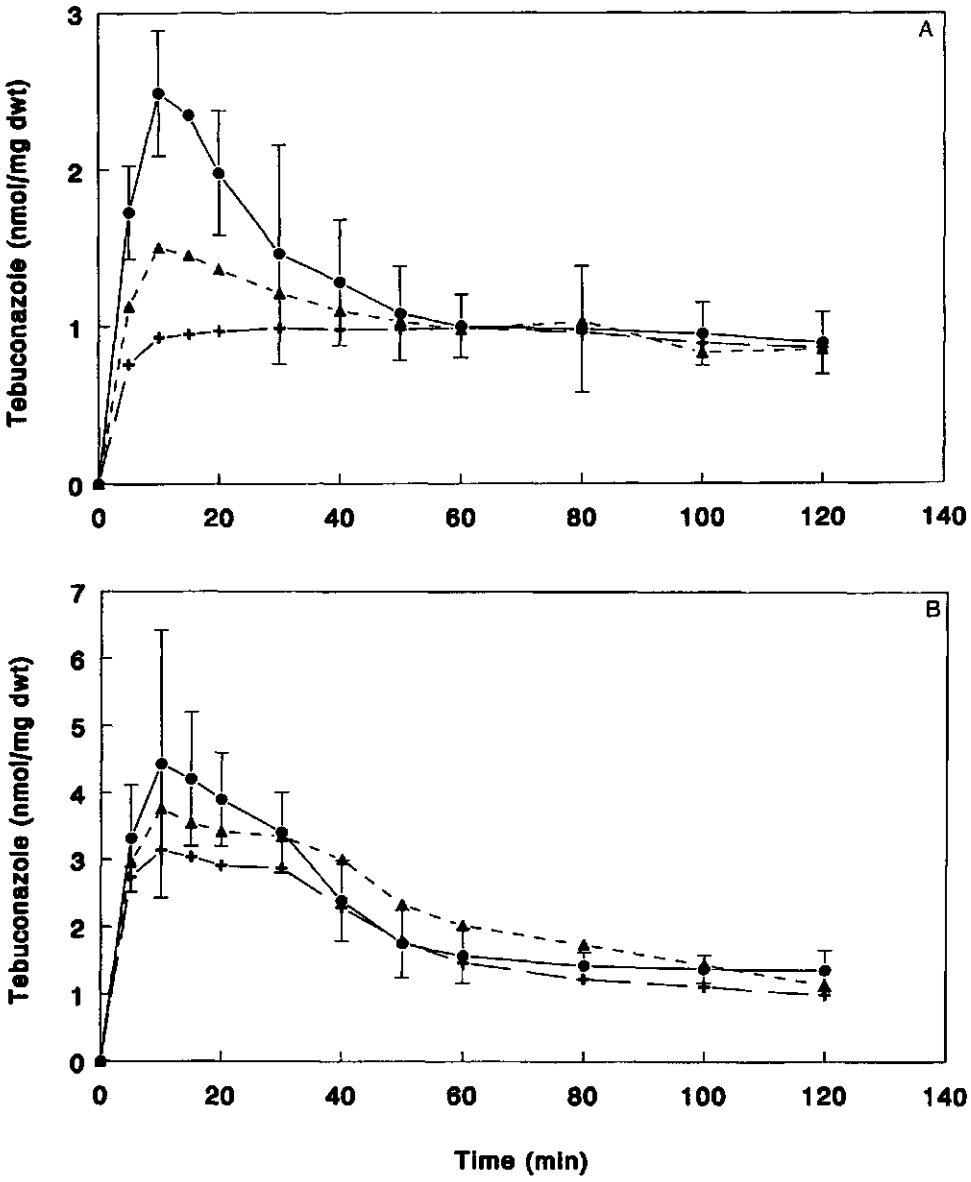

Fig. 7.1 Accumulation of tebuconazole (100 μM) by germlings of *Botrytis cinerea* **(A)** wild-type isolate B3 (●) and laboratory-generated DMI-resistant mutants G25 (+) and G66 (▲) and **(B)** wild-type isolate SAS56 (●) and field isolates SD29 (+) and D12 (▲) with reduced sensitivity to tebuconazole.

concentrations of tebuconazole resulted in higher accumulation levels (Table 7.2). Accumulation of tebuconazole in samples washed with buffer, which was supplied with the same concentration of tebuconazole as present in the incubation medium, or with buffer without the fungicide did not differ significantly (data not shown).

Table 7.2 Accumulation of tebuconazole by germlings of *Botrytis cinerea* SAS56.

External concentration (μM)	Incubation time (min)			
	10	30	60	120
10	0.1[1,2]	0.2	0.2	0.2
30	0.7	0.6	0.5	0.5
100	5.3	4.2	1.7	1.3
300	20.9	18.9	11.5	5.0
500	64.9	51.4	38.5	27.9

[1] Average of two experiments.
[2] nmol tebuconazole mg^{-1} dry weight mycelium.

Accumulation by less sensitive isolates

Accumulation of tebuconazole by the laboratory-generated mutants G25 and G66 was compared with that of the wild-type isolate B3. At initial external concentrations of 100 and 300 μM, G25 and G66 accumulated significantly lower amounts of tebuconazole than the wild-type B3 (Table 7.3 and 7.4). At an external concentration of 100 μM, accumulation by G25 was constant in time while accumulation by G66 was slightly transient (Fig. 7.1). At an external concentration of 300 μM, both mutants accumulated tebuconazole in a transient manner, but G66 accumulated significantly higher levels than G25 during the first 10 min of incubation (Table 7.4). Accumulation by the field isolates D12 and SD29 was compared with accumulation by the wild type SAS56. At initial external concentrations of 100 and 300 μM tebuconazole, the isolates showed transient accumulation patterns which did not differ significantly (Table 7.3 and 7.4).

Effect of test compounds on accumulation

The wild type isolate B3 and the DMI-resistant laboratory mutant G25 were selected to study effects of test compounds on accumulation of tebuconazole. At an initial external concentration of 100 μM the accumulation level in both isolates stabilized during the second hour of incubation at about 1 nmol tebuconazole mg^{-1} dwt

Table 7.3 Accumulation of tebuconazole (100 μM) by germlings of *Botrytis cinerea* isolates.

Isolate	Mycelial concentration (mg dwt ml^{-1})	Germtube length (μm)	Incubation time (min)			
			10	30	60	120
B3	2.9 ± 0.5[1]	106 ± 26[1]	2.4 ± 0.4 b[1,2,3]	1.7 ± 0.7 b	1.0 ± 0.2 a	0.9 ± 0.2 a
G25	3.0 ± 0.5	132 ± 32	0.9 ± 0.2 a	1.0 ± 0.2 a	1.0 ± 0.2 a	1.0 ± 0.2 a
G66	2.8 ± 0.4	130 ± 21	1.1 ± 0.1 a	1.1 ± 0.1 ab	1.0 ± 0.1 a	0.8 ± 0.1 a
SAS56	2.7 ± 0.3	119 ± 29	4.4 ± 2.0 c	3.4 ± 0.6 c	1.6 ± 0.4 b	1.4 ± 0.3 b
D12	2.6 ± 0.4	121 ± 31	3.8 ± 1.8 bc	3.4 ± 0.6 c	2.0 ± 0.3 c	1.1 ± 0.3 a
SD29	2.4 ± 0.2	125 ± 32	3.1 ± 0.8 bc	2.9 ± 0.6 c	1.5 ± 0.1 b	1.0 ± 0.2 a

[1] Average of three experiments.
[2] nmol tebuconazole mg^{-1} dry weight mycelium.
[3] Figures in the same column followed by the same letter(s) do not differ significantly ($P < 0.001$ for 10, 30 and 60 min, $P = 0.002$ for 120 min).

Table 7.4 Accumulation of tebuconazole (300 μM) by germlings of *Botrytis cinerea* isolates.

Isolate	Mycelial concentration (mg dwt ml^{-1})	Germtube length (μm)	Incubation time (min)			
			10	30	60	120
B3	2.0[1]	113 ± 31[1]	18.5[1,2]	17.4	13.2	8.4
G25	2.5	103 ± 21	5.7	3.7	2.9	2.3
G66	2.6	111 ± 49	12.3	6.6	4.1	2.4
SAS56	2.6	129 ± 25	20.9	18.9	11.5	5.0
D12	2.8	117 ± 25	19.3	14.5	12.6	8.0
SD29	2.6	134 ± 38	16.5	13.2	10.0	5.9

[1] Average of two experiments.
[2] nmol tebuconazole mg^{-1} dry weight mycelium.

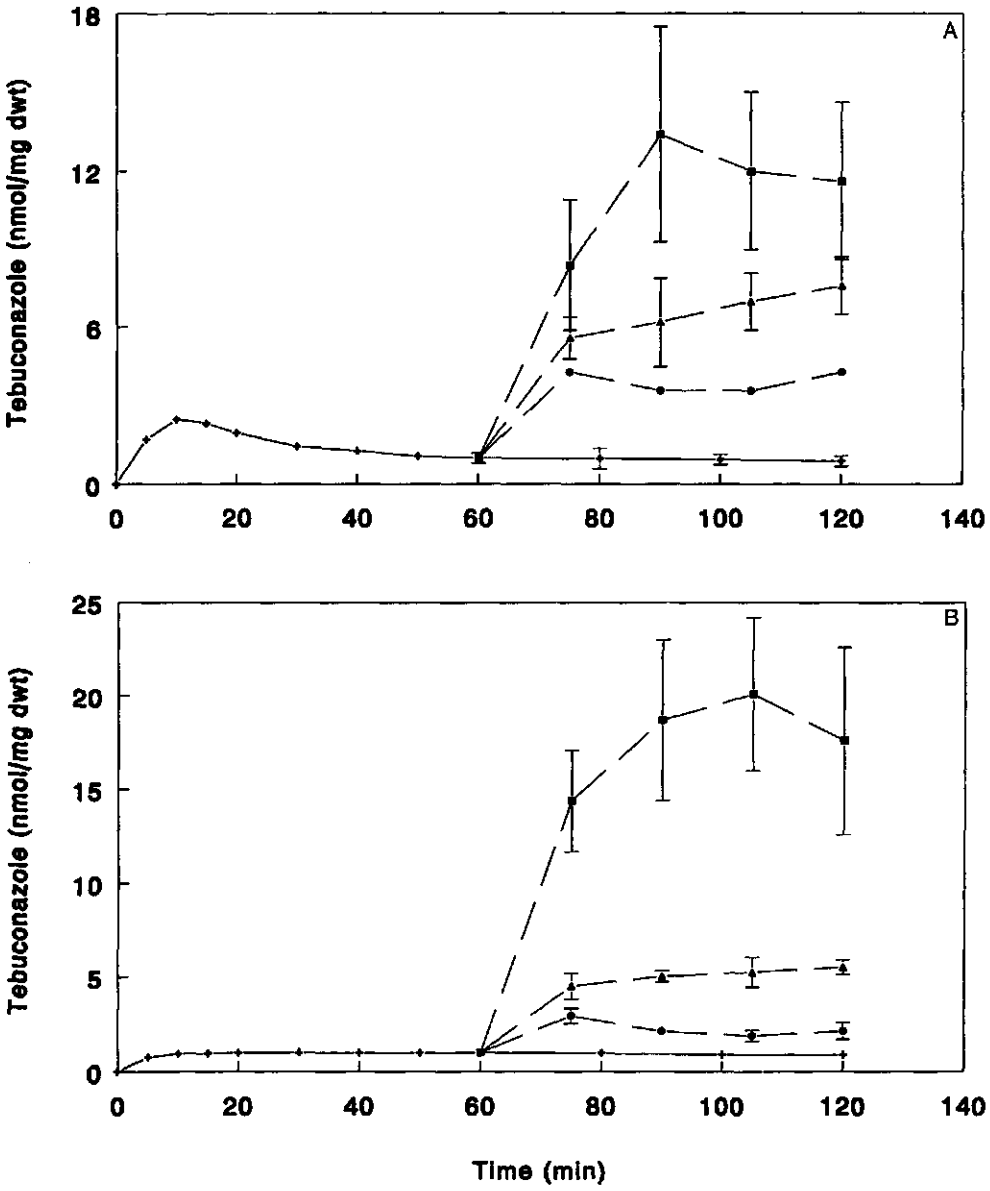

Fig. 7.2 Effect of test compounds BAS 490F (300 μM, ●), $CuSO_4$ (100 μM, ▲) and fluazinam (100 μM, ■) on accumulation of tebuconazole (100 μM, +) by *Botrytis cinerea* wild-type isolate B3 **(A)** and laboratory-generated DMI-resistant mutant G25 **(B)**. Test compounds were added 60 min after addition of tebuconazole.

Table 7.5 Effect of test compounds on accumulation of tebuconazole (initial external concentration 100 μM) by germlings of *Botrytis cinerea* isolates B3 and G25. Test compounds were added 60 min after addition of tebuconazole.

Test compound (100 μM)	B3						G25					
	15[1]		30		60		15		30		60	
Control	0.9 ± 0.2[2]		1.0 ± 0.2		1.0 ± 0.1		1.1 ± 0.1		1.0 ± 0.2		1.2 ± 0.4	
BAS 490F	1.2 ± 0.1	(1)[3]	1.1 ± 0.1	(1)	1.2 ± 0.1	(1)	n.d.[4]		n.d.		n.d.	
BAS 490F[5]	4.3 ± 0.7	(5)	3.6 ± 0.1	(4)	4.3 ± 0.1	(4)	2.9 ± 0.4	(3)	2.1 ± 0.2	(2)	2.1 ± 0.5	(2)
Captan	2.6 ± 1.0	(3)	2.7 ± 0.5	(3)	2.6 ± 0.2	(3)	2.6 ± 0.4	(2)	2.6 ± 0.4	(3)	2.9 ± 0.4	(2)
Chlorothalonil	1.0 ± 0.2	(1)	1.0 ± 0.2	(1)	1.1 ± 0.3	(1)	1.5 ± 0.3	(1)	1.2 ± 0.2	(1)	1.2 ± 0.1	(1)
CCCP	3.1 ± 0.8	(3)	3.7 ± 1.0	(4)	5.0 ± 1.6	(5)	1.9 ± 0.1	(2)	2.1 ± 0.5	(2)	2.8 ± 0.6	(2)
$CuSO_4$	5.6 ± 0.8	(6)	6.2 ± 1.7	(6)	7.6 ± 1.1	(8)	4.5 ± 0.7	(4)	5.0 ± 0.3	(5)	5.5 ± 0.4	(5)
Dichloran	0.9 ± 0.3	(1)	1.0 ± 0.2	(1)	1.1 ± 0.2	(1)	1.7 ± 0.5	(2)	1.2 ± 0.1	(1)	1.3 ± 0.2	(1)
Fenpiclonil	1.9 ± 0.2	(2)	1.9 ± 0.2	(2)	2.1 ± 0.5	(2)	1.9 ± 0.4	(2)	1.9 ± 0.4	(2)	1.6 ± 0.1	(1)
Fluazinam	8.4 ± 3.0	(9)	13.4 ± 4.1	(13)	11.6 ± 3.0	(12)	14.4 ± 2.7	(13)	18.7 ± 5.3	(19)	17.6 ± 6.0	(15)
Maneb	1.2 ± 0.2	(1)	1.2 ± 0.2	(1)	1.2 ± 0.3	(1)	2.0 ± 0.5	(2)	1.8 ± 0.5	(2)	1.7 ± 0.2	(1)
Pyrimethanil	1.0 ± 0.2	(1)	0.9 ± 0.2	(1)	1.0 ± 0.1	(1)	1.0 ± 0.1	(1)	0.9 ± 0.1	(1)	1.0 ± 0.2	(1)
Thiram	0.9 ± 0.2	(1)	1.0 ± 0.2	(1)	1.0 ± 0.2	(1)	1.9 ± 0.6	(2)	1.5 ± 0.4	(2)	1.4 ± 0.2	(1)

[1] Time (min) after addition of test compound.
[2] nmol tebuconazole mg^{-1} dry weight mycelium, average of three experiments.
[3] Between brackets: relative to control treatments.
[4] n.d. - not determined.
[5] Initial external concentration 300 μM.

mycelium (Table 7.5). Under these equilibrium conditions test compounds were added. Captan, CCCP, $CuSO_4$ and fluazinam significantly enhanced accumulation of tebuconazole in germlings of the sensitive and the resistant isolate (Fig. 7.2). In the presence of fenpiclonil accumulation of tebuconazole was doubled. BAS 490F enhanced accumulation only at an initial external concentration of 300 μM. Its effect on accumulation by the resistant isolate was minor. All other fungicides tested had hardly any or no effect on accumulation of tebuconazole under the test conditions used.

Discussion

The transient accumulation of tebuconazole by the wild-type isolates B3 and SAS56 of *B. cinerea* suggests that this is the result of passive influx and active efflux of the fungicide as described before for accumulation of other DMIs by *Aspergillus nidulans*,[14,24] *Candida albicans*,[17] *Monilia fructicola*,[18] *Nectria haematococca* var. *cucurbitae*[10] and *Penicillium italicum*.[15] Passive influx is probably determined by partitioning of the fungicide between the incubation medium and mycelial cell compartments, since it depended on the initial external concentration of the fungicide (Table 7.2). Such a passive accumulation of fungicides by fungal mycelium has been described also for dodine, glyodine and other fungicides.[25,26] The transient accumulation pattern indicates that passive influx of the fungicide is counteracted by an efflux with inducible character and corroborates previous results.[14] With the methods applied, the level of tebuconazole accumulation detected in this study may include fractions absorbed by the cell surface, which are not present in the cytoplasm.[27-30] Since the presence of tebuconazole in the washing buffer did not influence accumulation of tebuconazole and accumulation levels of tebuconazole did decrease during incubation time, it is concluded that the accumulation maximum of tebuconazole detected after 10 min of incubation is not caused by absorption by the cell-surface. Hence, the major amount of the fungicide appears to be present in the cytoplasm of fungal germlings.

The initial accumulation level of tebuconazole in *B. cinerea* is comparable to levels of other DMI fungicides accumulating under similar conditions in germlings of *A. nidulans*, *M. fructicola*, *N. haematococca* and *P. italicum* (Table 7.6).[10,13,14,24] It is assumed that a high initial accumulation of DMIs results in complex formation between the P450-dependent sterol 14α-demethylase ($P450_{14DM}$) and DMI fungicides as described before.[23,31-33] This complex formation is probably responsible for the high *in vitro* and *in vivo* activity of triazoles towards the *B. cinerea* isolate SAS56[23,34]

Therefore, limited field performance of triazoles towards *B. cinerea* can not be ascribed to low accumulation levels of these fungicides in the pathogen.

Table 7.6 Literature and present data on accumulation of triazole fungicides by DMI-sensitive isolates of filamentous fungi.

Fungus	Fungicide (μM)		Accumulation[1] (nmol mg^{-1} dwt mycelium)	Reference
Aspergillus nidulans	Fenarimol	30	1.65 ± 0.14	14
	Fenarimol	60	4.03 ± 0.2	14
	Fenarimol	120	8.26 ± 0.24	14
Botrytis cinerea	Tebuconazole	10	0.1	this paper
	Tebuconazole	30	0.7	this paper
	Tebuconazole	100	2.4 ± 0.4 - 4.4 ± 2.0	this paper
Monilia fructicola	Penconazole	10	5.0 - 10.0	18
	Etaconazole	10	0.6 - 0.8	18
	Etaconazole	30	2.5 - 2.7	18
Nectria haematococca	Fenarimol	30	2.0	10
	Fenarimol	90	4.5	10
Penicillium italicum	Fenarimol	90	2.7 - 4.4	15

[1] Incubation time 10 min.

Accumulation of tebuconazole by laboratory-generated mutants G25 and G66 was compared with that of the wild-type isolate B3. The two mutants were derived from B3 and showed significant levels of resistance in radial growth experiments (Table 7.1). Accumulation of tebuconazole by B3 was transient in time as described above, whereas accumulation by both mutants was significantly lower and almost constant in time. Results suggest, that low accumulation of tebuconazole in these mutants can be caused by an increased efflux capacity preventing a saturation of the target site as described before for laboratory-generated DMI-resistant mutants of other fungi.[10,13-15,17-19] At high external concentrations of tebuconazole (300 μM) all isolates accumulated tebuconazole in a transient manner, suggesting that the capacity of the efflux mechanism is not sufficient to prevent accumulation of the fungicide. G66, which is more resistant than G25, accumulated significantly lower amounts of tebuconazole than G25 (Table 7.4), suggesting that efflux capacity in these mutants is correlated with resistance. Similar results have been described for laboratory-generated mutants of *A. nidulans* with different levels of resistance to fenarimol.[14] Results indicate that reduced accumulation is a rather common

mechanism of resistance in laboratory-generated DMI-resistant mutants. Its relevance for field isolates with low sensitivity to DMI fungicides remains to be established.

For this reason accumulation of tebuconazole in the field isolates D12 and SD29 was studied. These isolates had a relatively low sensitivity to tebuconazole, comparable to that of the laboratory-generated mutants tested. Accumulation of tebuconazole in these field-isolates was compared with that of the wild-type isolate SAS56, since proper reference isolates from baseline studies are missing.[12] In fact, accumulation could have been compared with any wild-type isolate, including B3. Accumulation of tebuconazole in both wild-type isolates and both field isolates was transient in time and accumulation levels did not differ significantly. This suggests that the mechanism determining low sensitivity of D12 and SD29 to tebuconazole is different from the one operating in laboratory-generated mutants. However, this conclusion should be handled with care, since a representative isolate of the baseline population, from which D12 and SD29 originate, is not available.

The level of accumulated tebuconazole decreased in time until 60 - 100 min of incubation, when an equilibrium between influx and efflux is reached at a low level of accumulation. This equilibrium is reached in *B. cinerea* somewhat slower than in other fungi. Transient accumulation of tebuconazole was only observed at initial external concentration of 90 μM or higher. With other pathogens a transient accumulation of various DMIs was detected already at 10 and 30 μM (Table 7.6). This difference may suggest that intrinsic efflux activity in *B. cinerea* is higher than in other pathogens investigated. The physiological basis of such differences in efflux capacity of filamentous fungi is not yet understood.

The energy-dependent character of the efflux is confirmed by the observation that inhibitors of mitochondrial respiration (BAS 490F, CCCP and fluazinam) multisite-inhibiting fungicides ($CuSO_4$ and captan)[35-37] cause an instantaneous accumulation of the fungicide (Fig. 7.2, Table 7.5). Similar results were reported for other pathogens.[14,16] Such effects may result in a synergistic interactions.[16,38] Chemicals which enhance accumulation of DMI fungicides are therefore candidate compounds in mixtures with tebuconazole or other DMI fungicides.

Acknowledgements

This work was supported by Bayer AG (Leverkusen, Germany) and Sandoz AG (Basel, Switzerland). The authors are grateful to AgrEvo (Berlin, Germany), BASF AG (Limbugerhof, Germany), Bayer AG (Leverkusen, Germany), Ciba Geigy AG (Basel, Switzerland), Ciba Geigy Agro (Roosendaal, the Netherlands), ISK Biosciences Co.

(Mentor, Ohio, USA), Luxan BV (Elst, the Netherlands) and Dr. J.W. Eckert (California, USA) for generous supply of fungicides.

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Chapter 8

General Discussion

General Discussion

The aim of the present study was to describe factors involved in the limited field performance of triazole fungicides towards *Botrytis cinerea*. In most experiments tebuconazole, a triazole *in vitro* and *in vivo* highly active towards *B. cinerea* (*chapters 4 and 5*) and triadimenol, a triazole with low activity towards *B. cinerea* (*chapters 4 and 5*), were used as test compounds. Tebuconazole (10%), marketed in combination with dichlofluanid (40%), is the only triazole registered for grey mould control.[1]

A potential mechanism involved in limited performance of triazoles may be a relatively low sensitivity of the target enzyme, the cytochrome P450-dependent sterol 14α-demethylase ($P450_{14DM}$). To test the relevance of this mechanism, a cell-free assay able to synthesize C4-desmethyl sterols from the sterol precursor [2-^{14}C] mevalonate is a prerequisite. Ample attempts have been undertaken by various research groups to develop such assays for filamentous fungi but these were not successful. This is probably due to the rapid inactivation of the membrane-bound $P450_{14DM}$ during disruption of rigid cell walls from filamentous fungi.[2]

Cell-free assays capable of C4-desmethyl sterol synthesis from [2-^{14}C]mevalonate were first developed for the model fungus *Penicillium italicum*, *Moniliaceae* (*chapter 2*). The method used was based on mild mechanical disruption of mycelial germlings as described for *Aspergillus fumigatus*.[3] Radioactivity incorporated into C4-desmethyl sterols was on average 26% of total incorporation into non-saponifiable lipids (NSLs) and ergosterol was the only C4-desmethyl sterol identified. Ergosterol is also the main sterol present in intact mycelium of *P. italicum*.[4] C4,4-dimethyl sterols identified in cell-free assays were lanosterol and a trace amount of 24-methylene dihydrolanosterol (eburicol). Accumulation of lanosterol in cell-free assays from *P. italicum* was ascribed to a low conversion rate of the Δ^{24}-methyltransferase, resulting from a hampered transport of lanosterol to mitochondria, in which this reaction is believed to take place.[5]

In subsequent research, the cell-free assay developed for *P. italicum* was adapted for *B. cinerea* (*chapter 3*). In cell-free assays from *B. cinerea* C4-desmethyl and other sterols were synthesized only after relatively vigorous disruption of mycelial germlings. Protein concentrations of cell-free extracts were about 10 times higher than those described for *Aspergillus fumigatus*[3] and *P. italicum* (*chapter 2*), but comparable to cell-free assays derived from yeasts.[6-8] Results indicate, that the disruption of mycelial cells is a crucial step in the preparation of cell-free assays. Obviously, the disruption technique has to be adapted for each pathogen of interest. In cell-free assays from *B. cinerea* on average 39% of total NSLs synthesized was present in the C4-desmethyl sterol fraction. This was higher than reported for cell-free assays derived from other filamentous fungi and *Candida albicans*.[9] C4,4-

dimethyl sterols made up 29% of the NSLs synthesized, with lanosterol (58%) and eburicol (28%) as the main C4,4-dimethyl sterols. This may be due to the addition of L-methionine to the incubation mixture. Activation of this amino acid into *S*-adenosyl methionine may enhance C24 side-chain alkylation of lanosterol.[10,11] Therefore, the relatively low amount of eburicol in cell-free assays from *P. italicum* (*chapter 2*) may also be caused by a suboptimal concentration of methionine. Interestingly, the addition of *S*-adenosyl methionine to cell-free assays of *B. cinerea* resulted in an extremely low synthesis of total sterols (*chapter 3*). There is no explanation for this phenomenon. Cell-free assays from *P. italicum* and *B. cinerea* treated with DMI fungicides accumulated C4,4-dimethyl sterols, mainly eburicol, concomitant with a depletion of C4-desmethyl sterols (*chapters 2 and 3*). These results indicate, that C14-demethylation of sterols precedes C4-demethylation under cell-free conditions as demonstrated for ergosterol synthesis in intact mycelium of both pathogens.[4,12,13]

Table 8.1 Literature data on the potency of DMIs to inhibit $P450_{14DM}$ activity in cell-free assays.

Pathogen	DMI[1] and IC_{50} (nM)[2] reported			Reference
Aspergillus fumigatus	Itraconazole (33)	-	Fluconazole (1400)	19
Candida albicans	Ketoconazole (50)	-	Miconazole (350)	14;9
Botrytis cinerea	Itraconazole (1)	-	Triadimefon (170)	*chap. 3*
Penicillium italicum	Ketoconazole (7)	-	Triadimefon (70)	*chap. 2*; 15
Saccharomyces cerevisiae	Ketoconazole (50)	-	Buthiobate (300) Triadimefon (200)	16; 17 18

[1] Most - least potent DMI reported.
[2] Between brackets: concentration of DMI inhibiting cell-free C4-desmethyl sterol synthesis by 50%.

Commercial triazoles inhibited cell-free C4-desmethyl sterol synthesis of *B. cinerea* with different degrees of potency. IC_{50}s (concentrations of compounds inhibiting incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%) observed vary between 0.9 and 170 nM (*chapter 4*). Comparable IC_{50}s were determined in similar assays from other pathogens (Table 8.1). Hence, low field performance of DMI fungicides towards *B. cinerea* can not be ascribed to low sensitivity of $P450_{14DM}$ in the target pathogen.

Interestingly, azole derivatives with a large N1-substituent (*e.g.* the antimycotics itraconazole and ketoconazole) are the most effective inhibitors of cell-free C4-desmethyl sterol synthesis listed. These compounds probably bind more firmly to the substrate binding site of cytochrome $P450_{14DM}$ than compounds with a

relatively small N1-substituent. Stereoisomers of cyproconazole, SSF-109 and tebuconazole also inhibited cell-free C4-desmethyl sterol synthesis with different degrees of potency (*chapter 4*). These results confirm that not only the size but also the nature of the N1-substituent strongly affects inhibitory potency of DMI fungicides. This conclusion is consistent with current literature.[19-25]

EC_{50}s (concentrations of compounds inhibiting radial mycelial growth on PDA by 50%) of the triazoles tested range between 0.1 and 100 nM (*chapter 4*). This range of fungitoxicity is comparable to the *in vitro* activity of DMI fungicides towards other plant pathogens.[26-31] Some of these pathogens are controlled by DMIs in practice. Therefore, limited field performance of triazoles towards *B. cinerea* can not be ascribed to low *in vitro* sensitivity of the pathogen due to permeability barriers, increased efflux of the fungicide from mycelial cells, rapid metabolic breakdown or deposition in cellular compartments.

In further studies structure activity relationships for commercial triazoles, stereoisomers of cyproconazole and tebuconazole and experimental triazoles were performed with the cell-free assay from *B. cinerea* (*chapter 4*). In general, compounds and isomers most toxic to radial mycelial growth were also the most potent inhibitors of cell-free C4-desmethyl sterol synthesis (r_s 0.73 - 0.95), suggesting that intrinsic inhibitory potency and *in vitro* fungitoxicity of DMI fungicides are correlated. However, differences in IC_{50}s did not fully reflect the observed differences in fungitoxicity, as illustrated by the deviating ratios between EC_{50} and IC_{50} values of particular triazoles. The ratio is also relatively high for triazoles with low fungitoxicity. Thus, fungitoxicity of triazoles is not exclusively determined by their intrinsic inhibitory potency. Less active DMIs probably bind more readily to cytochrome P450-enzymes different from $P450_{14DM}$.[32,33] This hypothesis is supported by the observation that complex-formation of P450-isozymes with DMIs and fungitoxicity do not correlate.[15,26,27,34,35]

In vivo activity of the triazoles tebuconazole and triadimenol towards grey mould development on tomato plants and grape berries was compared with that of selected benzimidazole (benomyl and thiabendazole) and dicarboximide (iprodione and vinclozoline) fungicides (*chapter 5*). The latter fungicides are used in practice for grey mould control. *In vivo* experiments were performed with mature tomato plants or detached plant parts (tomato leaves, grape berries). Under these conditions, generally more than 90% of inoculated controls developed grey mould symptoms. In all *in vivo* experiments, tebuconazole was slightly less active than benomyl, but performed 10 to 60 times better than the other commercial botryticides tested. Triadimenol had generally a higher *in vivo* activity than iprodione. Hence, neither a low *in vivo* activity nor a high transfer ratio between *in vivo* and *in vitro* activity can account for limited field performance of triazoles towards *B. cinerea*.

Field rates recommended for control of target pathogens of triazoles such as powdery mildews and rusts in cereals are considerably lower than those recommended for benzimidazoles and dicarboximides in control of grey mould diseases on fruits.[1,36] Thus, low efficacy of triazoles may be due to low field rates recommended. However, application of higher rates of triazoles, which would allow better control of grey mould, may be limited by phytotoxicity (plant growth regulatory activity).[37-40]

Antagonistic effects of sterols and related compounds, phospholipids, acylglycerides, fatty acids and surfactants on activity of DMIs is well documented.[41-49] Little is known about the specificity of these effects, since until now such experiments were not performed with benzimidazoles and dicarboximides. Some of the compounds, tomato leaf homogenate and homogenized fungal mycelium antagonized toxicity of both triazoles and dicarboximides (iprodione and vinclozoline) to comparable extents, suggesting a non-specific interaction with these fungicides (*chapter 5*). Antagonism towards activity of benzimidazoles (benomyl and thiabendazole) was limited. This may be due to differences in lipophilicity of the fungicides. Tebuconazole has a log P of 3.7. Log P values of triadimenol, iprodione and vinclozoline are around 3.1. Those of benomyl and thiabendazole are 2.1 and 1.6, respectively. Therefore, it is likely that reduction of toxicity may be a consequence of partitioning of these fungicides into undissolved residues of the antagonist. This suggests a lack of specificity, which is not always observed.[41-43,45,48] Antagonistic effects of plant constituents may be relevant during pathogenesis, since wounded plant tissue, which may contain these antagonistic constituents, provides infection sites for *B. cinerea*.[50-56]

B. cinerea has traditionally been regarded as a very variable species,[57] presumably with heterokaryosis as an important source of variation.[57,58,59] Therefore, sensitivity of field isolates (121) of *B. cinerea* from France (1992), Germany (1979 - 1992), Israel (1990) and the Netherlands (1970 - 1989) to triazoles was studied in order to establish whether their limited field performance towards grey mould can be attributed to a significant variation in sensitivity of the pathogen population (*chapter 6*). The experiments were carried out with tebuconazole, a DMI fungicide *in vitro* and *in vivo* highly active towards *B. cinerea* (*chapters 4 and 5*) and registered for control of grey mould in combination with dichlofluanid.[1,60] The sensitivity distribution, based on EC_{50}s for inhibition of radial mycelial growth, was log-normal in character. The variation factor (ratio between highest and lowest EC_{50}) for tebuconazole of all isolates tested was 164, indicating a considerable variation in sensitivity within *B. cinerea* populations. Similar variations in sensitivity of *B. cinerea* to the same and other fungicides were demonstrated earlier.[58,61,62] Variation in sensitivity of other pathogen populations to DMI fungicides was also comparable.[62-73]

Hence, it is concluded that variation in sensitivity of *B. cinerea* populations to triazoles can not explain limited field performance of triazoles in grey mould control.

The average EC_{50} of tebuconazole for isolates collected in south west Germany in 1992 was significantly higher than the average EC_{50} of isolates sampled earlier in other countries (*chapter 6*). This may be attributed to quantitative shifts in sensitivity to DMI fungicides by treatments with tebuconazole or other DMIs. In Germany, tebuconazole (10%) is registered in combination with dichlofluanid (40%) for control of *B. cinerea*, *Uncinula necator* and *Plasmopara viticola* in grape vine. Tebuconazole and other DMIs (*e.g.* triadimenol) have also been used for control of other pathogens (*e.g.* powdery mildews) in other crops, which can be infected by *B. cinerea* as well. Similar observations and conditions led Elad (1992) assume that resistance to DMI fungicides occurred in *B. cinerea* populations in Israel.[62] Hence, resistance to DMIs may have also developed in south west Germany. Another explanation may be that *B. cinerea* populations differ in natural sensitivity to DMIs. Conclusive evidence for both hypotheses is lacking, since data on the baseline sensitivity of *B. cinerea* populations from the sampling regions are not available.

Grey mould development incited by isolates with a relatively low sensitivity to DMIs, may be less effectively controlled by DMI fungicides than when incited by the DMI-sensitive reference isolate SAS56 used in the present research project (*chapter 5*). If such less sensitive isolates dominate in *B. cinerea* populations, as in south west Germany, they may reduce field performance of triazoles towards *B. cinerea*. This is especially relevant, since field rates of DMIs recommended are relatively low for control of grey mould (*chapter 5*). Results emphasize the necessity to define reference isolates representing the mean or the lowest sensitivity of a variable pathogen population.

A putative mechanism of resistance to DMI fungicides, based on reduced accumulation of the fungicide in mycelium, was investigated in field strains and laboratory-generated mutants of *B. cinerea* with relatively low sensitivity to DMI fungicides (*chapter 7*). The transient accumulation of tebuconazole by the wild-type isolates SAS56 and B3 suggests that accumulation is a result of passive influx and induced active efflux as described already for *A. nidulans*,[74,75] *C. albicans*,[76] *Monilia fructicola*,[77] *Nectria haematococca* var. *cucurbitae*[78] and *P. italicum*.[79] Initial accumulation of tebuconazole by germlings of *B. cinerea* was comparable to levels of other azole fungicides present in germlings of the fungi mentioned.[74,75,78,80] These data form additional evidence for the conclusion that limited field performance of triazoles towards *B. cinerea* can not be ascribed to low accumulation of these fungicides by the pathogen (*chapter 4*) and corroborate the high *in vitro* and *in vivo* activity of triazoles towards *B. cinerea* (*chapters 4 and 5*). It is assumed that the initially high intracellular levels of tebuconazole result in complex formation with

$P450_{14DM}$ as demonstrated in cell-free assays (*chapter 4*) and CO-displacement studies.[15,81,82]

Accumulation of tebuconazole in laboratory mutants, derived from B3, was relatively low and constant in time (*chapter 7*). This low accumulation level is probably due to a relatively high efflux capacity as described for mutants of other fungi.[29,74,77-80] Increased efflux of the fungicide from fungal cells seems to be a rather common mechanism of resistance in laboratory-generated DMI resistant mutants. Its relevance for field isolates with low sensitivity to DMIs fungicides remains to be established.

Therefore, accumulation of tebuconazole in two field isolates with relatively low sensitivity to DMIs was studied (*chapter 7*). These isolates had a relatively low sensitivity to tebuconazole, which was comparable to that of the laboratory-generated mutants tested. Accumulation of tebuconazole in field isolates was compared with that of the wild-type isolate SAS56, since reference isolates from baseline studies are missing (*chapter 6*). Therefore, accumulation can also be compared with that of the wild-type isolate B3. Accumulation of tebuconazole by both wild-type isolates and both field isolates was transient in time and accumulation levels did not differ significantly. Results suggest, that relatively low sensitivity of field isolates can not be ascribed to reduced accumulation of the fungicide as reported for the laboratory-generated mutants. Other mechanisms as low affinity of tebuconazole to the target site may be involved.

Transient accumulation of tebuconazole in *B. cinerea* germlings was only observed at initial external concentrations of 90 μM or higher (*chapter 7*). With other pathogens a transient accumulation was detected already at 10 and 30 μM.[74,75,77,78,80] This difference suggests that efflux capacity of *B. cinerea* is stronger than that of other pathogens investigated. A high efflux capacity might explain the relatively low field performance of triazoles towards *B. cinerea*. This hypothesis is not supported by previous results (*chapters 4 and 5*). The physiological relevance of the diverse efflux capacities in different filamentous fungi is not yet understood.

Inhibitors of mitochondrial respiration (BAS 490F, CCCP and fluazinam)[83-85] and multisite-inhibiting fungicides ($CuSO_4$ and captan) caused an instantaneous accumulation of tebuconazole (*chapter 7*), confirming the energy-dependent character of fungicide efflux. Similar results were reported for other pathogens.[74,79,86] Such effects may result in synergistic interactions.[86,87] Fungicides interfering with energy generation in *B. cinerea* may therefore be promising compounds to be mixed with tebuconazole or other DMI fungicides. It is suggested that the development of such mixtures may result in DMI-containing products with a sufficient level of field performance towards *B. cinerea*.

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Summary

Botrytis cinerea Pers. ex Fr., the causal agent of grey mould, is one of the most ubiquitous plant pathogens. The fungus is of high economic importance, since it causes losses in various major crops. Post-harvest losses incited by grey mould diseases are also significant.

Antifungal activity of N1-substituted azoles was discovered in the late 1960s. Since then, a large number of azole derivatives has been developed as agricultural fungicides and antimycotics. The mode of action of these azoles is based on inhibition of the cytochrome P450-dependent sterol 14α-demethylase ($P450_{14DM}$), an enzyme of the sterol pathway. By now, sterol demethylation inhibitors (DMIs) represent with about 35 commercial products the most important group of agricultural fungicides. DMI fungicides are commonly applied in control of rusts, powdery mildews and scabs, but seldomly in control of grey mould (*chapter 1*). The aim of the study described in this thesis is to identify factors involved in the limited field performance of DMI fungicides towards *B. cinerea*. The study was restricted to the largest group of DMI fungicides, the triazoles.

A potential mechanism involved in limited field performance of triazoles towards *B. cinerea* may be a relatively low sensitivity of $P450_{14DM}$. A prerequisite to test this mechanism is the availability of a cell-free assay able to synthesize C4-desmethyl sterols from a sterol precursor. Such cell-free assays were described for yeasts and *Aspergillus fumigatus*, but lacked for filamentous plant pathogens. Therefore, such an assay was developed first for the model fungus *Penicillium italicum* (*Moniliaceae*), a species closely related to *Aspergillus fumigatus* (*chapter 2*). Cell-free assays capable of synthesizing C4-desmethyl sterols from [2-^{14}C]mevalonate were obtained by mild mechanical disruption of *P. italicum* germlings in a Bead-beater apparatus. Ergosterol was the only C4-desmethyl sterol synthesized and amounted to 26% of total non-saponifiable lipids (NSLs). Other sterols identified were lanosterol and a trace amount of 24-methylene dihydrolanosterol (eburicol). Treatment of cell-free assays with DMIs led to accumulation of eburicol, indicating an inhibition of $P450_{14DM}$ activity.

The method was then adopted for the filamentous plant pathogen *B. cinerea* (*chapter 3*). Extracts capable of synthesizing C4-desmethyl sterols from [2-^{14}C] mevalonate were obtained by relatively vigorous mechanical disruption of young conidial germlings. The C4-desmethyl sterol fraction consisted of three sterols and amounted 39% of NSLs formed. Ergosterol accounted for 63% of the C4-desmethyl sterols. Only small amounts of C4-monomethyl sterols were synthesized as in intact mycelium of *B. cinerea*. C4,4-dimethyl sterols amounted to 29% of NSLs with lanosterol (54%) and eburicol (28%) as the main C4,4-dimethyl sterols. Cell-free assays treated with imazalil depleted C4-desmethyl sterols concomitant with an accumulation of eburicol, indicating that C14-demethylation precedes C4-demethylation of sterols.

Summary

Commercial and experimental triazole fungicides inhibited cell-free C4-desmethyl sterol synthesis with different degrees of potency. IC_{50}s (concentrations of compounds inhibiting incorporation of (2-^{14}C)mevalonate into C4-desmethyl sterols by 50%) of the compounds tested ranged between 1 and 170 nM (*chapter 4*). Comparable IC_{50}s were found in similar assays for other pathogens. Hence, limited field performance of triazoles towards *B. cinerea* can not be ascribed to a low sensitivity of $P450_{14DM}$. Inhibitory activity of commercial and experimental triazole fungicides on radial growth of *B. cinerea* was tested to assess structure-activity relationships (*chapter 4*). EC_{50}s (concentrations of compounds inhibiting radial mycelial growth by 50%) of all triazoles tested ranged between 0.1 and 100 nM. This range of fungitoxicity is comparable to the *in vitro* activity of other DMI fungicides towards other plant pathogens. Some of them are controlled by DMIs in practice. This implies, that limited field performance of triazole fungicides towards *B. cinerea* is not due to low *in vitro* sensitivity of the fungus.

In vitro (radial growth on fungicide-amended agar) and *in vivo* (foliar-sprayed tomato plants and dip-treated grapes) activity of the triazoles tebuconazole and triadimenol towards *B. cinerea* were compared with that of the benzimidazoles benomyl and thiabendazole and the dicarboximides iprodione and vinclozoline (*chapter 5*). In all experiments benomyl and tebuconazole proved to be the most active fungicides. The transfer ratio, which is defined as the ratio between the EC_{50} of a particular fungicide determined *in vivo* and *in vitro*, was lowest for benomyl. The transfer ratio of tebuconazole was comparable to or slightly lower than those of dicarboximides. Hence, the magnitude of the transfer ratio can not explain limited field performance of triazoles towards *B. cinerea*. Field rates of tebuconazole recommended for control of *B. cinerea* are relatively low compared to those of benzimidazoles and dicarboximides. Therefore, limited field performance of triazoles towards *B. cinerea* may be due to relatively low field rates recommended. Application of higher rates of triazoles, which would allow better control of grey mould, may be limited by phytotoxic (plant growth regulatory) effects.

Tomato leaf homogenates and various biological compounds antagonized activity of triazoles and dicarboximides but did not affect inhibitory potency of benzimidazoles (*chapter 5*). This discrepancy may relate to the log P values of the fungicides. Antagonistic effects may be relevant during pathogenesis, since wounded plant tissue, which may contain such compounds, provides infection sites for *B. cinerea*. Hence, antagonism by natural compounds may contribute to limited field performance of triazoles towards *B. cinerea*.

Sensitivity of field isolates (121) of *B. cinerea* from France (1992), Germany (1979 - 1992), Israel (1990) and the Netherlands (1970 - 1989) to tebuconazole, triadimenol, benomyl and vinclozoline were tested in radial growth experiments (*chapter 6*). Resistance to benomyl (in 21 to 100% of isolates tested) and vinclozoline (in 25 to 71% of isolates tested) was common in most countries. This documents the need for

new disease control strategies and registration of new fungicides to control grey mould. EC_{50}s for tebuconazole and triadimenol ranged between 0.01 - 1.64 and 0.4 - 32.6 $\mu g\ ml^{-1}$, respectively, and were log-normally distributed. The variation factor (ratio between EC_{50}s of the least and most sensitive isolate tested) amounted 164 and 82 for tebuconazole and triadimenol, respectively. These values are comparable to those of other pathogens for DMI fungicides. Thus, variation in sensitivity to triazoles can probably not explain limited field performance of triazoles towards *B. cinerea*. Isolates from south west Germany (1992) were significantly less sensitive to tebuconazole than isolates collected earlier in Germany, Israel and the Netherlands (*chapter 6*). This may be due to a rapid development of resistance or to a variation in natural sensitivity to DMI fungicides of different populations of *B. cinerea*. These less sensitive populations can contribute to the limited field performance of DMI fungicides towards *B. cinerea*. This is especially relevant since recommended field rates of DMIs are relatively low for control of grey mould as compared to the recommended rates of other botryticides (*chapter 5*).

A putative mechanism of resistance to DMI fungicides based on reduced accumulation of the fungicide in fungal mycelium was investigated in laboratory-generated mutants and field isolates of *B. cinerea* with a relatively low sensitivity to DMI fungicides (*chapter 7*). Accumulation of tebuconazole in wild-type isolates was characterized by a high initial accumulation during the first 10 min of incubation and a subsequent gradual release of the fungicide within time. This indicates that accumulation of tebuconazole in *B. cinerea* is the result of passive influx and induced active efflux of the fungicide as described before for other fungi. Accumulation of tebuconazole by laboratory-generated mutants was significantly lower and constant in time, indicating that efflux capacity in resistant mutants is higher than in the corresponding wild-type isolate (*chapter 7*). In contrast, accumulation of tebuconazole by field isolates was transient in time and accumulation levels did not differ significantly from those of the wild-type isolates tested (*chapter 7*). These results suggest, that reduced accumulation of tebuconazole may account for resistance in laboratory-generated mutants but not in field isolates. Various chemicals such as BAS 490F (a strobilurin), carbonyl cyanide 3-chlorophenylhydrazone, copper sulphate and fluazinam enhanced accumulation of tebuconazole by a wild-type isolate and a laboratory-generated DMI-resistant mutant, indicating that accumulation of tebuconazole in both isolates is determined by energy-dependent efflux of the fungicide. Chemicals, which inhibit the energy-dependent efflux, may have synergistic effects on activity of tebuconazole and other DMI fungicides and be candidate companion compounds in mixtures with DMIs. The development of such mixtures may result in DMI-containing products with a better field performance towards *B. cinerea* than the single DMI.

Samenvatting

Botrytis cinerea Pers. ex Fr., de veroorzaker van de grauwe schimmel, is één van de meest voorkomende planteziekten. Het pathogeen veroorzaakt grote schade aan diverse belangrijke gewassen. Na-oogst ziekten veroorzaakt door de grauwe schimmel zijn eveneens belangrijk.

De fungitoxische werking van N1-gesubstitueerde azolen werd ontdekt aan het eind van de jaren zestig. Sindsdien is een groot aantal azoolderivaten ontwikkeld als landbouwfungiciden en als antimycotica. Het werkingsmechanisme van deze azolen is gebaseerd op remming van het cytochroom P450-afhankelijk sterol 14α-demethylase ($P450_{14DM}$), een enzym van de sterolbiosyntheseweg. Momenteel vertegenwoordigen de steroldemethylaseremmers (DMI's) met ongeveer 35 commerciële middelen de belangrijkste groep fungiciden. DMI's worden toegepast ter bestrijding van roesten, echte meeldauwen en schurft, maar zelden ter bestrijding van de grauwe schimmel. Het doel van het onderzoek, beschreven in dit proefschrift, is na te gaan welke faktoren verantwoordelijk zijn voor de zwakke werking van DMI's tegen *B. cinerea* in de praktijk. Het onderzoek werd beperkt tot de grootste groep van de DMI's, de triazolen.

Een mogelijk mechanisme, betrokken bij de beperkte werking van triazolen tegen *B. cinerea* in de praktijk, is een relatief lage gevoeligheid van het $P450_{14DM}$. Om dit mechanisme te toetsen is het noodzakelijk te beschikken over een celvrij systeem, dat in staat is vanuit een sterolprecursor C4-desmethylsterolen te synthetiseren. Dergelijke celvrije systemen zijn beschreven voor gisten en *Aspergillus fumigatus*, maar waren niet beschikbaar voor filamenteuze schimmels. Daarom werd eerst een celvrij systeem ontwikkeld voor de modelschimmel *Penicillium italicum* (*Moniliaceae*), die sterk verwant is aan *A. fumigatus* (*hoofdstuk 2*). Celvrije systemen, die in staat zijn vanuit [2-^{14}C]mevalonzuur C4-desmethylsterolen te synthetiseren, werden verkregen door mechanische desintegratie van *P. italicum* kiemlingen in een Bead-beater. Ergosterol was het enige C4-desmethyl sterol dat werd gesynthetiseerd en bedroeg 26% van de totale, niet-verzeepbare lipiden (NSL's). Andere geïdentificeerde sterolen waren lanosterol en een kleine hoeveelheid 24-methyleendihydrolanosterol (eburicol). Behandeling van celvrije systemen met DMI's leidde tot accumulatie van eburicol, hetgeen wijst op remming van $P450_{14DM}$ aktiviteit.

De methode werd vervolgens aangepast aan *B. cinerea* (*hoofdstuk 3*). Extracten die in staat waren om vanuit [2-^{14}C]mevalonzuur C4-desmethylsterolen te synthetiseren, werden verkregen door een relatief krachtige mechanische desintegratie van jonge kiemlingen. De C4-desmethylsterol fraktie bestond uit drie sterolen en bedroeg 39% van de gevormde NSL's. De hoeveelheid ergosterol bedroeg 63% van de C4-desmethylsterol fraktie. C4-monomethylsterolen werden, net als in intact mycelium van *B. cinerea*, slechts in kleine hoeveelheden gesynthetiseerd. De NSL's bestonden voor 29% uit C4,4-dimethyl sterolen met lanosterol (54%) en eburicol (28%) als de

belangrijkste vertegenwoordigers. C4-desmethylsterolen werden niet gevormd in celvrije systemen behandeld met imazalil, maar accumuleerden eburicol, hetgeen erop duidt dat de C14-demethylering van sterolen voorafgaat aan de C4-demethylering. Celvrije synthese van de C4-desmethylsterolen werd door commerciële en experimentele triazolen in verschillende mate geremd. De IC_{50} waarden van de geteste middelen (concentraties die de incorporatie van (2-^{14}C)mevalonzuur in C4-desmethylsterolen 50% remmen) lagen tussen 1 en 170 nM (*hoofdstuk 4*). In soortgelijke toetsen werden met andere pathogenen vergelijkbare IC_{50} waarden gevonden. De beperkte werking van triazolen tegen *B. cinerea* in de praktijk kan dus niet toegeschreven worden aan een lage gevoeligheid van het $P450_{14DM}$. De correlatie tussen structuur en aktiviteit van commerciële en experimentele triazolen werd vastgesteld door bepaling van de remming van radiale groei van *B. cinerea* (*hoofdstuk 4*). De EC_{50} waarden van de onderzochte triazolen (concentraties die de radiale groei 50% remmen) lagen tussen 0.1 en 100 nM. Deze variatie in fungitoxiciteit is vergelijkbaar met de *in vitro* aktiviteit van andere DMI's tegen andere plantepathogenen. In de praktijk worden enkele van deze pathogenen met DMI's bestreden. Dit betekent dat de zwakke werkzaamheid van triazolen tegen *B. cinerea* in de praktijk geen gevolg kan zijn van een relatief lage gevoeligheid van de schimmel.

De werking van de triazolen tebuconazool en triadimenol tegen *B. cinerea* is *in vitro* (radiale groei op agar met fungicide) en *in vivo* (bespoten tomateplanten en dompelbehandeling van druiven) vergeleken met die van de benzimidazolen benomyl en thiabendazool en de dicarboximiden iprodion and vinclozolin (*hoofdstuk 5*). In alle experimenten bleken benomyl en tebuconazool de meest werkzame fungiciden. De transferratio, gedefinieerd als de verhouding tussen de EC_{50} van een fungicide in de *in vivo* en *in vitro* experimenten, was het laagste voor benomyl. De transferratio voor tebuconazool was vergelijkbaar of iets lager dan die van de dicarboximiden. De grootte van de transferratio kan de beperkte werking van triazolen tegen *B. cinerea* in het veld dus niet verklaren. Aanbevolen doseringen van tebuconazool tegen *B. cinerea* zijn relatief laag vergeleken met die van benzimidazolen en dicarboximiden. De beperkte werkzaamheid van triazolen tegen *B. cinerea* zou daarom mede een gevolg kunnen zijn van de lage dosering die in de praktijk wordt aanbevolen. Toepassing van hogere doseringen die zouden kunnen leiden tot een betere werking tegen *B. cinerea*, kunnen echter fytotoxische effecten (groeistofwerking) veroorzaken.

Homogenaten van tomateblad en diverse natuurstoffen antagoneerden de activiteit van triazolen en dicarboximiden maar niet die van benzimidazolen (*hoofdstuk 5*). Dit verschil houdt mogelijk verband met de log P waarden van de fungiciden. De antagonistische effecten kunnen van betekenis zijn tijdens de pathogenese, omdat verwond planteweefsel, waarin de geteste verbindingen kunnen voorkomen, infektieplaatsen voor *B. cinerea* vormen. Antagonisme door

natuurstoffen kan dus één van de oorzaken zijn van de zwakke werking van triazolen tegen *B. cinerea* in de praktijk.

De gevoeligheid van veldisolaten van *B. cinerea* (121) uit Frankrijk (1992), Duitsland (1979 - 1992) en Nederland (1970 - 1989) voor tebuconazool, triadimenol, benomyl en vinclozolin werd bepaald in radiale groeiproeven (*hoofdstuk 6*). Resistentie tegen benomyl (in 21 tot 100% van de getoetste isolaten) en vinclozolin (in 25 tot 71% van de getoetste isolaten) kwam in de meeste landen algemeen voor. Dit duidt op de behoefte aan nieuwe bestrijdingstrategieën en de registratie van nieuwe botryticiden. De EC_{50} waarden van tebuconazool en triadimenol voor de veldisolaten varieerden respectievelijk tussen 0,01 - 1,64 en 0,4 - 32,6 $\mu g\ ml^{-1}$ en vertoonden een log-normale verdeling. De variatiefactor (verhouding tussen de EC_{50} van het minst en meest gevoelige isolaat) bedroeg 164 voor tebuconazool en 82 voor triadimenol. Deze waarden zijn vergelijkbaar met die van andere pathogenen voor DMI's. De variatie in gevoeligheid voor triazolen kan de zwakke werking van triazolen tegen *B. cinerea* in de praktijk waarschijnlijk niet verklaren. Isolaten uit het zuidwesten van Duitsland (1992) waren significant minder gevoelig voor tebuconazool dan isolaten die eerder verzameld waren in Duitsland, Israël en Nederland (*hoofdstuk 6*). Dit kan een gevolg zijn van resistentieontwikkeling of variatie in de natuurlijke gevoeligheid van verschillende populaties van *B. cinerea* voor DMI's. Dergelijke minder gevoelige populaties kunnen een rol spelen in de zwakke werking van DMI's tegen *B. cinerea* in de praktijk. Dit kan vooral van belang zijn omdat aanbevolen doseringen van DMI's voor de bestrijding van *B. cinerea* relatief laag zijn in vergelijking met die van andere botryticiden (*hoofdstuk 5*).

Een mogelijk resistentiemechanisme tegen DMI's, gebaseerd op verminderde accumulatie van de middelen in mycelium, is onderzocht in laboratoriummutanten en veldisolaten van *B. cinerea* met een relatief lage gevoeligheid voor DMI's (*hoofdstuk 7*). Accumulatie van tebuconazool in wild-type isolaten werd gekenmerkt door een hoge initiële accumulatie gedurende de eerste 10 minuten van de incubatie en een daarop volgende geleidelijke uitscheiding van het fungicide (transiente accumulatie). Dit wijst er op dat accumulatie van tebuconazool in *B. cinerea* het gevolg is van passieve influx en induceerbare actieve efflux van het fungicide, zoals eerder is beschreven voor andere schimmels. Accumulatie van tebuconazool door laboratoriummutanten was significant lager en constant in de tijd. Deze resultaten suggereren dat de laboratoriummutanten een hogere effluxcapaciteit voor het fungicide hebben dan het overeenkomstige wild-type isolaat (*hoofdstuk 7*). Accumulatie van tebuconazool door veldisolaten met een relatief lage gevoeligheid was transient en verschilde niet van dat van de wild-type isolaten (*hoofdstuk 7*). Deze resultaten suggeren, dat verminderde accumulatie van tebuconazool een verklaring kan zijn voor resistentie in de laboratoriummutanten, maar niet voor de relatief lage gevoeligheid in de veldisolaten. Diverse stoffen zoals BAS 490F (een strobilurine), carbonyl cyanide 3-chloorfenylhydrazon, kopersulfaat en fluazinam verhoogden de accumu-

latie van tebuconazool in zowel het wild-type isolaat als in een DMI-resistente laboratoriummutant. Dit betekent dat de accumulatie van tebuconazool in beide isolaten wordt bepaald door energie-afhankelijke efflux van het fungicide. Middelen die de energie-afhankelijke efflux remmen, kunnen de werking van tebuconazool en andere DMI's synergeren. Dit betekent dat deze middelen van potentiële betekenis zijn in combinatieprodukten met DMI's. De ontwikkeling van dergelijke mengsels kan resulteren in DMI-bevattende produkten die in de praktijk een betere werking tegen *B. cinerea* bezitten dan de afzonderlijke DMI fungiciden.

Epilogue

"Und wieder sehen wir betroffen,
der Vorhang ist geschlossen
und alle Fragen sind noch offen."
F. Dürrenmatt

But that seems to be science - by answering a question you create another. In my struggle to find answers I received support in many different ways. Therefore I am grateful to:

PROF. DR. FRANZ MÜLLER, Institut für Phytopharmakologie, Universität Hohenheim. He initiated the cooperation with Wageningen, but passed away before the project started. I appreciate his support for my plans to emigrate to "Tulip-Paradise".

PROF. DR. PIERRE DE WIT, my promotor after Prof. Müller passed away. I am grateful for your interest in scientific and personal problems and critical comments on my manuscripts.

DR. MAARTEN DE WAARD, mein Doktorvater. I owe you many thanks for the proposal of the research project, your confidence in my work, your marvellous supervision and your never ending energy in revising manuscripts.

PROF. DR. D. BERG, PROF. DR. U. GISI, DR. R. PONTZEN and DR. R. TIEMANN, meine Ansprechpartner bei Bayer und Sandoz. I am grateful for the contribution of stimulating ideas, the cooperation in experimental work and the financial support.

HELMUTH-AURENTZ-STIFTUNG and DEUTSCHER AKADEMISCHER AUSTAUSCH DIENST are acknowledged for additional financial support.

Eco's en MOLLIES, mijn collega's van Fyto. Bedankt voor de prettige werksfeer en de lekkere koffie, die 's ochtends altijd gezet was. In het bijzonder wil ik JANNY en TON bedanken voor de gezellige tijd buiten het werk (fitness, vakanties, ...) en de privé-lessen in oer-Hollandse gezelligheid.

DORETH en WILJAN VISSER, Sportinstituut Wageningen, are thanked for their contribution to a *mens sana* by ensuring a *corpus sanus*.

ANGELIKA, GERITH, LINUS und RAIMUND, meine Familie. Auch wenn das Verständnis für die abwesende Schwester und Tochter schwierig ist, auf Durchalteparolen und Aufmunterung aus Bickenbach war in kritischen Zeiten immer Verlaß.

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