Management of resistance to the fungicide fenpropimorph in *Erysiphe graminis* f.sp. *tritici*

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Management of resistance to the fungicide fenpropimorph in *Erysiphe graminis* f.sp. *tritici*

Proefschrift

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STELLINGEN

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1. Resistentie-ontwikkeling tegen genees- en gewasbeschermingsmiddelen in organismen die ziekten en plagen veroorzaken vormt een bedreiging voor de mensheid. De overheid schenkt hier te weinig aandacht aan.

2. Bij de bestrijding van tarwemeeldauw met fenpropimorf dient het frequent gebruik van lage doseringen vermeden te worden.

   - Dit proefschrift

3. Het risico van resistentie-ontwikkeling tegen fenpropimorf is gering.

   - Dit proefschrift

4. Het resistentiemechanisme tegen fenpropimorf in de modelschimmel *Aspergillus niger* is gerelateerd aan een verminderde gevoeligheid van sterol Δ^{14}-reductase voor het fungicide.

   - Dit proefschrift

5. Ziogas *et al.* (1991) concluderen dat fenpropimorf naast sterol Δ^{14}-reductase en sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase een derde enzym remt in de sterol biosynthese van *Nectria haematococca* var. *cucurbitae*, namelijk squaleen epoxidase. Deze conclusie is onjuist.

   - Ziogas et al. (1991) Pesticide Biochemistry and Physiology 39, 74-83

6. Een te smal gewasbeschermingsmiddelenpakket werkt het illegaal gebruik van gewasbeschermingsmiddelen in de hand.

7. Advisering op maat is essentieel voor een optimaal en verantwoord gebruik van gewasbeschermingsmiddelen.

8. Het recht op leven gaat boven het recht op bezit.

- Mgr. M. Muskens

9. Afgezien van het feit dat het klonen van de mens ethisch onverantwoord is, is het onmogelijk aangezien ieder mens een onkloonbare ziel heeft.

10. De combinatie van sociaal leven en 24-uurs economie brengt sociale onrust.

11. De zwarte dood van varkens leidt tot de paarse dood van varkenshouders.

12. Als je met schimmels werkt, heb je je sporen snel verdiend.

13. Netheid is voor de domme, de wijze beheerst de chaos.

14. Gezien de verregaande internationalisering is het aan te bevelen om Engels te promoten.

Stellingen behorende bij het proefschrift

Management of resistance to the fungicide fenpropimorph
in *Erysiphe graminis* f.sp. *tritici*

Wageningen, 29 mei 1998 A.J.G. Engels

Voor Vader en Moeke

Niemand alleen

Alleen uit de gemeenschap komt de wijsheid.
Eén hand kan geen touw om een bundel knopen.
Wie alleen loopt raakt de weg kwijt.
Wie dan valt heeft niemand om te helpen.
Wie dan schreeuwt, heeft niemand die hoort.
Niemand deelt vreugde of verdriet.
In de gemeenschap heeft ieder duizenden handen,
heeft iemand duizenden voeten,
loopt niemand ooit alleen.

Patice Kayo

VOORWOORD

Wat een vreugde, wat een feest! Het proefschrift is af. Hier ligt dan, kort gezegd, het produkt van vier jaren literatuur lezen, experimenten doen, experimenten opnieuw doen, literatuur opnieuw lezen, manuscripten schrijven en manuscripten opnieuw schrijven. Aha, vandaar dat wetenschap met herhalingen te maken heeft.

Dit proefschrift is niet het werk van één persoon. Graag wil ik een ieder die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift van harte bedanken. Een aantal mensen wil ik met name noemen.

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Tonnie

CONTENTS

Chapter 1

Outline of this thesis

Background

Crops and natural vegetations are attacked by numerous fungal pathogens. They cause plant diseases which have been the subject of study for several centuries. Plant diseases have quite some impact on society and economy since a number of pathogens causes considerable yield losses worldwide. Losses in the production of the eight principal food and cash crops caused by pathogens is estimated at 13.3% (Oerke *et al.*, 1995).

Fungicides have been used for about a century to reduce crop losses to an economically acceptable level. Before 1960, nearly all fungicides used in agriculture acted at multiple subcellular targets of the pathogens and possessed limited biochemical specificity, which restricted their application to external plant therapy. Two distinct classes of these fungicides with a multiple site of action can be distinguished. The first one includes the inorganic Bordeaux mixture, sulphur and copper compounds. The second one comprises organic compounds such as the phthalimides, dithiocarbamates, dinitrophenols and aromatic hydrocarbons. From 1960 onwards, the discovery of systemic fungicides with specific modes of action brought about the benefit of internal plant therapy. These compounds have both protective and curative properties. The recently developed systemic fungicides generally have a highly potent action against plant pathogens and can, therefore, be applied in relatively small amounts. The sterol biosynthesis inhibitors (SBIs) belong to this class of fungicides. SBIs comprise azoles and related compounds which inhibit P450-dependent 14α-demethylation of sterols and morpholines which inhibit other steps in sterol biosynthesis.

The morpholine fenpropimorph

The most important representative of the morpholine fungicides is fenpropimorph, introduced in 1979 by BASF and Maag (Bohnen and Pfiffner, 1979; Pommer and Himmele, 1979). It is intensively used as a cereal fungicide, especially for control of powdery mildews. Fenpropimorph is a systemic fungicide, being absorbed through the leaves and roots. It is translocated acropetally and has both protective and curative properties. Fenpropimorph also has a strong vapour phase activity. The fungicide is applied both as a single product and in mixtures.

Sterols are essential for all eukaryotes as membrane components (maintaining optimal membrane fluidity and integrity) and regulatory compounds (*e.g.* steroid hormone synthesis) (Bloch, 1983; Hall, 1987; Van den Bossche, 1990). A suboptimal sterol composition of membranes can lead to an increased permeability resulting in a severe leaking of the membranes. Fenpropimorph interferes with sterol biosynthesis by inhibition of the enzymes sterol Δ^{14}-reductase and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase (Baloch *et al.*, 1984; Mercer, 1991). Fenpropimorph probably binds to the sterol binding site of both enzymes and exerts its

inhibitory effect by mimicking the carbocationic high-energy intermediates of both the Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization (Köller, 1992). Inhibition of sterol synthesis leads to depletion of demethyl sterols (*e.g.* ergosterol) and accumulation of sterols which are normally not present or only in small amounts. These biochemical effects are regarded as the cause of the fungistatic action of fenpropimorph.

Fungicide resistance

Plant pathogens can develop resistance to fungicides which have a site-specific mode of action. This phenomenon, described as fungicide resistance, can be defined as a stable, inheritable adjustment of a fungus to a fungicide, resulting in a less than normal sensitivity to that fungicide (Anonymous, 1988). The terms 'resistance in practice' or 'practical resistance' can be used to refer to a situation in which development of resistance leads to failure of disease control. These terms should only be used when both the following criteria have been met (Anonymous, 1988):

(1) a significant reduction in disease control is observed under practical conditions, in the presence of natural inoculum, following application of a product correctly and in accordance with the label;

(2) a demonstration that such significantly reduced disease control is due to the presence of pathogenic strains with reduced sensitivity.

Fungicide resistance is one of the main problems in chemical control of plant diseases. Resistance development made the use of a number of systemic fungicides ineffective against plant pathogens of major importance. Resistance builds up through the survival and spread of initially rare mutants upon selection pressure by fungicide treatment.

Factors involved in fungicide resistance can be classified as biological factors (genetic and epidemiological) and operational factors (type of compound and method of application) (De Waard, 1993). Resistance, characterized by a sudden loss of effectiveness and the presence of clearcut sensitive and resistant subpopulations, is usually referred to as 'qualitative', 'single-step' or 'monogenic' resistance. When a gradual decline in disease control and sensitivity is observed, resistance is usually referred to as 'quantitative', 'multi-step' or 'polygenic' resistance. Biochemical mechanisms of resistance involved are alternation of the target site, circumvention of the target site by developing an alternative metabolic pathway, metabolic breakdown of the fungicide (detoxification), increased efflux of the fungicide, lack of conversion to active compound, and compensation (Henry, 1992).

The present study

The aim of the present research is to develop strategies to avoid or decrease the risk for resistance development to fenpropimorph in *Erysiphe graminis* f.sp. *tritici* and to study putative mechanisms of resistance. In order to put these results in perspectives, this thesis starts with an overview on integrated control of cereal mildews and its impact upon fungicide use (Chapter 2). In many countries integrated control programs have been initiated in order to reduce pesticide use and their side-effects on the environment. In this context, farmers started using fenpropimorph in cereals at rates lower than recommended. The adopted approaches are use of split applications which is defined as a treatment in which the recommended dosage is divided over a number of applications, and use of reduced dosages. The effect of such spray regimes on resistance development in *E. graminis* f.sp. *tritici* is not known. Experiments designed to assess the resistance risks of these strategies are described in Chapter 3 and 4. An important parameter influencing resistance development in fungi to fungicides is the fitness of resistant subpopulations. An analysis of the fitness of isolates of *E. graminis* f.sp. *tritici* with a reduced sensitivity to fenpropimorph is given in Chapter 5. Changes in sterol composition may play a role in the reduced sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph. Experiments designed to test this hypothesis are described in Chapter 6. Genetic and biochemical studies on resistance in *E. graminis* f.sp. *tritici* are very difficult, if not impossible, because of its obligate character. As an alternative, fenpropimorph-resistant mutants of *Aspergillus niger* were selected. In some of these mutants, the genetic (Chapter 7) and biochemical (Chapter 8) mechanisms of resistance were studied. Chapter 9 concludes the thesis with a general discussion.

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Chapter 2

Integrated control of powdery mildew in cereals and its impact upon fungicide use

A.J.G. Engels

Modified version of an invited paper in: *A vital role for fungicides in cereal production*, H.G. Hewitt, D. Tyson, D.W. Hollomon, J.M. Smith, W.P. Davies, K.R. Dixon (Eds.), Oxford: BIOS (1995), 117-128

Abstract. Powdery mildew is one of the most common and severe foliar diseases of cereals in Europe. In this chapter, integrated control of powdery mildew in cereals and its impact upon fungicide use is discussed. Possible control methods such as the use of reduced fungicide dosages, warning systems, cultural practices, biological control, systemic acquired resistance, biotechnology, and variety mixtures are outlined. Several methods should be combined to develop strategies for integrated control. It is likely that fungicide use will decrease when these strategies become common practice. On the other hand, such practices may significantly enhance the life-time of modern systemic fungicides because resistance in target pathogens will develop less readily.

Introduction

In the last decades, plant disease control has become heavily dependent on fungicides. This practice increased yield significantly but had also adverse side-effects on the environment. In 1991, the Dutch government introduced the Multi-Year Crop Protection Plan to set standards for the reduction of the negative effects of pesticides (Anonymous, 1991). The plan states three policy objectives: (I) reduction of dependence on pesticides, (II) reduction of volume of pesticides used and (III) reduction of emission on pesticides. A major solution for the structural dependence on pesticides may be the change from conventional farming systems to integrated farming systems. Integrated crop protection is part of these integrated farming systems. In this chapter, an integrated approach to powdery mildew control in cereals and its impact upon fungicide use is discussed. The first part of this chapter deals with the importance of *Erysiphe graminis*, the causal agent of powdery mildew in cereals, and its life cycle. Possible control methods and their effect on fungicide use are given in the middle sections of this paper. First, the use of fungicides in cereals is discussed. In this context, resistance development, reduced fungicide use, and novel fungicides are addressed. Second, integrated control is discussed in more detail. Results obtained through using integrated cultural practices, warning systems and models appear promising. Third, possibilities for biological control are outlined briefly. Fourth, developments in systemic acquired resistance (SAR) are described. The latter control method might become of practical importance in the near future. Fifth, future prospects of breeding for resistance are discussed. In this context, classical breeding, the impact of biotechnology, the phenomenon of *Mlo* resistance in barley and the use of mixtures are briefly considered. In the final sections of this chapter, some policy affairs are briefly commented on and prospects concerning fungicide use are outlined.

Importance of powdery mildew in cereals

Powdery mildew occurs over the whole of Europe with the most affected region being the north-west of Europe. It is one of the most common and severe foliar diseases of cereals infecting leaves, stems and ears. Disease intensity fluctuates from year to year depending on weather conditions. In the second half of the 20th century the economic significance of powdery mildew has increased. The main reason is the intensification of cereal production: highly productive varieties, higher seed rates and higher amounts of nitrogen fertilizer. The most important fungal diseases in wheat are shown in Table 2.1. The principle fungal diseases of barley are *E. graminis* f.sp. *hordei*, *Rhynchosporium secalis* and *Helminthosporium* leaf spot diseases (= *Pyrenophora* spp and *Cochliobolus sativus*), *Puccinia graminis*, *Puccinia hordei* and *Fusarium* spp. (Oerke *et al.*, 1994).

Table 2.1. The major fungal pathogens of wheat and their regional distribution (Oerke *et al.*, 1994).

Pathogen	Asia	Europe		America		Oceania	Africa
		Western	Eastern	Northern	Latin		
Tilletia spp.	++	+	+	+	+	+	+
Puccinia spp.	++	++	++	++	++	+	+
Ustilago spp.	++	+	+	++	+	+	+
Septoria spp.	+	++	+	++	+	+	+
E. graminis	+	++	++	+	+	+	+
P. herpotrichoides	o	++	+	+	o	+	+
G. graminis	+	+	+	+	+	++	+
C. sativus	+	o	+	+	++	++	+

++, +, o: ranking in descending order of importance

At present 42 million hectares of land in Western Europe are cultivated with cereals of which 60-80% is treated with fungicides (Bigler *et al.*, 1992). The 1980s witnessed rising concerns about the sustainability of agricultural systems that depend, increasingly, on inputs of pesticides. Several countries have responded to these concerns by adopting policies for reducing pesticide use. Some countries like the Netherlands, Denmark and Sweden have set legislative targets to be met in specified time frames (Evans and Rowland, 1993). The Netherlands is targeting a 35% reduction in pesticide use to the year 1995 and 50% to the year 2000 (Anonymous, 1991). Also other European countries and countries in other continents have initiated programs to reduce pesticide use. In those continents farmers deal with additional problems. For instance in the Commonwealth of Independent States, one of the most important tasks is to increase cereal production (Susidko *et al.*, 1988).

Nevertheless, they advocate the use of integrated control systems in the protection of cereal crops. Also in the U.S.A., extension services support integrated wheat management (Cuperus *et al.*, 1993). Even in developing countries projects have been initiated to develop integrated control. The situation in these countries is completely different from the situation in Western Europe since farms in these areas are small and farmers have insufficient resources to apply extensive chemical treatments.

Life cycle of *Erysiphe graminis*

The most important factor in powdery mildews' success is the life cycle of the pathogen (Wolfe, 1984). Therefore, one should take this into account to innovate control methods. The biology of powdery mildew has been described extensively by Jørgensen (1988). *E. graminis* is an obligate pathogen and can infect cereals and grasses. Special forms (the so-called *formae speciales*) exist, each being restricted to its own particular host. Wheat is attacked by *E. graminis* f.sp. *tritici*, barley by f.sp. *hordei*, rye by f.sp. *secalis* and oat by f.sp. *avenae*. *E. graminis* overwinters as cleistothecia on straw and, in milder climates, also as mycelium and conidia. Wind-borne ascospores or conidia are primary inocula. Cleistothecia which produce ascospores are formed in midsummer and conidia are most frequent in spring. Cleistothecia serve two purposes. First, they represent the sexual stage in the life cycle of the fungus and are therefore a mean by which recombination of genetic material can occur and provide opportunity for production of new races of the fungus. Second, cleistothecia are means by which the fungus may survive the period just before and during harvest when little or no green plant material is available (Gair *et al.*, 1978). Volunteer wheat plants are opportune hosts and also support survival of the pathogen between summer and winter crops. Ascospores, released after rains, are sparse relative to conidia but sometimes infect cereals in autumn. Conidia are produced in great numbers and are epidemiologically most important. They survive only a few days but withstand dispersal over several kilometres (Gair *et al.*, 1978).

Factors affecting epidemics

The initial stage of polycyclic epidemics can be described by an exponential growth curve:

$$x_t = x_0 e^{rt}$$

where x_t is the amount of disease at time t, x_0 is the amount of initial disease (at $t = 0$), e is the base of the natural logarithm ($= 2.73...$), r is the apparent infection rate of the disease and t is time. For the development of powdery mildew epidemics three interrelated factors have to be considered: the fungus, the state of the host (crop) and the environment. The main infection source of the fungus in autumn is conidia from volunteer plants. Ascospores from cleistothecia on straw can serve as a possible additional source (x_0). The

more initial inoculum present in the crop, the greater the spread within a crop during favourable conditions in spring.

Cultural techniques involving, for example, the destruction of infected volunteer cereals or restrictions in nitrogen fertilization, only offer partially effective options for the control of powdery mildew. More effective measures are the planting of (partially) resistant varieties and chemical control, both of which affect the apparent infection rate. Both are essential components of modern cereal husbandry.

Environmental factors also affect the apparent infection rate of the disease, notably temperature. Infection and development of pustules takes place over a wide range of temperatures but optimal development occurs in the range 15-22°C. Temperatures above 25°C severely restrict powdery mildew development. Also rainfall has an effect on powdery mildew development since water inhibits colony formation and conidia production. Furthermore, wind facilitates the dispersal of conidia.

Fungicide use in cereals

In the Netherlands, azoles and morpholines (both sterol biosynthesis inhibitors = SBIs) are commonly used to control powdery mildew in cereals. The azoles were introduced in the 1970s. Barley powdery mildew in the UK became field resistant to some azoles (Fletcher and Wolfe, 1981), whereas similar compounds have remained effective in other European countries. Recently introduced azoles are highly effective against powdery mildews. This may be due to their relatively high activity and to differences in level of cross resistance to other azoles (De Waard, 1992). In the late 1960s, the morpholine tridemorph was introduced as a fungicide for the control of powdery mildew in barley. Chemical synthesis and biological screening led to the development of another morpholine, fenpropimorph, which has a high efficacy against powdery mildew. This fungicide was introduced in 1980. In comparison with tridemorph, fenpropimorph has a wider spectrum of antifungal activity and a greater activity against powdery mildew. Fungicides used in wheat in the Netherlands are shown in Table 2.2. In practice, fungicide mixtures are commonly used since farmers have to control various diseases in one spray application (*e.g.* powdery mildew, rusts and *Septoria* spp). An additional advantage of mixtures is their value in anti-resistance strategies.

Resistance development

One of the main problems in chemical control of plant diseases is resistance development in target organisms. Factors involved in fungicide resistance can be classified as biological factors (genetic and epidemiological) and operational factors (type of compound and method of application) (De Waard, 1993b). Resistance development made the use of a number of

systemic fungicides ineffective against plant pathogens of major importance. Because of these developments, chemical control by modern systemic fungicides is severely hampered or only dependent on a limited number of chemical groups. This is a highly undesirable situation and it stresses the need for new classes of fungicides (De Waard, 1993a).

Table 2.2. Common fungicides used for powdery mildew control in cereals in the Netherlands.

Trade name	Active ingredient
Bayfidan	triadimenol
Corbel	fenpropimorph
Tilt Gel	propiconazole
Alto	cyproconazole
Sportak Delta	cyproconazole and prochloraz
Matador	tebuconazole and triadimenol
Opus Team	epoxyconazole and fenpropimorph
Septor	difenoconazole and fenpropimorph
Granit Ace	bromuconazole and fenpropimorph
Amistar	azoxystrobin
Allegro	kresoxim-methyl and epoxyconazole

The risk of resistance development also applies to azole and morpholine fungicides in the control of *E. graminis*. The development of azole resistance in cereal powdery mildews (Fletcher and Wolfe, 1981), stimulated the design of anti-resistance strategies. A small decrease in the sensitivity of powdery mildew populations to morpholines has been found in a number of Western European countries (De Waard *et al.*, 1992). This reduction in sensitivity did not effect field performance of fenpropimorph and hence, field resistance is still absent.

To prevent the occurrence of fungicide resistance, agrochemical companies cooperate in the Fungicide Resistance Action Committee (FRAC). The aims of FRAC are (Russell, 1995):

- To foster a responsible attitude towards fungicide resistance from all concerned with crop health;
- To seek and promote effective cooperation both amongst agrochemical companies and between companies and the public and private research sectors at national and international levels;
- To promote and communicate a better understanding of the causes, risks and management strategies surrounding the phenomenon of fungicide resistance.

Reduced fungicide dosages

In Denmark, reduced dosages of azoles and morpholines have been used against fungal diseases in cereals for several years. The mixtures Tilt Turbo (propiconazole and tridemorph) and later, Tilt Top (propiconazole and fenpropimorph) are most commonly used (Nielsen, 1995). The average dosage of Tilt Top used, decreased from 0.87 l ha^{-1} in 1987 to 0.33 l ha^{-1} in 1994. The number of applications was about 2 in 1987, 3.3 in 1990 and 2.2 in 1994 (Jørgensen, 1995). Reduced dosages often perform with similar efficacy to the recommended ones.

In other countries such as the UK reduced fungicide dosages are advocated in barley (Wale, 1990) and wheat (Wale and Oxley, 1992). They found that low dosages morpholine and azole mixtures could control powdery mildew as effectively as full dosages morpholine fungicides and thereby increase profitability. Timing, however, is crucial when reduced dosages are used.

The effect of low dosages on resistance development is not well documented. In theory, it is possible that reduced dosages increase the rate of resistance development. Engels *et al.*, (1996) showed that split application increases the development of reduced sensitivity.

Novel fungicides for the control of powdery mildew in cereals

Modern fungicides have a specific target site and improved characteristics with respect to environmental criteria. Some new fungicides for cereal crops have been developed and will be marketed in the near future. Among these are the anilinopyrimidine fungicide cyprodinil (Heye *et al.*, 1994), the benzamide fungicide XRD-563 (Arnold *et al.*, 1992) and the strobilurin analogues BAS 490 F (Allegro) (Ammermann *et al.*, 1992) and ICIA5504 (Amistar) (Godwin *et al.*, 1994).

Integrated control

Integrated control is an economically justified and sustainable system of crop protection, consisting of a combination of cultural, biological, genetic and chemical control methods, that aims at the maximum productivity with the least possible adverse consequences for the environment. Integrated control is not new. Before pesticides were available, farmers had to survive with only the help of their common sense, so they used cultural practices. When pesticides became available, a number of these practices became less important, since farmers relied on those pesticides. Nowadays, most farmers recognize the importance of reduced pesticide usage. A major solution for the structural dependence on pesticides may be the change from conventional farming systems to integrated farming systems. In these farming systems monitoring and supervised control play an important role. Model systems

have been developed which estimate the future state of crop diseases and pests, crop damage and evaluate the situation with a cost/benefit analysis. The main point of these forecast systems for the farmer is to reduce his uncertainty in taking control decisions. A well known decision-support system is EPIPRE (Rabbinge and Rijsdijk, 1983), but also other systems are available nowadays. Substantial reduction in fungicide use can be achieved by those systems.

Wijnands and Darwinkel (1990) formulated a strategy for wheat production within an integrated farming system. Prevention of diseases, pests and weeds should result in a reduced dependence on pesticides. An integrated crop protection strategy for cereal diseases in winter wheat was formulated. The most important cultural practices are the use of resistant varieties, decreased nitrogen application and late sowing of winter wheat. In the Netherlands, pilot farms started with integrated arable farming in 1987. Fungicide use in winter wheat decreased by ± 80% (Table 2.3). This reduction became possible by the above mentioned cultural practices, use of damage thresholds and use of only systemic fungicides (Wijnands *et al.*, 1995). The number of applications decreased from on average more than two to one in 1992 and 1993 (Table 2.3). Spring barley was grown on about half of the farms, especially on clay soils. On average one application was needed, but by using modern fungicides, the amount of active ingredients decreased by 50%. In accordance with the concepts of integrated control, chemical treatments were only applied when other (preventive) methods failed.

Table 2.3. Number of applications, amount of fungicides used (a.i.) and the reduction in fungicide use in winter wheat (WW) and spring barley (SB) on pilot farms in the Netherlands (Wijnands *et al.*, 1995).

Crop	Year	Number of applications	Amount (kg ha^{-1})	Reduction (%)[1]
WW	1987-1989	2.6	2.7	
	1990-1991	1.3	0.9	66
	1992-1993	1.1	0.5	82
SB	1987-1989	1.0	0.5	
	1990-1991	1.0	0.2	59
	1992-1993	1.1	0.3	43

[1] % use compared to 1987-1989

Biological control

Control of powdery mildew with biological control methods is very difficult to achieve in a field situation. Biological control can be direct (mycoparasitism, competition and antibiosis) or indirect via the plant (systemic acquired resistance or induced resistance). Direct control on barley powdery mildew has been found with leaf application of actinomycetes (Lee, 1990). Cell suspensions of *Streptomyces* spp. act curatively against barley powdery mildew. The use of microorganisms can also have an indirect effect via the plant (*e.g. Bacillus subtilis*). These indirect effects, often described as systemic acquired resistance can also be regarded as biological control.

Systemic acquired resistance

Many plant species can be protected against diseases by a challenge inoculation with microorganisms. This induces a systemic defense response resulting in systemic acquired resistance (SAR). SAR may confer long-lasting disease resistance of the whole plant towards subsequent infections by a broad spectrum of pathogens. Early reviews by Chester (1933) brought together many descriptions of the phenomenon. Induction of SAR in plants by chemicals has been reviewed by Kessmann *et al.* (1994). SAR can also be activated by treatment with compounds from biological or synthetic origin, *e.g.* salicylic acid or 2,6-dichloroisonicotinic acid. SAR in barley has frequently been reported over the past twenty years using various biotic and abiotic inducers and powdery mildew as the challenged pathogen. Results concerning SAR in barley against powdery mildew have been described by Wei *et al.* (1993). A recent overview of SAR in monocots is given by Steiner and Schönbeck (1995). Induction of resistance in barley plants with microbial metabolites produced by the bacterium *B. subtilis* has been studied in field trials in Germany over several years. Results show that infection by *E. graminis* f.sp. *hordei* is never inhibited completely but mildew that developed did not affect yield (Steiner *et al.*, 1988). Modern molecular techniques are being used to elucidate the mechanism of SAR.

The first compound (CGA 245704) of a new generation of plant protection products, plant activators which act as SAR, has been introduced in 1995 (Kessmann *et al.*, 1995). This compound, a benzothiadiazole, acts indirectly against pathogens by activating the plant's own defense mechanisms. Since it has no direct fungicidal or fungistatic activities, it is not a fungicide. The plant activator induces the same set of proteins as observed in plant tissue after local activation with a necrotizing microorganism, but without producing necrotic lesions. The compound can be applied like conventional fungicides. In Germany, the plant activator (trade name: Bion) was used in winter wheat in 1996. In the long term, prospects for the use of SAR-inducing agents are good. SAR-inducing chemicals can

provide novel benefits for disease control although these agents should be used in a preventative way. SAR-inducing compounds will probably reduce the use of fungicides and their resistance risk.

Breeding for resistance

Classical breeding

Resistant varieties have been developed and are still being developed by classical breeding. The major problem is that powdery mildews are able to overcome resistance quite easy. Monitoring surveys, which are carried out all over Europe, give insight in the virulence pattern of the powdery mildew populations. Breeders are now facing the need for durable, high-level resistance. Recently, more effort is put into the development of horizontal resistance which may be more durable, but also more complex.

Biotechnology

Biotechnology can help to exploit more diverse and durable sources of plant resistance. Up till now, however, it has been a very difficult task to genetically engineer cereals. Latest results are quite promising and have been described by Scott (1994). Rapid progress has been made in developing procedures for routine production of transgenic cereals in the last three years. Identification of resistance genes will be the next step in future research. These genes will be used as a source of genetically engineered resistance. Transfer of these resistance genes in commercial varieties will then become very important. In the years to come, molecular genetic technologies will probably result in the development of genetic modified varieties resistant to several plant pathogens.

Mlo resistance in barley

Three types of resistance of barley to the powdery mildew fungus are at present accessible for barley breeding and production: i) horizontal, ii) vertical and iii) *Mlo* resistance (Jørgensen, 1993). *Mlo* resistance to barley powdery mildew is a relatively new kind of resistance. It was originally described in a powdery mildew resistant barley mutant in 1942. Mutation at the *mlo* locus confers resistance to powdery mildew which is expressed at the primary penetration stage of the fungus. Molecular components of papillae are involved in *Mlo* resistance to barley powdery mildew. A lot of research has been carried out to elucidate the mechanism of resistance, but researchers have not succeeded yet. Most important characteristics of *Mlo* resistance are (Jørgensen, 1992):

- *Mlo* resistance does not conform to the gene-for-gene system;
- It is effective against all isolates of *E. graminis* f.sp. *hordei*;
- Resistance is caused by a rapid formation of large cell wall appositions, preventing

penetration by the fungus;
- *Mlo* resistance is considered very durable since no powdery mildew isolates have been found in nature with the virulence to overcome this resistance.

The exploitation of *Mlo* resistance has been hampered by pleiotropic effects of the *mlo* genes, but they have been overcome by recent breeding work. During the 1980s *Mlo* resistant spring barley varieties have become cultivated extensively in several European countries.

Variety mixtures
The use of variety or species mixtures has been considered many times. It was first recorded by Tozzetti (1767). More recently, Wolfe and Barrett (1979) looked more closely at the use of variety mixtures for disease control. Promising results have been obtained in Eastern Europe. In Western Europe, however, very few mixtures are used since high yielding varieties are available and new varieties have been marketed. Furthermore, the malting and food industry does not accept mixtures. In the Netherlands, for instance, spring barley variety Prisma has been grown on more than 80% of the barley acreage for several years till 1994. In 1995, Prisma has almost completely been exchanged by the new variety Reggae.

Policy affairs

Environmental and public considerations
The availability of pesticides is under pressure due to increasing development costs, the increasing environmental demands and public perception. Ragsdale (1992) stated that public education is of the highest priority in order to change the negative perception of disease control with chemicals that meet the latest toxicological and environmental standards. The negative public perception of pesticides influences politics, which has an impact on legislation of compounds.

EU Cost Action 817
COoperation in Science and Technology of the European Union provides the opportunity for experts in a certain area to meet and discuss their research. Cost Action 817 entitled "Population studies of airborne pathogens on cereals as a means of improving strategies for disease control" started in the end of 1993. Aim of this Cost Action is to understand better the behaviour of the powdery mildew and rust population and to improve predictions on performance of resistant varieties and fungicides. In that way, the need to use fungicides for disease control can perhaps be reduced. This would decrease both environmental hazards

and farmers' costs. In the framework of this Cost Action working groups have been formed dealing with:

- Surveys on virulence, aggressiveness and fungicide resistance in Europe;
- Variety and species mixtures to control airborne diseases of cereals;
- Cereal rust and mildew genome database;
- Nomenclature of powdery mildew resistance and virulence genes;
- Epidemiological parameters.

Prospects

An overview of the effect of the integrated control strategies discussed is given in Table 2.4.

Table 2.4. Effect of strategies to integrated control of powdery mildew upon fungicide use.

Strategy		Impact upon fungicide use
Reduced dosages		++
Warning systems and models		+++
Cultural practices	- N application	++
	- resistant varieties	+++
	- sowing date	+
Biological control		+
Systemically acquired resistance		+++
Transgenic varieties (biotechnology)		++
Variety mixtures		+

+++, ++, +: ranking in descending order of importance

Several strategies should be combined to develop strategies for integrated disease control. Some strategies have shown to be effective such as the use of resistant varieties and warning systems. Other approaches are promising such as SAR and transgenic varieties. Biological control may become an important strategy in the long run. Because of these developments, it is likely that fungicide use will decrease. However, fungicides will not be abolished but used in the context of integrated control within a sustainable agriculture.

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Chapter 3

Effect of split applications of fenpropimorph-containing fungicides on sensitivity of *Erysiphe graminis* f.sp. *tritici*

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Abstract. A field trial was conducted in the south of the Netherlands over a period of three years (1992-1994) in order to test the effect of fenpropimorph-containing fungicides on the sensitivity of *Erysiphe graminis* f.sp. *tritici* to fenpropimorph. In general, use of split applications resulted in a better protection against wheat powdery mildew than use of normal applications. This is ascribed to a better timing of the split applications. The sensitivity to fenpropimorph of the mildew population in plots treated with normal applications of Corbel and Tilt Top did not change during the growing seasons of the years investigated. In contrast, the sensitivity of the mildew population from plots treated with split applications of Corbel became significantly lower. A significantly lower sensitivity was also found in the mildew population from plots treated with split applications of Tilt Top in 1993 and 1994. These results indicate that split applications can increase the rate at which *E. graminis* f.sp. *tritici* can become less sensitive to fenpropimorph. Because maintenance of sensitivity of the pathogen population is valued highly, the split application strategy of fenpropimorph-containing fungicides is not recommended for wheat powdery mildew control.

Introduction

Erysiphe graminis f.sp. *tritici*, the causal agent of wheat powdery mildew, is one of the most common and severe foliar pathogens of wheat in Europe. On average, 70% of winter wheat fields are infected by powdery mildew in the Netherlands (Daamen *et al.*, 1992). Non-chemical disease control strategies are usually not sufficient to reduce mildew infestation to acceptable levels. In most wheat growing areas of Europe, fungicides are needed to achieve this goal. One of the main problems in chemical control of wheat powdery mildew is resistance development in the target organism, *E. graminis* f.sp. *tritici*. Factors involved in fungicide resistance can be classified as biological (genetic and epidemiological) or operational (type of compound and method of application) (De Waard, 1993). The risk of resistance development also applies to morpholine fungicides in the control of *E. graminis* f.sp. *tritici*. The most important representative of the morpholine fungicides is fenpropimorph, introduced in 1980. A small decrease in the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph has been found in a number of western European countries (De Waard *et al.*, 1992; Lorenz *et al.*, 1992; Felsenstein, 1994; Readshaw and Heaney, 1994).

Morpholines belong to the sterol biosynthesis inhibitors (SBIs). This group of fungicides is generally considered to have a dual mode of action. They inhibit, to different degrees, both sterol Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization, depending on the

fungicide and the fungal species (Mercer, 1991). Fenpropimorph is regarded as a strong inhibitor of sterol Δ^{14}-reductase and a weak inhibitor of $\Delta^{8}\rightarrow\Delta^{7}$-isomerase (Baloch *et al.*, 1984; Berg *et al.*, 1984). Ziogas *et al.* (1991) showed that fenpropimorph inhibits an additional enzyme in the sterol pathway in *Nectria haematococca* var. *cucurbitae*: squalene epoxidase.

Fenpropimorph is commonly used in cereals against mildews, both as a single product and in mixtures. The fungicide can be applied at the recommended (normal) rate or at a reduced rate in a 'split application', which is defined as a treatment in which the recommended rate is divided over a number of applications. Little data are available on the effect of split applications on the rate of resistance development.

The aim of the present research is to study the effect of split application of fenpropimorph-containing fungicides in wheat on the sensitivity of *E. graminis* f.sp. *tritici*. Experiments were carried out during the growing seasons of 1992 to 1994 in large farm fields, and were designed in such a way that effects of flux of conidia between fields were avoided as much as possible.

Materials and Methods

Field trials were conducted in Heerlen in the south of the Netherlands from 1992 through 1994. This region usually has environmental conditions favourable to the development of wheat powdery mildew epidemics. The commercial field (6 ha) selected was surrounded by forests and was, thus, isolated from neighbouring fields. One half of the field was used in 1992 and 1994 as the experimental field; in 1993, the other half of the field was used (Figure 3.1).

A major concern was the possible risk of inter-plot interference, for example the flux of conidia and fungicide movement between plots (Jenkyn *et al.*, 1979). To minimize these risks as much as possible, large plots of 300 x 16 m were used as the experimental units and the arrangement of the plots was such that the predominant wind direction was parallel with the length of the plots. As a consequence, there was a limit to the number of experimental units (plots) that could be managed. The need of large plots conflicts with the desirability of replicating experiments, but the use of non-replicated designs can be valuable, for example to study the effect of different disease levels on yield (Teng, 1985; Shaw and Royle, 1987). De Waard (1992) found that, in the province of Limburg, fungicide sensitivity was similar in populations of different fields and could be regarded as one large population hardly influenced by environmental conditions. Hence, the risk that differences in sensitivity of populations from different plots can be attributed to factors other than the treatments is practically absent. Still, however, in 1993 and 1994, the control treatment was replicated to confirm the validity of this assumption. In addition, adequate

care was taken to select a uniform site for experimentation: soil type, fertilization and microclimate was the same for all plots.

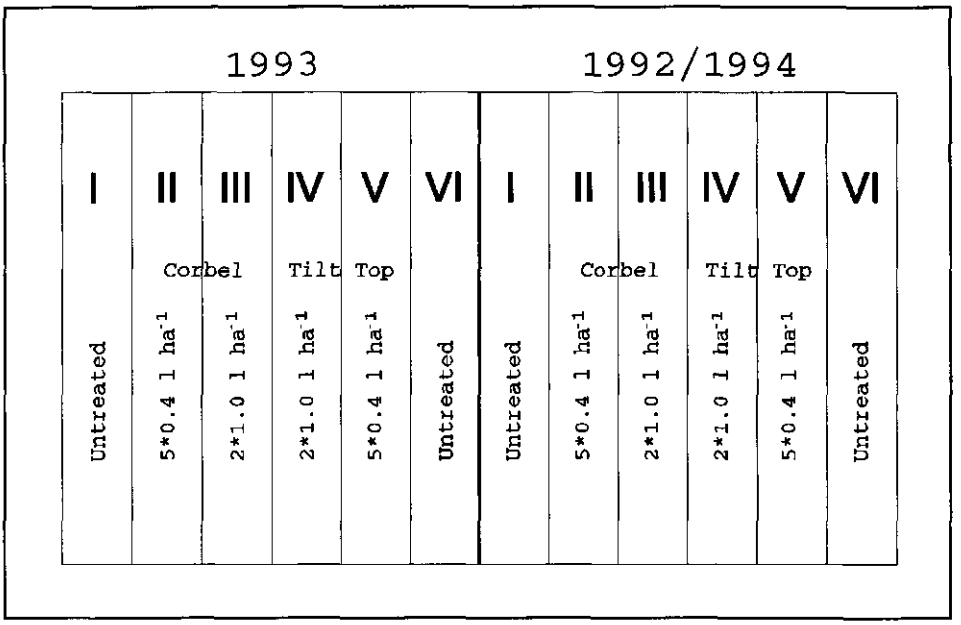

Figure 3.1. Design of the field trial to test the effect of split and normal applications of fenpropimorph-containing fungicides on disease control and sensitivity of *Erysiphe graminis* f.sp. *tritici* to fenpropimorph.

The arrangement of the plots was similar in each year and was designed in such a way that the split application treatments were situated in between a control plot and a plot with a normal application rate. This was done to avoid a possible gradient in sensitivity in the mildew population in the field as a result of factors (flux of conidia, soil fertility) other than the treatment effects.

The wheat cultivar Pagode was used in 1992, whereas Apollo was selected in 1993 and 1994. Pagode has two resistance genes (*Pm2* and *Pm5*) and is moderately resistant to wheat powdery mildew. Apollo carries three powdery mildew resistance genes (*Pm2*, *Pm4b* and *Pm8*) but is susceptible to most of the wheat powdery mildew races present in the Netherlands. Apollo was used in 1993 and 1994 because of its field resistance against *Septoria* spp. This characteristic is important because *E. graminis* f.sp. *tritici* can be strongly inhibited in the presence of *Septoria nodorum* (Weber *et al.*, 1994). Fertilizers and herbicides were applied according to normal practice during all three years.

Two fungicides, Corbel (750 g fenpropimorph l^{-1}) and the mixture Tilt Top (375 g

fenpropimorph l^{-1} and 125 g propiconazole l^{-1}), were applied. Treatments used were:

I: control; untreated
II: split application of Corbel; 5 applications of 0.4 l ha^{-1}
III: normal application of Corbel; 2 applications of 1.0 l ha^{-1}
IV: normal application of Tilt Top; 2 applications of 1.0 l ha^{-1}
V: split application of Tilt Top; 5 applications of 0.4 l ha^{-1}
VI: control; untreated (1993 and 1994)

The total amount of active ingredient of fenpropimorph applied was 1500 g in plots II and III and 750 g in plots IV and V.

Wheat powdery mildew development was assessed five times in 1992 and six times in 1993 and 1994. From every plot, random samples of 40 tillers were taken and the coverage by mildew on the upper three leaves was estimated. Wheat powdery mildew severity data were used to calculate the Area Under the Disease Progress Curve (AUDPC) for the flag leaf per plot. AUDPC is the disease intensity integrated over time between two observations dates, using the trapezoidal integration method (Campbell and Madden, 1990). In 1993 and 1994, severity of other diseases and pests, for example *Septoria* spp, brown rust (*Puccinia recondita*), cereal leaf beetle (*Lema cyanella*) and aphids (*Sitobion avenae*) was assessed as well.

The first fungicide treatment was applied when the disease coverage on flag-3 leaves was, on average, 2%. The flag leaf was not yet present at this stage. The growth stages of the crops present at the time-points of applications and the number of days after planting are given in Table 3.1.

Table 3.1. Timing of split and normal applications of Corbel and Tilt Top to control *Erysiphe graminis* f.sp. *tritici* in wheat. (Between parenthesis: days after planting)

Treatment[1]	Year	Growth stage (DC)						
		31	32	34	45	59	65	71
II, V	1992		X(238)		X(248)	X(257)	X(263)	X(270)
III, IV	1992				X(248)		X(263)	
II, V	1993	X(217)	X(229)		X(240)		X(252)	X(262)
III, IV	1993	X(217)					X(252)	
II, V	1994		X(226)	X(237)	X(246)	X(259)	X(267)	
III, IV	1994			X(237)		X(259)		

[1] II: Corbel (5x0.4 l ha^{-1}), III: Corbel (2x1.0 l ha^{-1}), IV: Tilt Top (2x1.0 l ha^{-1}) and V: Tilt Top (5x0.4 l ha^{-1}).

To assess the sensitivity of the wheat powdery mildew population to fenpropimorph, four bulk samples per plot were collected before the fungicide treatments and ten samples per plot were collected after the fungicide treatments. Each bulk sample consisted of three mildewed leaves collected within 10 m of each other within a plot. These samples were kept overnight at 4°C and the next day mildew conidia were transferred to one-week-old seedlings of the susceptible cultivar Okapi (1992 and 1993) or Florida (1994). Neither cultivars carry wheat powdery mildew resistance genes. Cross-contamination between isolates was avoided by covering each pot (6x6 cm) containing seedlings (± 25) with a cellophane bag. Covered seedlings were incubated in a climate room at 18°C under fluorescent light (Philips TLMF 40W/35 RS; 7000 Lux) for 16 hours per day and at 80% RH. After two weeks, pots were shaken to spread inoculum all over the wheat seedlings and then incubated for another week. The infected seedlings were then used for the production of inoculum. Each pot was shaken above and gently tapped against seven-day-old seedlings (± 70) in two inoculum pots (diameter 12.5 cm). After 14 days of incubation, these pots with bulk isolates were used for the inoculation of seedlings used in foliar spray tests.

Foliar spray tests were used to assess the sensitivity of bulk isolates to fenpropimorph; in 1993, the sensitivity to propiconazole was also tested. Foliar spray tests were carried out in a spray cabinet. One-week-old wheat seedlings (± 25) in pots (6x6 cm) were sprayed with a Corbel or Tilt solution (25 ml) for two minutes at a pressure of 0.8 bar, untill run-off. The optimal concentration range of fenpropimorph to assess the sensitivity of isolates was determined using the statistical program 'Cademo' (Rasch *et al.*, 1987). The optimal concentration range of fenpropimorph for testing field isolates was 0, 5, 10 and 25 μg ml^{-1} and for the reference wild-type isolate LH 0, 1, 5 and 10 μg ml^{-1}. The optimal concentration range of propiconazole for testing field isolates was 0, 2.5, 5 and 10 μg ml^{-1}, and for testing the reference wild-type isolate LH 0, 0.25, 0.5 and 1 μg ml^{-1}. In each foliar spray test, all concentrations were tested in duplicate. In one day, 11-13 isolates were tested simultaneously: two isolates from each plot and the reference isolate LH (twice). This procedure avoided 'day effects' on the sensitivity of the isolates. After spraying, seedlings were grouped per concentration with a minimum distance of 3 m between groups in order to avoid vapour phase activity of fenpropimorph, and left to dry for at least two hours.

Seedlings were inoculated by shaking infected seedlings above sprayed seedlings. In this way, the seedlings received a dense inoculum of powdery mildew conidia. Immediately after inoculation, inoculated seedlings were covered with a cellophane bag, grouped per concentration and incubated in a climate room under the conditions mentioned above.

After 10-12 days of incubation, infection by wheat powdery mildew was estimated by assessing powdery mildew coverage of ten randomly chosen leaves per pot. Assessment criteria were based on the disease simulation program 'Distrain' (Tomerlin and Howell, 1988). Average powdery mildew coverage of the ten leaves was calculated for each

concentration. These averages were expressed as percentages of powdery mildew severity in the control treatment and then transferred to probits. Linear regression was carried out to calculate EC_{50} and Q_{50} values. The Q_{50} value is the degree of reduced sensitivity and was calculated by dividing the EC_{50} value of a field isolate by the average EC_{50} value of the reference isolate LH tested in the same test. All calculations were carried out with the software package 'Lotus 123'. The statistical procedure used was a Student's *t*-test. The statistical analysis was carried out with 'Genstat'.

Results

Powdery mildew development

In all three years, differences in mildew infestation between plots at the start of the treatments (DC31-32) were negligible. During the course of the season plants in untreated plots became more infected than plants of other plots. Mildew severity in untreated plots was highest in 1993 when the average percentage of mildew coverage of flag, flag-1 and flag-2 leaves at growth stage DC72-77 was about 5, 14 and 45%, respectively. These percentages amounted 4, 10 and 7% in 1992, and 2, 11 and 22% in 1994, respectively. The AUDPC of the flag leaf was highest in untreated plots (Table 3.2). In general, the AUDPC was lowest in plots treated with split applications. Mildew control seemed slightly higher in plots treated with Tilt Top than with Corbel.

Table 3.2. Wheat powdery mildew severity, transformed to AUDPC, on the flag leaf of wheat in plots of disease control experiments carried out in 1992, 1993 and 1994.

Treatment	AUDPC (%.days)[1]		
	1992	1993	1994
Untreated (plot I)	86.7	78.0	36.7
Untreated (plot VI)	n.d.[2]	62.3	38.6
Corbel (5x0.4 l ha^{-1})	6.4	2.4	2.5
Corbel (2x1.0 l ha^{-1})	10.9	12.9	7.5
Tilt Top (5x0.4 l ha^{-1})	3.9	0.5	0.6
Tilt Top (2x1.0 l ha^{-1})	8.5	6.2	5.7

[1] Time range was 36, 34 and 31 days in 1992, 1993 and 1994, respectively.
[2] Not determined.

Development of other diseases and pests

At DC75-77, *P. recondita* was present in the untreated plots on the upper leaves and absent in the treated plots. In 1993, *Septoria* was observed in all plots, but plots treated with Tilt Top were less infected than the other plots. In 1994, hardly any *Septoria* was present. In 1993, damage of the flag and flag-1 leaf by *L. cyanella* was 0.6 and 0.2%, respectively. The average number of *S. avenae* present on the flag and flag-1 leaf was about 0.8 and 0.2, respectively. In 1994, very few insects were observed.

Sensitivity to fenpropimorph

At the start of the experiments, the average Q_{50} value of isolates in the experimental field selected was 4.7 (1992), 6.8 (1993), and 6.7 (1994), indicating that the mildew population tested had a decreased sensitivity to fenpropimorph as compared to the wild-type reference isolate LH. In all three years, these Q_{50} values did not differ significantly between the plots ($P=0.90$, 0.81 and 0.91 in 1992, 1993 and 1994, respectively). During the growing season of these years, the mean Q_{50} value of isolates from untreated plots did not change significantly (Table 3.3). In contrast, the mean Q_{50} value of isolates from plots treated with split applications of Corbel increased.

Table 3.3. Q_{50} values of fenpropimorph of isolates of *Erysiphe graminis* f.sp. *tritici* collected from untreated plots and plots treated with split and normal applications of Corbel and Tilt Top before (May) and after (July) treatments were applied in the field trials of 1992-1994.

Year	Treatment[1]					
	I	II	III	IV	V	VI
May 1992	4.6 a	4.4 a	4.4 a	5.0 a	5.2 a	n.d.[2]
July 1992	3.5 a	6.0 b	5.8 a	4.5 a	4.5 a	
T-value[3]	1.4	2.2	1.7	0.9	1.2	
May 1993	6.8 a	6.1 a	7.1 a	7.0 a	7.2 a	6.6 a
July 1993	6.2 a	11.0 b	8.0 a	7.8 a	10.0 b	5.3 a
T-value	1.0	4.1	1.0	0.9	2.9	1.9
May 1994	7.1 a	6.4 a	6.3 a	7.3 a	6.6 a	6.3 a
July 1994	6.3 a	8.6 b	7.4 a	8.0 a	8.5 b	6.1 a
T-value	0.9	2.5	1.1	0.7	2.5	0.2

[1] I and VI = Untreated, II = Corbel 5x0.4 l ha^{-1}, III = Corbel 2x1.0 l ha^{-1}, IV = Tilt Top 2x1.0 l ha^{-1} and V = Tilt Top 5x0.4 l ha^{-1}.

[2] n.d.: not determined.

[3] Critical T-value=2.2; P=0.05.

Means followed by the same letter in the same column in the same year do not differ significantly.

The most pronounced increase in Q_{50} value (from 6.1 to 11.0) of isolates from these plots was detected in 1993. A significant increase in Q_{50} values was also found for isolates from plots treated with split applications of Tilt Top during the growing season of 1993 and 1994. Normal applications of both fungicides did not lead to a shift in Q_{50} values.

Sensitivity to propiconazole

In 1993, all isolates collected were also used to test the sensitivity to propiconazole. Q_{50} values for isolates collected at the end of the season in plot I to VI were 17.7, 16.9, 19.3, 17.3, 20.8 and 13.2, respectively. The average Q_{50} value of propiconazole was 17.4 ($\pm$ 10.8). A correlation between the sensitivity to fenpropimorph and propiconazole was absent (Figure 3.2). The slope of the regression line did not differ significantly from zero (P=0.05).

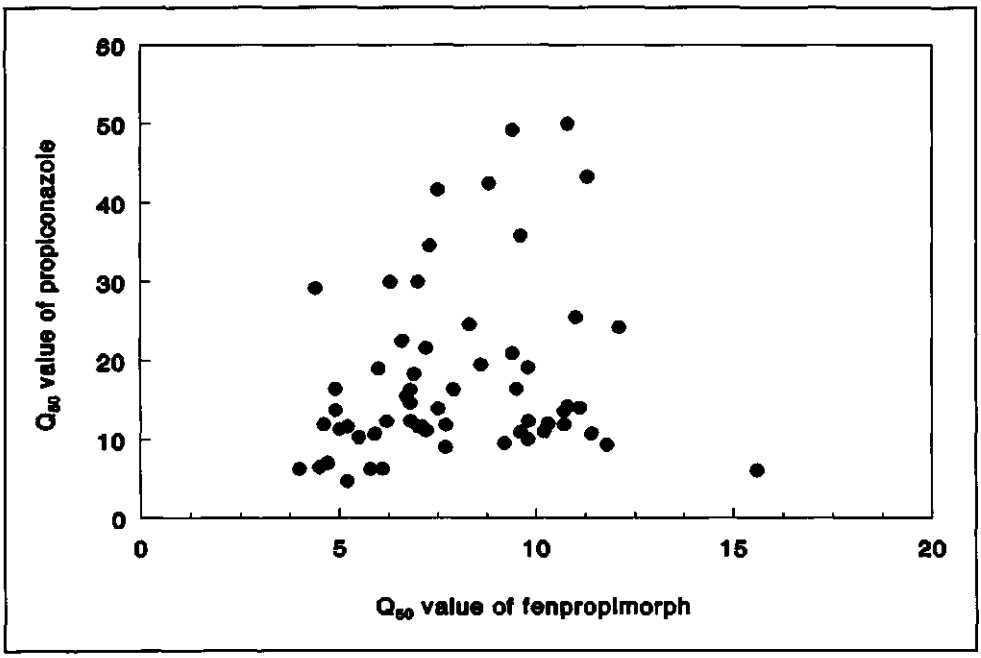

Figure 3.2. Sensitivity of isolates of *Erysiphe graminis* f.sp. *tritici* between fenpropimorph and propiconazole in foliar spray tests.

Discussion

In all years tested (1992-1994), less powdery mildew was observed in plots treated with split applications than in plots treated with normal applications. The good performance of

split applications may be explained by the timing of the sprays; length of intervals between subsequent sprays and initiation of applications may play a role. In 1992 and 1994, the split applications were initiated earlier than the normal applications, whereas, in 1993 - the year with the highest disease incidence - all treatments were initiated at the same date. The main explanation for the good performance of split applications seems to be the relatively short intervals between sprays, which results in a better timing of application in relation to leaf emergence and epidemic onset. An early initiation of split applications seems of minor importance. Similar results were obtained for control of *E. graminis* f.sp. *tritici*, *Puccinia striiformis* and *Septoria* spp in wheat in Denmark (Jørgensen and Nielsen, 1992) and for control of *E. graminis* f.sp. *tritici* in Germany (Forster *et al.*, 1994; Schulz, 1994). Good control is not always achieved when reduced dosages are applied (Hardwick *et al.*, 1994; Jørgensen, 1994). They showed that reduced rates of fenpropimorph were not as effective as full rates when the mildew epidemic was rather severe. Wale (1994) stated that reduction in fungicide dose is possible but that timing has to be optimal and careful consideration should be given to factors that influence the success of reduced fungicide use.

In all years, isolates collected from the various plots showed no significant difference in sensitivity to fenpropimorph before the start of the treatments. No shifts in sensitivity were observed in the untreated plots at the end of the growing season. After the fungicide applications, Q_{50} values of isolates from plots treated with split applications of Corbel had shifted significantly towards higher values. This was consistent in all three years. A significant shift towards higher Q_{50} values was also found in the mildew population in plots treated with split applications of Tilt Top in 1993 and 1994. The sensitivity of the mildew population in plots treated with normal applications of both fungicides did not change significantly. This was also consistent in all three years. These results suggest that split applications favoured a reduction in sensitivity to fenpropimorph. Shifts in Q_{50} values observed in plots treated with split applications may have been a consequence of an earlier initiation of applications as carried out in 1992 and 1994. However, in 1993, shifts in Q_{50} values were most pronounced whilst initiation of all treatments was at the same day. As a consequence, timing of the initial application is probably not the main cause of the results observed. In our opinion, efficient selection of a less-sensitive mildew population is the main determinant for shifts in Q_{50} values. Under conditions of a high disease intensity, as present in 1993, and an effective selection pressure by split applications of the fungicide in space and time, a relatively fast development of reduced sensitivity to fungicides in fungi with a high apparent infection rate will occur. This has been predicted in models described by Georgopoulos and Skylakakis (1986). Because maintenance of the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph is of prime importance, we do not recommend the use of split applications of fenpropimorph-containing fungicides for wheat powdery mildew control. Reductions in sensitivity to fenpropimorph of *E. graminis* f.sp. *tritici* after split applications of fenpropimorph-containing fungicides were also observed by Forster *et al.*

(1994). Schulz (1994) stated that it is not the split applications themselves but the degree of performance of fungicide programmes that is the decisive factor influencing sensitivity. Zziwa and Burnett (1994) found no significant differences between full and reduced dosages in their effects on the sensitivity of *Erysiphe graminis* f.sp. *hordei* to fenpropimorph.

The results on resistance development in *E. graminis* f.sp. *tritici* to fenpropimorph described in this and a previous paper (De Waard, 1992) indicate that resistance development proceeds stepwise and is probably linked to the continuous sensitivity distribution of the pathogen population and the polygenic nature of reduced sensitivity to fenpropimorph. The latter statement may well relate to the multisite action of the fungicide, but this is not necessarily a prerequisite because resistance to a related group of fungicides (DMIs), which have a single site of action, is also polygenic (Butters *et al.*, 1984; Wolfe, 1985; Heaney, 1988). Polygenic resistance to fenpropimorph has indeed been observed in genetic studies with *N. haematococca* var. *cucurbitae* (Demakopoulou *et al.*, 1989) and *Pseudocercosporella herpotrichoides* (Hocart and McNaughton, 1994). However, in *E. graminis* f.sp. *hordei* only one single gene seems to control a low degree of reduced sensitivity to fenpropimorph (Brown, 1994).

It is suggested that the risk of development of polygenic resistance in plant pathogens is relatively low (Georgopoulos, 1994). This assumption has been corroborated by practical experience, as resistance to DMIs developed only gradually (Schepers, 1985; Heaney, 1988; Köller, 1995). The same is true for morpholines, because cases of field resistance to this group of fungicides are virtually absent after about 25 years of practical use.

Assumptions have been made that split applications and low dosages enhance resistance development in cases of polygenic controlled resistance (Georgopoulos, 1994; Gressel, 1995). The present results provide experimental data to support this hypothesis. Similar results were obtained in experimental studies with *Venturia inaequalis* (Köller, 1995). Isolates from this pathogen collected from trees treated with half rates of fenarimol showed a significant shift towards a higher frequency of resistance, whereas isolates collected from trees treated with full rates did not differ from isolates collected from untreated trees. Computer models either confirm these experiences (Josepovits, 1989) or predict that, in the case of polygenically controlled fungicide resistance, shifts in fungicide sensitivity are approximately independent of the fungicide concentration applied, excluding dosages producing no biological effect (Shaw, 1989).

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Chapter 4

Effect of reduced dosages of fenpropimorph on sensitivity of *Erysiphe graminis* f.sp. *tritici*

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Abstract. The effect of reduced dosages of the fungicide Corbel on sensitivity of *Erysiphe graminis* f.sp. *tritici* to fenpropimorph was tested in one field trial carried out in 1995. Plots treated with the lowest reduced dosage tested (2x0.25 l ha^{-1}) or treated with split applications (5x0.4 l ha^{-1}) showed a pronounced decrease in sensitivity of isolates in the course of the season. Sensitivity to fenpropimorph of the isolates from the untreated plots and the plot treated twice with 0.4 l ha^{-1} did not change significantly. Control of powdery mildew and brown rust in plots treated with reduced dosages of the fungicide was relatively low. Therefore, the results indicate that the use of Corbel at reduced dosages should be discouraged since it may lead to enhanced selection of mildew populations less sensitive to fenpropimorph and to insufficient disease control of foliar pathogens.

Introduction

Wheat powdery mildew, caused by *Erysiphe graminis* f.sp. *tritici*, is an important disease of wheat in Europe. In most wheat growing areas of Europe, azole and morpholine fungicides are needed to control the disease. Morpholines belong to the sterol biosynthesis inhibitors and have a dual mode of action. They inhibit, to different degrees, both sterol Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization, depending on the fungicide and the fungal species (Mercer, 1991).

A risk of morpholine fungicides is resistance development in the target organisms. This risk also applies to the morpholine fenpropimorph in the control of *E. graminis* f.sp. *tritici*. A small decrease in the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph has already been found in a number of western European countries (De Waard *et al.*, 1992; Lorenz *et al.*, 1992; Felsenstein, 1994; Readshaw and Heaney, 1994). In practice, fenpropimorph is applied in cereals not only at the recommended dosage (1.0 l ha^{-1}) but also at reduced dosages. Reduced dosages are commonly used in various parts of Europe to decrease the fungicide input in agriculture.

The aim of the present study is a preliminary investigation on resistance development in *E. graminis* f.sp. *tritici* in relation to the dosage of application. This was studied in a single field trial, carried out in 1995.

Materials and Methods

The experiment was carried out in Heerlen in 1995 in the same area as used for previous field trials (Engels *et al.*, 1996). A plan of the field trial is drawn in Figure 4.1. The area

per plot was about 0.75 ha. Wheat cultivar Apollo, susceptible to most of the wheat powdery mildew races present in the Netherlands, was used. The fungicide Corbel (750 g fenpropimorph l^{-1}) was applied in a field trial with the following treatments:

I: control; untreated
II: 5 applications of 0.4 l Corbel ha^{-1}: split application (100%)
III: 2 applications of 1.0 l Corbel ha^{-1}: normal application (100%)
IV: 2 applications of 0.4 l Corbel ha^{-1}: reduced dosage (40%)
V: 2 applications of 0.25 l Corbel ha^{-1}: reduced dosage (25%)
VI: control; untreated

The quantity of active ingredient of fenpropimorph applied was 1500 g ha^{-1} in plot II and III, 600 g ha^{-1} in plot IV and 375 g ha^{-1} in plot V.

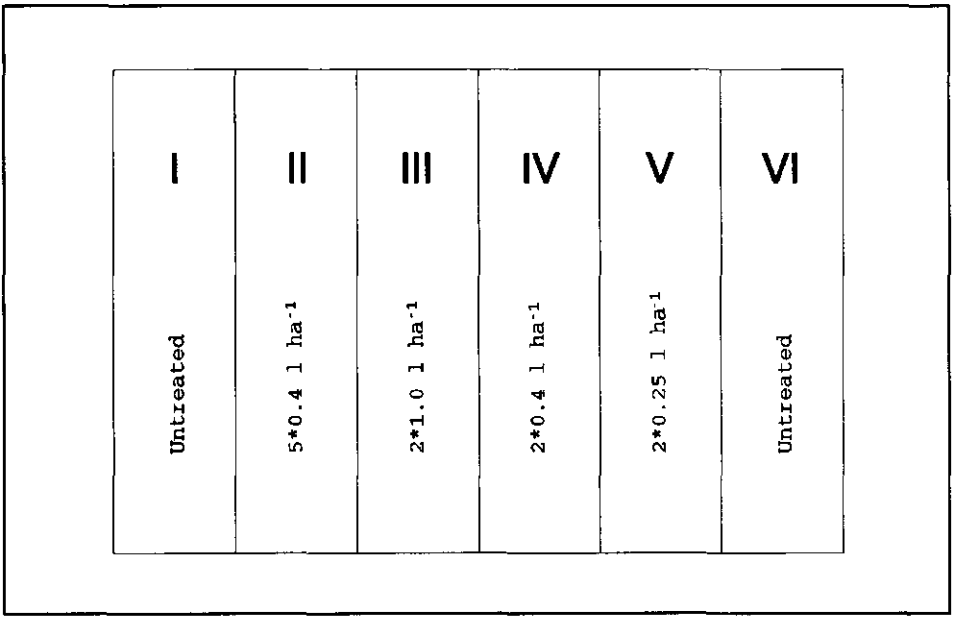

Figure 4.1. Design of the field trial to test the effect of reduced dosages of fenpropimorph on sensitivity of *Erysiphe graminis* f.sp. *tritici*.

Wheat powdery mildew development was assessed six times. From each plot a sample of 40 tillers was taken randomly and the mildew coverage on the upper three leaves was estimated. Wheat powdery mildew severity data were used to calculate the Area Under the Disease Progress Curve (AUDPC). AUDPC is the disease intensity integrated over time between two observation dates, using the trapezoidal integration method (Campbell and Madden, 1990). On the last observation date, not only mildew severity was assessed but also the severity of brown rust (*Puccinia recondita*) and *Septoria* spp. The first fungicide

treatment was applied when the disease coverage on the flag-3 leaf was on average 2%. The flag leaf was not yet present at this stage. The growth stages of the wheat present at the time points of applications and the number of days after planting are given in Table 4.1.

Table 4.1. Timing of applications of Corbel to control *Erysiphe graminis* f.sp. *tritici* in wheat.

Treatment[1]	Growth stage (DC)				
	31-32 (203)[2]	34 (212)	45 (226)	59 (242)	73 (252)
II	X	X	X	X	X
III		X		X	
IV, V		X			X

[1] II: 5x0.4 l ha^{-1}, III: 2x1.0 l ha^{-1}, IV: 2x0.4 l ha^{-1} and V: 2x0.25 l ha^{-1}.

[2] Between brackets: days after planting.

To assess the sensitivity of the wheat powdery mildew population to fenpropimorph, four bulk samples per plot were collected before and ten samples per plot after the fungicide treatments. Each bulk sample consisted of three mildewed leaves collected within a mutual distance of 10 m within a plot. Mildew samples were treated as described in the previous chapter. Foliar spray tests for the assessment of the sensitivity of the field isolates to fenpropimorph were carried out in a spray cabinet as described by Engels *et al.* (1996). One-wk-old seedlings were sprayed with a Corbel solution at a pressure of 0.8 bar for two minutes, till run off. The concentration range of fenpropimorph used for assessing the sensitivity of field isolates was 0, 5, 10 and 25 μg ml^{-1}, and for the reference wild-type isolate LH 0, 1, 5 and 10 μg ml^{-1}. In each foliar spray test, all concentrations were tested in duplicate. Three hours after spraying, seedlings were inoculated by shaking infected seedlings above sprayed seedlings. Inoculated seedlings were covered with a cellophane bag, grouped per concentration and incubated in a climate room at 18°C under fluorescent light during 16 hours a day and 80% RH.

After 10 days of incubation, wheat powdery mildew severity of seedlings was estimated by assessing powdery mildew coverage of ten randomly chosen leaves per pot. Average powdery mildew coverage of the ten leaves was calculated for each concentration. These averages were expressed as percentages of powdery mildew severity in the control treatments and then transferred to probits. Linear regression was performed to calculate EC_{50} and Q_{50} values. The Q_{50} value is the degree of resistance and was calculated by

dividing the EC_{50} value of a field isolate by the EC_{50} value of the reference isolate LH in the same test. Sensitivity data were statistically analysed by comparing within a plot the Q_{50} values of isolates collected before and after fenpropimorph treatments. A t-test was used to detect shifts in sensitivity within a plot. All calculations were carried out with the software package "Lotus 123". The statistical analysis was carried out with "Genstat".

Results

Powdery mildew development

At the start of the treatments (DC 31-32), no significant differences in mildew infestation between the plots were found. After the treatments, the average percentage of mildew coverage of flag (DC 77), flag-1 (DC 77) and flag-2 (DC 73) leaves was 2, 8 and 17%, respectively. The AUDPC of the flag leaf was highest in untreated plots and lowest in plots treated with 2 l of the fungicide (Table 4.2).

Table 4.2. Wheat powdery mildew severity, transformed to AUDPC, on the flag, flag-1 and flag-2 leaves of wheat in plots of disease control experiments carried out in 1995.

Treatment	AUDPC (%.days)[1]		
	Flag	Flag-1	Flag-2
Untreated (plot I)	21.8	151	284[2]
Untreated (plot VI)	19.7	114	228[2]
Corbel (5x0.4 l ha^{-1})	3.5	14.5	51.4
Corbel (2x1.0 l ha^{-1})	7.3	19.1	56.1
Corbel (2x0.4 l ha^{-1})	11.8	39.2	114
Corbel (2x0.25 l ha^{-1})	8.0	45.2	150

[1] Time range for the flag, flag-1 and flag-2 leaves was 31, 54 and 54 days, respectively.

[2] Time range for untreated flag-2 leaves was 40 days since these leaves were dead at the last observation date.

Development of other diseases

At DC 77-83, *P. recondita* was present in untreated plots and plots treated with reduced dosages of Corbel. The average leaf coverage with *P. recondita* on the upper two leaves was 2.2% in the untreated plots, 0.7% in the plot treated with 2x0.4 l ha^{-1} and 1.2% in the

plot treated with 2x0.25 l ha^{-1}. *P. recondita* was not present in the plots which received 2 l of the fungicide per hectare. *Septoria* was hardly present in any of the plots.

Sensitivity to fenpropimorph

At the start of the experiments, the mean Q_{50} value of isolates collected in the experimental field was 7.3, indicating that the mildew population had a decreased sensitivity to fenpropimorph as compared to the wild-type reference isolate LH. The Q_{50} value did not differ significantly between the plots (P=0.90). During the growing season, the mean Q_{50} value of isolates from untreated plots did not change significantly (Table 4.3). Application of 2x0.4 l ha^{-1} did also not lead to a significant shift in Q_{50} values. In contrast, a significant increase in Q_{50} value was found for isolates from plots treated with normal applications (2x1.0 l ha^{-1}), split applications (5x0.4 l ha^{-1}) and the lowest dosage used (2x0.25 l ha^{-1}).

Table 4.3. Q_{50} values of fenpropimorph of isolates of *Erysiphe graminis* f.sp. *tritici* collected from untreated plots and plots treated with normal and reduced dosages of Corbel before (May) and after (July) treatments were applied in the field trial in 1995.

Month	Treatment[1]					
	I	II	III	IV	V	VI
May	7.8 a	7.2 a	6.5 a	7.7 a	7.1 a	7.3 a
July[2]	6.8 a	11.3 b	8.8 b	9.2 a	11.8 b	7.4 a
T-value[3]	1.3	3.8	3.0	1.7	3.0	0.0

[1] I and VI = Untreated, II = Corbel 5x0.4 l ha^{-1}, III = Corbel 2x1.0 l ha^{-1}, IV = Corbel 2x0.4 l ha^{-1} and V = Corbel 2x0.25 l ha^{-1}.

[2] Standard deviations of the Q_{50} values of isolates collected in July in plot I to VI were 0.9, 1.9, 1.4, 1.5, 3.0 and 1.3, respectively.

[3] Critical T-value=2.2; P=0.05

Discussion

The data described in this chapter should be regarded as preliminary since they only relate to one field trial, carried out in 1995. Conformation of the results discussed will need another two years of similar trials. The preliminary results obtained demonstrate that the sensitivity of isolates from the plots treated twice with 0.25 l Corbel ha^{-1} decreased significantly. In contrast, sensitivity of the mildew isolates from plots treated twice with 0.4

l Corbel ha^{-1} and control plots remained the same. Mildew severity in the plot treated with 2x0.25 l ha^{-1} (plot V) was relatively high. Treatment with 2x0.4 l ha^{-1} (plot IV) resulted in a relatively better mildew control, especially on the flag-2 leaves. Therefore, the pronounced decrease in sensitivity of isolates from the plot treated with 2x0.25 l ha^{-1} may possibly be explained by a model for quantitative resistance development to fungicides in fungi, described by Georgopoulos and Skylakakis (1986) (Figure 4.2).

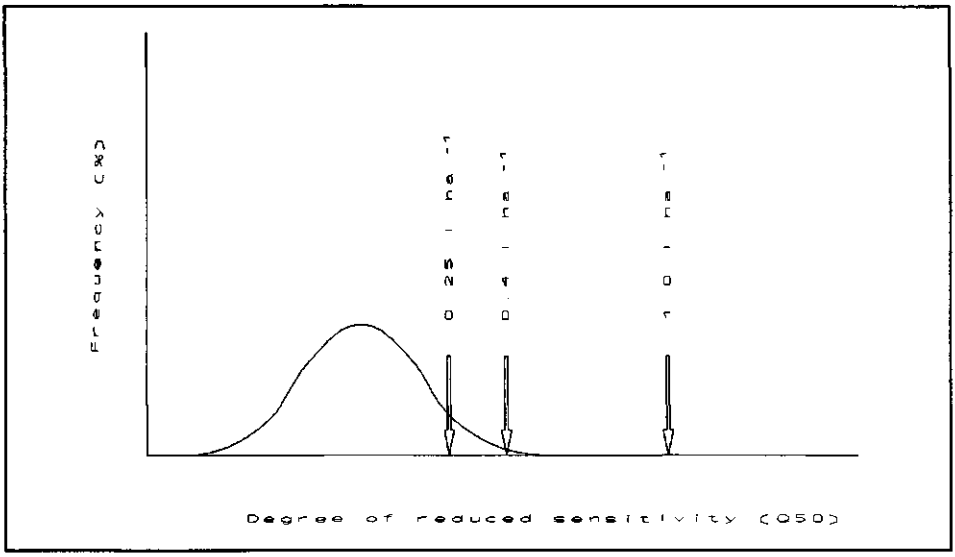

Figure 4.2. Model for the effect of reduced dosages on quantitative resistance development in fungi against fungicides. (Modified after Georgopoulos and Skylakakis, 1986).

According to this model, surviving mildew populations consist of escapes (proportion of population escaping fungicide contact) which have a normal sensitivity distribution, and of isolates with relatively high Q_{50} values. It is possible that, at the lowest dosage tested (0.25 l ha^{-1}), isolates with a relatively high Q_{50} value survive more frequently and multiply to a relatively high extent. This may cause the shift to a population with higher Q_{50} values as observed in plot V. An additional indication that this model is valid is the high standard deviation of the mean Q_{50} value of isolates from this plot indicating that isolates with relatively low and high Q_{50} values were found. Surviving mildew in the plot treated with 2x0.4 l ha^{-1} also consists of escapes and isolates with relatively high Q_{50} values. However, the proportion of the latter isolates is probably relatively low. Therefore, the ratio between escapes and isolates with a relatively high Q_{50} value will be high. In other words, the frequency of less-sensitive isolates in plot IV is relatively low. This may explain why these isolates were hardly detected.

In plot III, treated with normal applications of Corbel (2x1.0 l ha^{-1}), mildew was almost fully controlled. The model described above should also be applicable to quantitative resistance development in the mildew population of this plot and would suggest that resistance development in the surviving population is absent. However, a slight decrease in sensitivity was found. This is contradictory to results found in a three-year-experiment (1992-1994) in which no significant shifts in sensitivity were observed in plots treated with normal applications of Corbel (Engels *et al.*, 1996). Therefore, the change in Q_{50} values in plot III in 1995 can probably be explained by the relatively low Q_{50} value of the isolates sampled in this plot before fungicide application (Table 4.3). No significant change in sensitivity is found when the Q_{50} values of the mildew population from plot III are tentatively compared with the Q_{50} values of isolates from all other plots collected before the start of the treatments. This circumstantial evidence suggests that the observed change in sensitivity of isolates from plot III is not reliable.

Plot II, treated with split applications of Corbel (5x0.4 l ha^{-1}) caused a relatively high decrease in sensitivity of the population. This result has been reported before (Engels *et al.*, 1996) and can be explained by the assumption that with each spray the ratio between escapes and isolates with a high Q_{50} value will decrease. After five sprays the number of escapes will be very low and mainly less-sensitive isolates will survive. In other words, the frequency of less-sensitive isolates increases and these isolates become easier to detect. With split applications, timing probably plays a role in resistance development as well, since the fungus is exposed to the fungicide for a longer period of time.

The results of this one-yr-study suggest that the use of Corbel at dosages lower than recommended should be discouraged for two reasons. First of all, it will lead to enhanced selection of mildew populations less sensitive to fenpropimorph. Secondly, disease control of powdery mildews and other foliar pathogens may become insufficient.

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Chapter 5

Fitness of isolates of *Erysiphe graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph

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Abstract. Under crowded infection conditions, isolates of *Erysiphe graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph displayed a lower competitive ability than wild-type isolates. The lower competitive ability could not be ascribed to changes in germination, latent period, colony forming ability or production of conidia, but seems to be a complex change in phenotype that cannot be assessed easily by characterization of individual fitness components. Using mildew isolates containing fungicide-sensitivity and virulence markers to study survival, some isolates with reduced sensitivity to fenpropimorph survived the winter. The competitive ability of these isolates seemed to be slightly lower than that of the mildew population which migrated naturally into the experimental field. Results described indicate that in practice, in periods without selection pressure, the frequency of isolates with reduced sensitivity to this compound may decrease.

Introduction

Wheat powdery mildew, caused by the obligate pathogen *Erysiphe graminis* f.sp. *tritici*, is an economically important disease of wheat in Europe. The most common control strategies are the use of resistant cultivars and fungicides. One of the main problems of modern systemic fungicides is resistance development in target organisms. Fenpropimorph, a morpholine, is an important fungicide for the control of powdery mildews in cereals. The compound belongs to the class of sterol biosynthesis inhibitors (SBIs) and inhibits both sterol Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization (Baloch *et al.*, 1984; Mercer, 1991). This dual mode of action was predicted to reduce the risk of resistance developing in target pathogens, and, so far, only isolates of *E. graminis* f.sp. *tritici* with a low degree of decreased sensitivity to fenpropimorph have been found in western European countries (De Waard *et al.*, 1992; Lorenz *et al.*, 1992; Felsenstein, 1994; Readshaw and Heaney, 1994).

Development of resistance to fungicides is influenced by the fitness of populations to all the factors favourable to the production of progeny (Hartl, 1980). Dekker (1995) states that the fitness of fungicide-resistant strains seems to relate to the mechanism by which the pathogen becomes resistant to a fungicide. Some genetic changes conferring resistance are linked to lower fitness in the absence of the fungicide while others are not. If resistance is correlated with reduced fitness, it may slow down resistance development significantly (Chin, 1987; Milgroom *et al.*, 1989; Shaw, 1989).

To date, the study of fitness characteristics of fenpropimorph-resistant isolates has been limited to laboratory-resistant mutants of non-target pathogens (Nuninger-Ney *et al.*, 1989; Lasseron *et al.*, 1991). Fitness of naturally occurring isolates with a reduced sensitivity to morpholines sampled from field populations has been investigated in only a

limited number of studies (Walmsley *et al.*, 1979; Laws *et al.*, 1982). Experimental data on fitness of field isolates of *E. graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph are virtually absent. In view of this situation, it was decided to study a broad range of fitness components, such as germination, latent period, colony forming ability and production of conidia, since no consensus exists on the importance of the different components. In addition, the competitive ability and survival in winter was studied.

Materials and Methods

Plants. Wheat seedlings or leaf segments of cultivar 'Okapi' and 'Florida' were used in the experiments. Both cultivars do not carry powdery mildew resistance genes. Seeds were not treated with fungicides. Plants were grown in a greenhouse at 18°C for eight days.

Fungal isolates. Two wild-type isolates, LH and 67 (FS), and two isolates with reduced sensitivity to fenpropimorph, 3a and 16c (FR), of *E. graminis* f.sp. *tritici* were used. These four monospore isolates were isolated in the Netherlands: LH in 1982, 67 in 1983 and 3a and 16c in 1989. Since their isolation, the isolates have been maintained on 'Okapi' in a climate room at 10°C under fluorescent light (Philips TLMF 40W/35 RS; 7000 Lux) during 16 hours a day and 80% RH. In foliar spray tests, the EC_{50} value of fenpropimorph for the sensitive isolates LH and 67 is about 1.5 $\pm$ 0.4 μg ml^{-1}. The EC_{50} value for isolates 3a and 16c is about 12.0 $\pm$ 2.2 and 13.5 $\pm$ 2.8 μg ml^{-1}, respectively. Ten field isolates with EC_{50} values varying between 2.0 and 16.0 μg fenpropimorph ml^{-1} were used in competition experiments. These bulk isolates were collected in the Netherlands in 1994.

Germination. To determine the germination percentage of isolates, eight-day-old wheat seedlings were inoculated with mildew conidia ($\pm$ 25-50 conidia per cm^2) and incubated in a climate room at 18°C. After 24 hours, a collodion solution in ethanol-ether (1:1) was gently poured onto the leaves and left to dry. Collodion strips were taken from leaves and stained with cotton blue. Germination was observed with a binocular. Collodion strips from 15 different leaves were examined per isolate. The germination percentage was defined as the percentage conidia with germ tube(s) longer than 3 μm.

Latent period. To assess the latent period of isolates (the time from inoculation to the first appearance of a new conidium), 52 conidia per isolate were transferred to the adaxial site of leaf segments, using an eyelash attached to a cocktail stick. The segments were placed with this site upwards on 0.8% water agar amended with a concentration of 30 mg benzimidazole l^{-1} (Limpert *et al.*, 1988) in a Petri dish. Benzimidazole was added to delay the senescence of the leaf segments. One conidium was placed on each leaf segment. The

leaf segments were incubated in a climate room at 18°C. The end of the latent period was scored 112, 112.5, 113 and 113.5 hours after transfer of the conidia and then at intervals of four hours after these time points. This experiment was repeated four times.

Colony forming ability. The proportion of conidia which form a colony was measured to assess the colony forming ability (mean number of colonies which develop on untreated plants from a standard amount of inoculum) of each isolate. Leaf segments were placed on 0.8% water agar amended with 30 mg benzimidazole l^{-1} in a clear plastic box. 25 leaves were inoculated per isolate. Using a binocular, the number of conidia per cm^2 was estimated by counting the conidia on a glass slide, placed next to the leaf segments during inoculation. Boxes with infected leaf sections were kept in a climate room at 18°C. After seven days, the number of colonies per leaf segment was counted and the colony forming ability was calculated. The density of conidia allowed the development of 20 to 50 colonies per cm^2 leaf area.

Production of conidia. Production of conidia (number of conidia produced by a colony formed by one conidium) was assessed by inoculating wheat seedlings with conidia at low densities (± 5 conidia per cm^2) and these were incubated in a climate room at 18°C. For each isolate, a leaflet with five colonies was cut and transferred into an Eppendorf tube containing 1 ml of a 1% NaCl solution with 7.5 μl Tween 80 l^{-1}. This was duplicated at 139, 144.5, 150, 163, 168.5, 174 and 187 hours after inoculation. The conidia were dislodged from the leaves by gently shaking the Eppendorf tube. Three droplets (10 μl) were removed from each tube and placed on a Petri dish and the conidia in the droplets were counted under a microscope. The experiment was carried out twice.

Competitive ability. Six experiments were conducted to study the competitive ability of FS- and FR-isolates in mixed-isolate inoculations on wheat seedlings (Table 5.1). Mixtures of about 50% FS- and 50% FR-isolates were obtained by inoculating the isolates simultaneously onto eight-day-old wheat seedlings in 12.5 cm diameter pots. Seedlings with mixtures and the separate FS- and FR-monospore isolates were incubated in a climate room under fluorescent light (Philips TLMF 40W/35 RS; 7000 Lux) during 16 hours a day and 80% RH (standard conditions) and transferred every 7 or 14 days to unsprayed wheat seedlings. The experiments were carried out using inoculum levels (250 conidia per cm^2) that assured more than 80% mildew coverage on wheat seedlings.

The composition of the mixtures in the various experiments differed. In the first experiment, simple mixtures consisting of one FS- and one FR-isolate were used, whereas in following experiments mixtures of two FS- and two FR-isolates were studied under different experimental conditions. In the final experiment, complex mixtures consisting of ten field isolates were tested.

Table 5.1. Experimental design of competition experiments in climate rooms with isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph.

No	Mixture[1]	Temperature (°C)	Transfer[2] (days)	Duration[3] (weeks)	Repetitions
1	LH/3a, LH/16c, 67/3a, 67/16c	18	7	17	2
2	LH/67/3a/16c	15	14	15	4
3	LH/67/3a/16c	20	7	15	4
4	LH/67/3a/16c	18	7	15	4
5	LH/67/3a/16c	18	14	14	4
6	Field isolates (10)	18	14	15	4

[1] LH and 67: isolates with a wild-type sensitivity to fenpropimorph (FS-isolates); 3a and 16c: isolates with a reduced sensitivity to fenpropimorph (FR-isolates).

[2] Number of days between transfer of conidia to disease-free seedlings.

[3] Duration of experiment.

The composition of the wheat powdery mildew population with respect to fenpropimorph sensitivity in experiments 1 to 5 was assessed by transfer of 50 colonies to wheat seedlings sprayed with 11 μg fenpropimorph ml^{-1}. This concentration inhibits colony development of sensitive isolates while it allows growth of isolates with a reduced sensitivity. These sensitivity tests were done at the start of the experiment (week 0) and at regular intervals till 14 to 17 weeks after the start of the experiment. At the end of the experiments, EC_{50} values of fenpropimorph of monospore isolates and mixtures were assessed in foliar spray tests in experiment 1, 4, 5 and 6. Foliar spray tests were carried out as described by Engels *et al.* (1996).

Survival in winter. In order to study the effect of low temperatures on the survival of isolates 3a and 16c, field experiments were carried out in the winter of 1992/1993 and 1993/1994. Both winters were mild with an average temperature from November 1992 to April 1993 of 5.9°C with a minimum of -10.9°C and from November 1993 to April 1994 4.9°C with a minimum of -10.0°C.

In a field covered with grass, winter wheat (cv Okapi) was sown in autumn in eight clusters each consisting of four plots of one m^2 (Figure 5.1). A major concern was the possible risk of inter-plot interference, *e.g.* flux of conidia between clusters and influx from outside the field (Jenkyn *et al.*, 1979). To minimize the risk of flux of conidia between clusters as much as possible, non-inoculated plots were arranged between inoculated plots. Clusters inoculated with FS-isolates were located as far as possible from the clusters inoculated with FR-isolates.

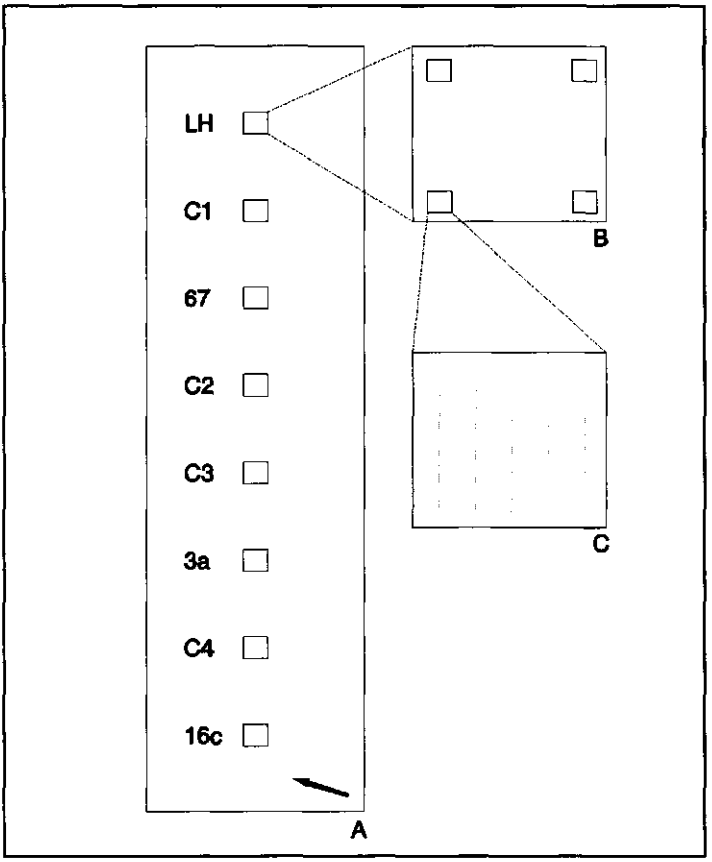

Figure 5.1. Experimental design of field experiments to study the overwintering of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph.
A: Plan of the field with the eight clusters of wheat plots. Plots in clusters LH, 67, 3a and 16c were inoculated with the corresponding isolate in autumn. C1...C4 are clusters which were not inoculated (controls). Distance between clusters was 25 m.
B: Plan of a cluster with four plots of 1 m^2. Distance between plots was 5 m.
C: One wheat plot of 1 m^2 with five rows of wheat plants.
The arrow indicates the predominant wind direction.

The arrangement of the clusters was chosen in such a way that the predominant wind direction was perpendicular with the length of the field. This was also done to avoid a flux of mildew between clusters. Each plot consisted of five plant rows of one meter. In the beginning of November, two pots with diseased seedlings were shaken above the middle row of a plot and planted in this row. In this way, four plots were inoculated with the FS-isolates LH and 67 and four with the FR-isolates 3a and 16c (Figure 5.1). The other 16

plots were non-inoculated controls. At the end of April, mildew coverage of 20 leaves per plot was assessed and one mildew sample per plot was collected to test the sensitivity to fenpropimorph. EC_{50} values were assessed in foliar spray tests.

In 1994, the virulence of the April isolates was assessed on wheat lines carrying resistance genes *Pm1* or *Pm4a*. These genes were chosen since FS-isolates LH and 67 are virulent on wheat lines carrying *Pm1*, *Pm2* and *Pm8*, and FR-isolates 3a and 16c on lines carrying *Pm2*, *Pm4a*, *Pm4b* and *Pm8*. To study the virulence, six monospore isolates per plot were collected and used to inoculate primary leaf segments (1.5 cm) of cultivar Florida (no resistant genes), the near-isogenic lines Axminster x 8 back crosses Chancellor (*Pm1*) and Khapli x 8 back crosses Chancellor (*Pm4a*) (Briggle, 1969). Leaf segments were placed in a clear plastic box containing 0.8% water agar with 30 mg benzimidazole l^{-1}. Two replicates of each line were used per isolate. Each isolate was inoculated onto the differential set of wheat lines in a miniature settling tower. Conidia were allowed to settle for 90 s. The density of conidia ranged between 250 and 400 conidia cm^{-1}. Boxes of infected leaf sections were kept in a climate room at 18°C.

After seven days the percentage of leaf coverage was estimated for each leaflet. A test was valid when coverage of the leaflet with no resistance genes was at least 20%. When the coverage of a leaflet with resistance genes was 50% or more compared to the most heavily attacked segment of the set *i.e.* the leaflet with no resistance genes, the monospore isolate was scored as virulent.

Data analysis. Mean EC_{50} values, mean germination percentages, mean number of colonies and mean production of conidia were calculated for each isolate and significance was determined by the Tukey test following Analysis of Variance (ANOVA). Mean latent period of each isolate was compared with Wilcoxon's rank correlation test. Survival in winter was tested by Student's *t*-test by comparing the EC_{50} value of the isolate which was inoculated in a cluster in November with the EC_{50} values of the isolates collected in the same cluster in April.

Results

Germination. Germination percentages of conidia from different isolates on leaves of wheat seedlings varied significantly (Table 5.2). Isolate LH had the highest percentage of germinated conidia with a germ tube longer than 3 μm. No significant difference in germination was found between FS-isolate 67 and the FR-isolates.

Table 5.2. Germination and colony forming ability of conidia of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph on fungicide-free wheat leaves.

Isolate	EC_{50} value ($\mu g\ ml^{-1}$)	Germination[1] (%)	Colony forming ability[2] (%)
LH	1.5 ± 0.4	73.3 b	44.4 b
67	1.5 ± 0.4	53.1 a	47.8 b
3a	12.0 ± 2.2	54.1 a	45.9 b
16c	13.5 ± 2.8	53.0 a	32.4 a
$Tukey_{0.05}$		18.5	8.2

[1] Percentage of conidia with germ tube(s) longer than 3 μm.

[2] Percentage of conidia able to form a colony.

Means followed by the same letter in the same column do not differ significantly, Tukey, $P < 0.05$.

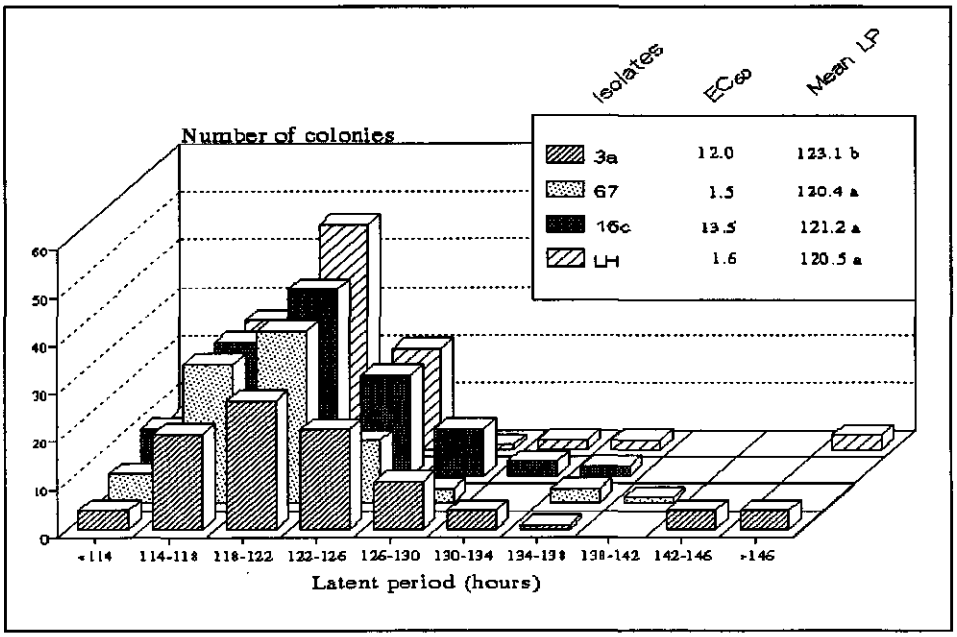

Figure 5.2. Latent period of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph; EC_{50} value in μg fenpropimorph ml^{-1}; mean LP is the arithmetic mean latent period; means followed by the same letter in this column do not differ significantly (5%), Wilcoxon's rank correlation test.

Latent period. The average latent period of FS-isolates LH and 67 was 120.5 and 120.4 h, respectively and did not differ significantly from FR-isolate 16c (121.1 h). The latent period of isolate 3a was significant longer (123.1 h) (Figure 5.2).

Colony forming ability. The ability to form colonies on seedlings did not differ for the FS-isolates LH and 67 and the FR-isolate 3a. FR-isolate 16c produced a significantly lower number of colonies (Table 5.2).

Production of conidia. FS-isolate LH significantly produced the highest number of conidia 187 hours after inoculation (Figure 5.3). However, the variability in this experiment was high (standard deviation $\geq$ 0.5 * mean) and no significant differences among isolates 67, 3a and 16c could be established.

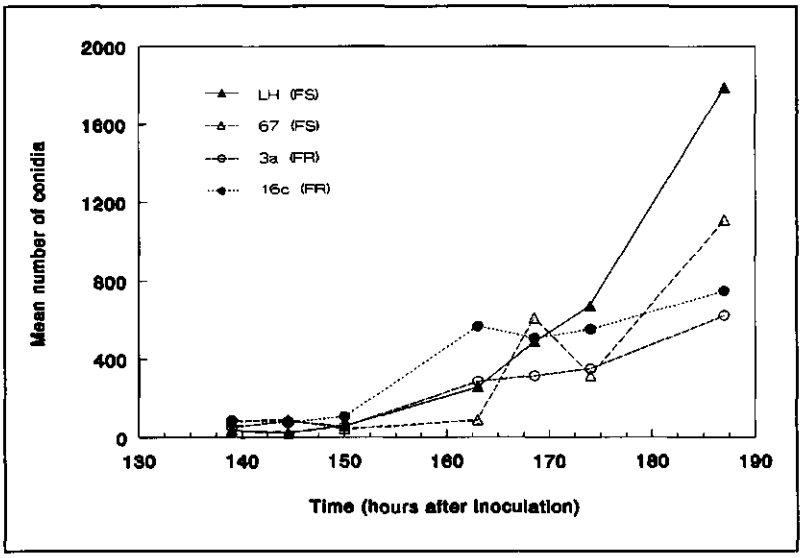

Figure 5.3. Production of conidia of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph.

Competitive ability. The initial proportion of FR-isolates in the competition experiments on wheat seedlings always varied between 50 and 75%. In all competition experiments, the frequency of FR-isolates in the mixtures gradually decreased during the course of the experiments reaching a level of 10-20% after 14-17 weeks (Figure 5.4a and 5.4b).

EC_{50} values of the mixtures after these weeks were similar to those of the FS-isolates LH and 67 (Table 5.3). The sensitivity of the FS- and FR- monospore isolates did not change significantly during the course of the experiments.

Table 5.3. EC_{50} values of monospore isolates and mixtures of *Erysiphe graminis* f.sp. *tritici* after 15-18 transfers on non-sprayed wheat seedlings in competition experiments at 18°C under climate room conditions as described in Table 5.1.

Isolate	Exp. 1	Exp. 4	Exp. 5
LH	1.5 a	2.0 a	2.0 a
67	1.9 a	1.7 a	1.6 a
3a	9.8 b	10.5 b	12.2 b
16c	10.9 b	13.0 b	14.3 b
LH/3a	1.7 a	-	-
LH/16c	2.0 a	-	-
67/3a	1.4 a	-	-
67/16c	1.5 a	-	-
LH/67/3a/16c	-	2.0 a	2.7 a
$Tukey_{0.05}$	4.2	3.3	3.4

Means followed by the same letter in the same column do not differ significantly, Tukey, $P < 0.05$.

EC_{50} values of field isolates used in competition experiment 6 (Table 5.1) were 2.0, 5.6, 8.9, 10.2, 10.8, 11.3, 12.4, 13.7, 14.1 and 16.0 μg fenpropimorph ml^{-1}. Hence, the major proportion of the inoculum originated from isolates with reduced sensitivity to fenpropimorph. After seven transfers to unsprayed wheat seedlings (15 weeks), the EC_{50} value of the mixtures was 1.4 $\pm$ 0.3 μg fenpropimorph ml^{-1}. FS- and FR- monospore isolates maintained during the experimental period under similar conditions retained their original sensitivity.

Survival in winter. In both winters tested (1992/1993 and 1993/1994), mildew development was higher in the inoculated plots than in the non-inoculated control plots (Table 5.4). Obviously, migration of conidia within the field and/or from outside sources took place. Isolates from all plots were collected in April of 1993 and 1994. The isolates collected in April from plots inoculated with a FS-isolate showed similar EC_{50} values as the corresponding FS-isolate used for inoculation in November in that plot. The same was true for FR-isolates after the winter of 1993/1994. After the winter of 1992/1993, the EC_{50} values of isolates collected in plots inoculated with FR-isolates was significantly lower than the corresponding FR-isolate (Table 5.4).

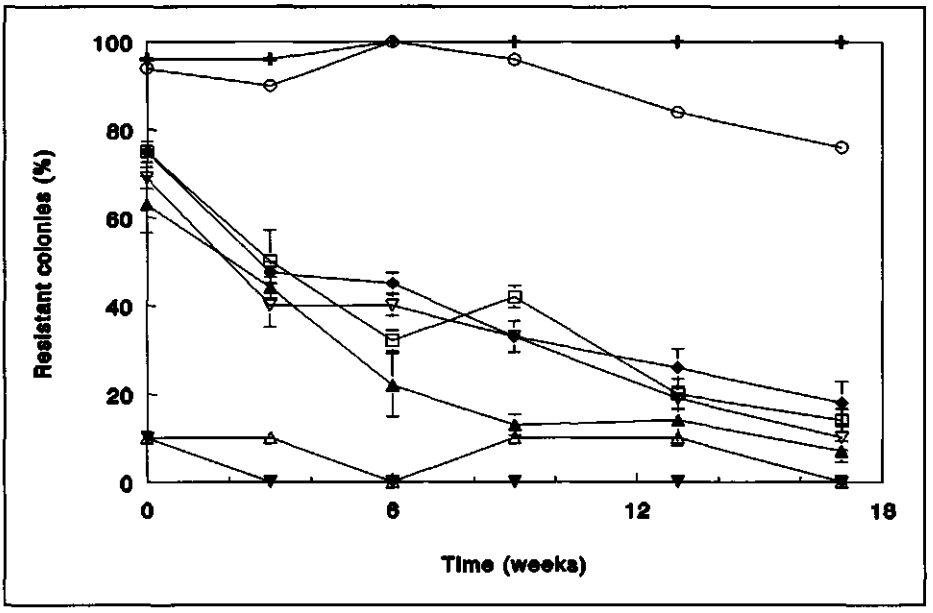

Figure 5.4a

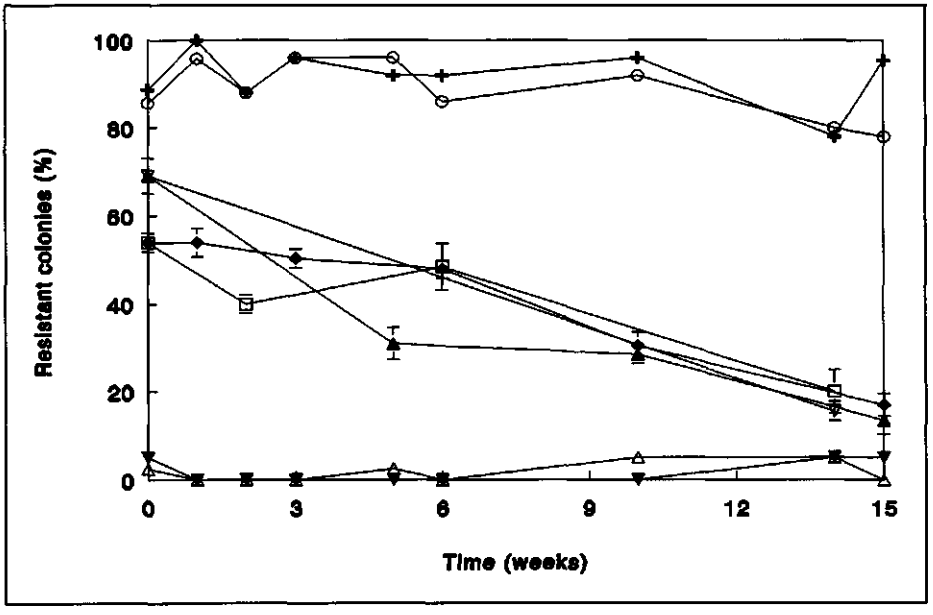

Figure 5.4b

Figure 5.4a. Changes in the composition of mixtures of FS- (LH and 67) and FR- (3a and 16c) isolates of *Erysiphe graminis* f.sp. *tritici* (LH/3a: ▲; LH/16c: ▽; 67/3a: □; 67/16c: ◆) grown on wheat seedlings in competition experiments in the absence of fenpropimorph as described in Table 5.1 (Exp. 1). Controls: percentage of resistant colonies in single isolates (LH: ▼; 67: △; 3a: o; 16c: +).
Figure 5.4b. Changes in the composition of mixtures of FS- (LH and 67) and FR- (3a and 16c) isolates of *Erysiphe graminis* f.sp. *tritici* (Exp. 2: □; Exp. 3: ◆; Exp. 4: ▲; Exp. 5: ▽) grown on wheat seedlings in competition experiments in the absence of fenpropimorph as described in Table 5.1. Controls: percentage of resistant colonies in single isolates (LH: ▼; 67: △; 3a: o; 16c: +).

Table 5.4. Mildew coverage and EC_{50} values of isolates of *Erysiphe graminis* f.sp. *tritici* from different clusters in field experiments as described in Figure 5.1 during the winter period of 1992/1993 and 1993/1994.

Cluster	Isolate	Coverage (%)		EC_{50} value (μg fenpropimorph ml^{-1})		
		1992/1993	1993/1994	Single isolate[1]	1992/1993	1993/1994
1	LH	6.7	6.6	1.5 a	1.7 a	2.7 a
2	-	1.9	1.7		5.3	5.9
3	67	10.3	5.0	1.5 a	1.6 a	3.2 a
4	-	3.0	1.6		4.9	7.5
5	-	2.5	1.5		4.0	8.5
6	3a	6.6	4.3	12.0 b	5.5 a	10.4 b
7	-	1.5	1.3		4.0	9.3
8	16c	9.7	6.4	13.5 b	5.6 a	11.3 b

[1] EC_{50} value of single isolates used to inoculate experimental plots.
Means followed by the same letter in the same row do not differ significantly, t-test, $P < 0.05$.

In order to check whether isolates collected in April 1994 would be identical to the isolates used for inoculation, the virulence pattern of the FS- and FR-isolates was used as a marker for identification (Table 5.5). It is expected that isolates from plots inoculated with FS-isolates (LH and 67) are virulent on wheat lines with *Pm1* (+) but not on *Pm4a* (-). The percentage of isolates from plots that match this assumption is 75% for LH and 83% for 67. In plots inoculated with FR-isolate 3a, 71% of the isolates showed the FR-virulence pattern. For isolates from plots inoculated with 16c, this percentage is 46%. However,

isolates with other virulence patterns were also found in these plots, indicating that an influx of conidia from outside sources had occurred. The EC_{50} values of isolates collected in the non-inoculated plots were similar to those of the isolates from the plots inoculated with a FR-isolate. The virulence pattern in these non-inoculated plots was diverse. More than 30% of the isolates had a virulence pattern different from both the FS- and FR-isolates.

Table 5.5. Virulence pattern of isolates of *Erysiphe graminis* f.sp. *tritici* collected from plots in the field experiment of 1993/1994 as described in Figure 5.1.

R-gene[1]		Cluster[2]							
Pm1	*Pm4a*	LH	C1	67	C2	C3	3a	C4	16c
+	-	75[3]	38	83	12	4	4	8	12
-	+	0	4	0	42	62	71	42	46
other		25	58	17	46	34	25	50	42

[1] FS-isolates LH and 67 grow on lines with *Pm1* and not on lines with *Pm4a*; Virulence pattern of FR-isolates 3a and 16c is vice versa.

[2] Cluster LH, 67, 3a and 16c were inoculated with isolate LH, 67, 3a and 16c, respectively. C1...C4 were not inoculated.

[3] Figures indicate percentage of isolates with corresponding virulence pattern.

Discussion

The methods described in this paper provide useful tools to characterize the comparative fitness of isolates of *E. graminis* f.sp. *tritici* with a reduced sensitivity to fenpropimorph. The methods are based on analysis of separate fitness components, competitive ability and survival in winter. The best method to assess fitness seems to be the competitive ability since results obtained in the various competition experiments were consistent. The method for assessing competitive ability is simple and even complex mixtures can be tested easily. Using this method, we found that the competitive ability of FS-isolates was higher compared to FR-isolates in the absence of fenpropimorph, regardless of the composition and complexity of the mixture. This conclusion is based on observations that the proportion of FR-isolates in mixtures decreased during the course of the experiments and that at the end of the experiments EC_{50} values of mixtures were similar to those of the wild-type isolates. Even mixtures composed of one fenpropimorph-sensitive and nine -less sensitive field isolates had at the end of the experiment an EC_{50} value comparable with those of the FS-isolates. This suggests that in periods without fenpropimorph application and with a high

mildew infection the frequency of FR-isolates may decrease in practice. Similar results were found by Buchenauer and Hellwald (1985) and Al-Mughrabi and Gray (1995) who state that the competitive ability of DMI-resistant isolates of *E. graminis* f.sp. *tritici* were inferior to those of sensitive ones in the absence of triadimefon.

Analysis of fitness components of individual isolates demonstrated that the reduced competitive ability cannot be ascribed to a specific reduction of either germination, latent period, colony forming ability or production of conidia. These fitness components vary for the two FS- and FR-isolates tested and no relation between single fitness components and sensitivity was observed. Hence, it seems as if various mechanisms account for the reduced competitive ability of FR-isolates. In order to gain more insight in the fitness of FS- and FR-isolates in genetically diverse field populations, a much larger number of isolates needs to be tested. This, however, is hardly feasible. In tests in which a more limited number of fitness components was studied, similar results were found for field isolates of *E. graminis* f.sp. *hordei* resistant to tridemorph (Laws *et al.*, 1982), and triadimefon (Butters *et al.*, 1984), DMI-resistant field isolates of *Sphaerotheca fuliginea* (Schepers, 1985) and *Pyrenophora teres* (Peever and Milgroom, 1994), and triadimefon-resistant field isolates of *E. graminis* f.sp. *tritici* (Buchenauer and Hellwald, 1985).

All experiments discussed above were carried out under controlled conditions. Therefore, two field experiments were carried out during the winters of 1992/1993 and 1993/1994 to gain insight in the overwintering of isolates. Influx of conidia into the experimental field occurred, since virulence patterns, different from the patterns of isolates used for inoculation, were found. This implies that not only the survival in winter was tested but, most likely, also the competitive ability of the inoculated isolates with the natural population. As a consequence, results are influenced both by survival capacity under adverse climate conditions and competition. Results of these experiments show that FS-isolates survived the winter and had a higher competitive ability than the mildew population originating from sources outside the field. This conclusion is based on the fact that the virulence pattern and EC_{50} values of isolates collected from plots inoculated with a FS-isolate were similar to the corresponding isolate which had been inoculated in that plot. The FR-population also seemed to be able to overwinter since isolates with a FR-virulence pattern were found after the winter. However, this FR-pattern was only detected in a proportion of the population. After the winter, the EC_{50} value of isolates collected in plots inoculated with FR-isolates was similar to the EC_{50} values of isolates collected in the non-inoculated plots. Hence, it appears that part of the FR-population was replaced by the natural population. The discrepancy between the field and climate room experiments can probably be ascribed to differences in infection density and number of generations.

Field studies monitoring the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph revealed that the sensitivity decreases during the spring under selection pressure of fenpropimorph (Engels *et al.*, 1996). However, in the absence of selection the sensitivity

seems to increase since isolates collected in spring before application of fenpropimorph have a relatively high sensitivity compared to the isolates tested in the foregoing summer. We propose that this phenomenon can be explained by the reduced comparative fitness of isolates with a reduced sensitivity to fenpropimorph as observed in the present studies.

In general, the risk for resistance development to fenpropimorph is regarded to be low since fenpropimorph has a dual mode of action (Baloch *et al.*, 1984; Mercer, 1991). In addition, resistance development in *E. graminis* f.sp. *tritici* to fenpropimorph proceeds stepwise and is probably linked to the continuous sensitivity distribution of the pathogen population and the polygenic nature of reduced sensitivity to fenpropimorph (De Waard, 1992; Engels *et al.*, 1996). Polygenic resistance to fenpropimorph has been observed in genetic studies with *Nectria haematococca* var. *cucurbitae* (Demakopoulou *et al.*, 1989) and *Pseudocercosporella herpotrichoides* (Hocart & McNaughton, 1994). Based on these characteristics and the decreased comparative fitness observed for isolates with a reduced sensitivity to fenpropimorph, we propose that resistance in *E. graminis* f.sp. *tritici* to fenpropimorph under field conditions is not likely, provided that proper resistant management is applied. The main elements of such a management are the use of recommended dosages at the full rate, alternation of fungicides with a different mode of action and the use of mixtures (Russell, 1995).

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Chapter 6

Sterol composition of isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph

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Pesticide Science **52** (1998) 258-262

Abstract. Isolates of *Erysiphe graminis* f.sp. *tritici* with wild-type or reduced sensitivity to fenpropimorph were similar in sterol composition, viz. ergosta-5,24(28)-dien-3β-ol ($\pm$90%) and episterol ($\pm$10%). Following treatment with fenpropimorph, the relative content of episterol increased in conidia of all isolates tested while that of ergosta-5,24(28)-dien-3β-ol decreased. These results suggest that fenpropimorph, under the test conditions used, does not inhibit activity of sterol Δ^{14}-reductase or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase but likely interferes with the final part of the demethyl sterol synthesis. However, modifications in this part of the pathway are probably not responsible for the decreased sensitivity of the pathogen to the fungicide.

Introduction

The obligate fungus *Erysiphe graminis* f.sp. *tritici*, the causal agent of wheat powdery mildew, is an important pathogen causing considerable yield reductions in wheat worldwide. For this reason, chemical industry has intensively searched for compounds active against this fungus and most cereal fungicides that have been developed are effective against powdery mildews. The most prominent group of systemic fungicides for mildew control are the sterol biosynthesis inhibitors (SBIs).

The morpholine fenpropimorph, a representative of the SBIs, has been successfully used in agriculture against powdery mildews of cereals, cereal rusts and leaf blotch of barley (Schulz and Scheinpflug, 1988). Morpholines are generally considered to have a dual mode of action. They inhibit both sterol Δ^{14}-reduction and $\Delta^{8}\rightarrow\Delta^{7}$-isomerization to different degrees, depending of the structure of the inhibitor and the fungal species under investigation (Mercer, 1991). Cell-free studies with yeast reveal that tridemorph specifically inhibits activity of sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase while fenpropimorph and fenpropidin strongly inhibit both sterol Δ^{14}-reductase and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase activity (Baloch and Mercer, 1987).

One of the main risks of modern fungicides is resistance development in the target organisms. This risk of resistance development also applies to fenpropimorph in the control of *E. graminis* f.sp. *tritici*. A small decrease in the sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph has been found in a number of western European countries (De Waard *et al.*, 1992; Lorenz *et al.*, 1992; Felsenstein, 1994; Readshaw and Heaney, 1994).

To date, the biochemical mechanism responsible for reduced sensitivity to fenpropimorph in powdery mildews has not been investigated. Elucidation of this mechanism is important for designing anti-resistance strategies and for development of fungicides active against fungicide resistant populations.

Previous studies have revealed that the sterol biosynthesis pathway of *E. graminis*

f.sp. *tritici* and f.sp. *hordei* is slightly different from other fungi (Loeffler *et al.*, 1984; Loeffler *et al.*, 1992; Senior *et al.*, 1995). Mildew fungi do not synthesize ergosterol but the related demethyl sterols ergosta-5,24(28)-dien-3β-ol and its Δ^7 analogue, ergosta-7,24(28)-dien-3β-ol (episterol) (Figure 6.1).

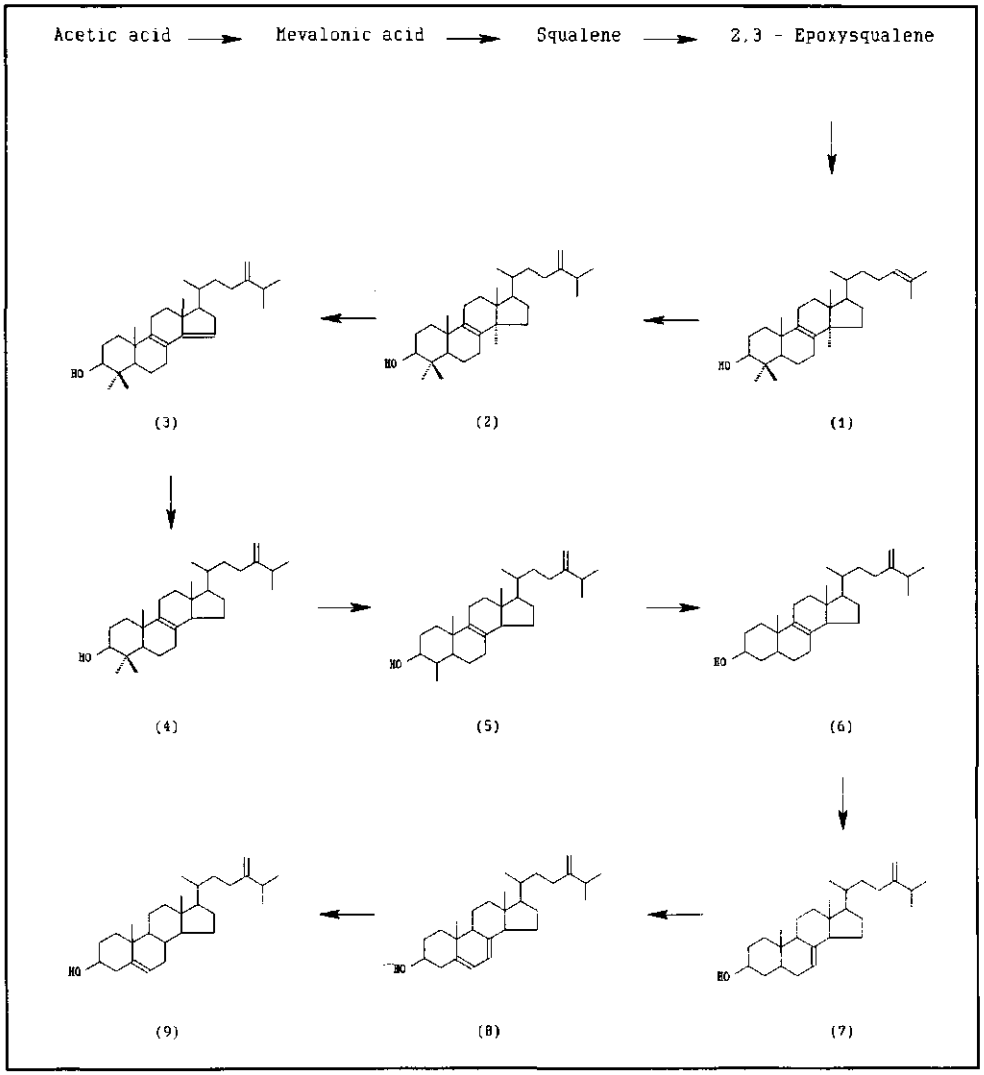

Figure 6.1. Sterol biosynthesis pathway of *Erysiphe graminis* f.sp. *tritici* (Senior, 1991). 1: lanosterol; 2: 24-methylene-dihydrolanosterol (eburicol); 3: 4,4-dimethylergosta-8,14,24(28)-trien-3β-ol; 4: 4,4-dimethylergosta-8,24(28)-dien-3β-ol; 5: 4-methylergosta-8,24(28)-dien-3β-ol; 6: ergosta-8,24(28)-dien-3β-ol (fecosterol); 7: ergosta-7,24(28)-dien-3β-ol (episterol); 8: ergosta-5,7,24(28)-trien-3β-ol; 9: ergosta-5,24(28)-dien-3β-ol.

The aim of this study is to investigate whether conidia from isolates of *E. graminis* f.sp. *tritici* with a reduced sensitivity to fenpropimorph differ in sterol composition from those of wild-type isolates. In addition, the sterol composition of conidia from these isolates, collected from fenpropimorph-treated wheat plants, was analyzed.

Abbreviations: f.sp. = forma specialis; SBI = sterol biosynthesis inhibitor; FS-isolate = isolate with a wild-type sensitivity to fenpropimorph; FR-isolate = isolate with a reduced sensitivity to fenpropimorph; EC_{50} value = effective concentration 50% (fungicide concentration at which the growth of the fungus is reduced by 50%); TMSi = trimethylsilyl; GC = gas chromatography; GC-MS = gas chromatography linked to mass spectrometry; MSD = mass selective detector; RR_t = relative retention time (cholesterol TMSi ether = 1.000); ANOVA = analysis of variance; LSD = least significant difference; *m/z* = mass/charge ratio; SC = sterol side chain; DMIs = sterol 14α-demethylation inhibitors.

Materials and Methods

Plants and fungal isolates. Wheat seedlings of cultivar 'Okapi' were used. This cultivar carries no genes for resistance to powdery mildew. Plants were grown in a greenhouse at 18°C for eight days.

Two wild-type isolates of *E. graminis* f.sp. *tritici*, LH and 67 (FS), and two isolates with reduced sensitivity to fenpropimorph, 3a and 16c (FR) were used. They were isolated in the Netherlands in 1982 (LH), 1983 (67), and 1989 (3a and 16c) and have been maintained on 'Okapi' in a climate room at 10°C under fluorescent light (Philips TLMF 40W/35 RS; 7000 Lux) during 16 hours a day and 80% RH. In foliar spray tests, the EC_{50} value of fenpropimorph for the FS-isolates LH and 67 is about 1.5 μg ml^{-1} and for the FR-isolates 3a and 16c about 12 and 13.5 μg ml^{-1}, respectively.

Preventive foliar spray test. Seedlings were sprayed with fenpropimorph prior to inoculation with *E. graminis* f.sp. *tritici*. Eight-day-old wheat seedlings ($\pm$ 25) in 0.2 l pots (6x6 cm) were sprayed to run off in a spray cabinet with 25 ml of a Corbel (a.i. fenpropimorph) solution for two minutes at a pressure of 0.8 bar. The concentrations used were 0, 1.5, 10 and 25 μg fenpropimorph ml^{-1}. After spraying, seedlings were grouped per concentration and 3 m apart in order to avoid cross-contamination from vapour phase activity of fenpropimorph, and left to dry for two hours.

Seedlings were inoculated with mildew by shaking infected seedlings, abundantly sporulating, above fenpropimorph-sprayed seedlings. In this way, the seedlings received a dense inoculum of powdery mildew conidia. FS-isolates were inoculated onto seedlings sprayed with 0 (control) and 1.5 μg fenpropimorph ml^{-1} ($\pm$ EC_{50} value) whereas FR-isolates were inoculated onto seedlings sprayed with 0, 10 ($\pm$ EC_{50} value) and 25 μg fenpropimorph

ml^{-1}. For each isolate and concentration, 30 pots with seedlings were inoculated. Immediately after inoculation, seedlings were covered with a cellophane bag, grouped per concentration and incubated in a climate room at 18°C.

After 10 days of incubation, conidia of the fungus were harvested using a cyclone-type spore collector connected to a small vacuum pump. The conidia were freeze-dried and stored at -80°C in the dark. Control and fenpropimorph treatments were carried out twice and three times, respectively.

Curative foliar spray test. Seedlings were first inoculated and five days later sprayed with fenpropimorph. Seedlings inoculated with FS-isolates were sprayed with 0 and 1.5 μg whereas seedlings inoculated with FR-isolates with 0 and 10 μg fenpropimorph ml^{-1}, respectively. Six days after inoculation, conidia were collected and treated in the same way as described for the preventive foliar spray test. All treatments were carried out in duplicate.

Sterol extraction. Sterol extraction was performed by alkaline saponification (Ellis *et al.*, 1991). Conidia (0.1 g dry weight) were acid-labilized by resuspending in 0.1 N HCl (2 ml), and heated at 100°C for 20 minutes in screw-capped tubes. Cholesterol was added as an internal standard. After heating, conidia were washed in distilled water and resuspended in 5 ml 4.3 M KOH in 60% methanol and 2 ml 40 mM pyrogallol in methanol and refluxed for 1-2 hours at 60-80°C (Parks *et al.*, 1985).

After cooling, the saponification mix was extracted by addition of heptane (3 ml). The phases were separated by centrifugation at 500 *g* for 1 min. The top heptane layer was collected and the remaining solution was re-extracted twice. Any emulsions occurring were dispersed by adding methanol (0.5 ml). Anhydrous sodium sulphate was added to remove any water in the sample. The combined heptane extracts were evaporated to dryness under nitrogen at 40°C and sterol residues were stored at -20°C.

GC and GC-MS. Sterol residues were dissolved in a mixture of anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane (7:2:1, by vol.) and left overnight at room temperature. The resulting trimethylsilyl (TMSi) sterol derivatives were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC was performed on a Hewlett-Packard 5890 gas chromatograph, fitted with a flame ionization detector, using a DB-17 fused silica capillary column (30 m x 0.241 mm i.d., film thickness: 0.25 μm). Analyses were conducted isothermically at an oven temperature of 260°C and an injection temperature of 300°C. The carrier gas was hydrogen with a flow rate of 1 ml min^{-1}. Peak areas were determined using a data processor. GC-MS was performed on a Hewlett-Packard 5870 series Mass Selective Detector (MSD) coupled to a

Hewlett-Packard 5890 gas chromatograph. TMSi derivatives were separated on a BP-17 fused silica capillary column (30 m x 0.25 mm i.d., film thickness: 0.25 μm) at 260°C. The carrier gas was helium and the pressure at the column inlet was 3.2 bar. An on-column injector was utilized and the column was coupled directly to the ion source of the MSD. The ion source temperature was 200°C, electron energy 70 eV and electron current 100 μA. The cholesterol internal standard was used to calculate relative retention times (RR_t) and total amount of sterols. Identification was based on comparison with references to published data on mass spectra and relative retention times of known sterols.

Data analysis. The ratio between the Δ^7 and Δ^5 sterols was analyzed using Analysis of Variance (ANOVA). Whenever significant differences were found ($P<0.05$), treatment means were separated using the Least Significant Difference (LSD) test.

Results

Identification of sterols. Total sterol content was about 4 $\mu g\ mg^{-1}$ dry weight of conidia. Both GC and GC-MS analysis revealed two peaks with a RR_t (cholesterol TMSi ether = 1.000) of 1.313 and 1.529, respectively. Mass spectra ($[M]^+$ (*m/z*)) and relative intensity (% of base peak) of the peak with a RR_t of 1.313 was 470 $[M]^+$ (31), 455 $[M\text{-}CH_3]^+$ (22), 387 (32), 386 $[M\text{-}C_6H_{12}]^+$ (100), 380 [M-TMSiOH]$^+$ (56), 371 (26), 365 $[M\text{-}CH_3\text{-}TMSiOH]^+$ (38), 344 [M-(SC+H)]$^+$ (23), 343 [M-(SC+2H)]$^+$ (55), 342 (31), 341 $[M\text{-}(TMSiOH{=}CHCH_2CH_3]^+$ (60), 340 (21), 296 (65), 281 (37), 257 (29), 255 [M-TMSiOH-SC]$^+$ (18), 253 [M-TMSiOH-(SC+2H)]$^+$ (44), 213 [M-TMSiOH-SC-42]$^+$ (26) and of the peak with a RR_t of 1.529 470 $[M]^+$ (8), 455 $[M\text{-}CH_3]^+$ (17), 386 $[M\text{-}C_6H_{12}]^+$ (34), 344 [M-(SC+H)]$^+$ (31), 343 [M-(SC+2H)]$^+$ (100), 255 [M-TMSiOH-SC]$^+$ (19), 253 [M-TMSiOH-(SC+2H)]$^+$ (13), 227 (18), 213 [M-TMSiOH-SC-42]$^+$ (21). These results and comparison with data from literature (Loeffler *et al.*, 1992) identified the compounds with RR_t 1.313 and 1.529 as ergosta-5,24(28)-dien-3β-ol and episterol, respectively. No differences in sterol composition were observed between isolates with wild-type or reduced sensitivity to fenpropimorph.

Sterol analysis of conidia from preventive foliar spray tests. Analysis of conidia from FS- and FR-isolates produced after a preventive treatment with fenpropimorph demonstrated that they had the same sterol composition as conidia present in control treatments, *i.e.* ergosta-5,24(28)-dien-3β-ol (89-91%) and episterol (9-11%) (Table 6.1). However, after fenpropimorph treatment, the content of episterol increased and of ergosta-5,24(28)-dien-3β-ol decreased. As a result, the ratio between Δ^7 and Δ^5 sterols increased as well. This increase was statistically significant for the FS-isolate LH.

Table 6.1. Effect of fenpropimorph on sterol composition of conidia from isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph in preventive foliar spray tests.

Isolate[1]	Fenpropimorph (μg ml^{-1})	Ergosta-5,24(28)-dien-3β-ol (%)	Episterol (%)	Ratio Δ^7:Δ^5 sterols	Sterol content (μg mg^{-1} dry wt.)
LH	0	89.0	11.0	0.12 a[2]	4.1
LH	1.5	83.2	16.8	0.20 b	3.8
67	0	88.3	11.7	0.13 a	3.8
67	1.5	85.4	14.6	0.17 a	3.9
3a	0	88.7	11.3	0.13 a	3.6
3a	10	86.9	13.1	0.15 a	4.3
3a	25	85.1	14.9	0.18 a	3.8
16c	0	88.0	12.0	0.14 a	4.2
16c	10	86.7	13.3	0.15 a	4.6
16c	25	85.6	14.4	0.17 a	5.0

[1] LH and 67: isolates with wild-type sensitivity to fenpropimorph; 3a and 16c: isolates with reduced sensitivity to fenpropimorph.

[2] Means followed by the same letter do not differ significantly, $P < 0.05$.

Sterol analysis of conidia from curative foliar spray tests. After curative treatment with fenpropimorph, conidia from FS- and FR-isolates also contained the two sterols, ergosta-5,24(28)-dien-3β-ol and episterol, already detected in conidia collected from isolates growing on untreated seedlings. The increase of episterol (%) after the curative treatment was obvious in all treatments (Table 6.2). As a result, the ratio between Δ^7 and Δ^5 sterols increased significantly in sterol extracts of all isolates tested.

Table 6.2. Effect of fenpropimorph on sterol composition of conidia from isolates of *Erysiphe graminis* f.sp. *tritici* differing in sensitivity to fenpropimorph in curative foliar spray tests.

Isolate[1]	Fenpropimorph (μg ml^{-1})	Ergosta-5,24(28)-dien-3β-ol (%)	Episterol (%)	Ratio Δ^7:Δ^5 sterols	Sterol content (μg mg^{-1} dry wt.)
LH	0	91.4	8.6	0.09 a[2]	4.0
LH	1.5	86.2	13.8	0.16 b	4.0
67	0	88.1	11.9	0.14 a	3.8
67	1.5	83.2	16.8	0.20 b	1.9
3a	0	90.2	9.8	0.11 a	3.8
3a	10	86.2	13.8	0.16 b	2.0
16c	0	87.9	12.1	0.14 a	4.2
16c	10	73.2	26.8	0.37 b	3.0

[1] LH and 67: isolates with a wild-type sensitivity to fenpropimorph; 3a and 16c: isolates with a reduced sensitivity to fenpropimorph.

[2] Means followed by the same letter do not differ significantly, $P < 0.05$.

Discussion

Sterols from conidia of FS- and FR-isolates of *E. graminis* f.sp. *tritici* were identified as ergosta-5,24(28)-dien-3β-ol and episterol and the proportion of the two sterols was $\pm$90% and $\pm$10%, respectively. Similar sterol compositions have been described for the same and related mildew pathogens, although in some species also traces of other sterols were identified (Loeffler *et al.*, 1984). In the absence of fenpropimorph, FS- and FR-isolates displayed no difference in sterol composition. This indicates that an altered sterol composition cannot explain the reduced sensitivity to fenpropimorph in FR-isolates. Similar results were found for isolates of *E. graminis* f.sp. *hordei* and f.sp. *tritici* differing in sensitivity to DMIs (Senior *et al.*, 1995).

In three out of four isolates studied in the curative foliar spray test with fenpropimorph, the sterol content was almost halved. In both the preventive and curative treatment no accumulation of $\Delta^{8,14}$-sterols such as ergosta-8,14,24(28)-trien-3β-ol and of Δ^8-

sterols such as fecosterol was observed, indicating that Δ^{14}-reductase and $\Delta^{8}\rightarrow\Delta^{7}$-isomerase, described as the target enzymes of morpholines in literature (Mercer, 1991), were not affected under the test conditions used. However, the proportional content of episterol increased. This resulted in a significant increase in the ratio between Δ^{7} and Δ^{5} sterols in conidia of all isolates tested, especially in the curative foliar spray tests. An increase of episterol was unexpected since this sterol is an intermediate in the final stage of the biosynthesis pathway (Figure 6.1). It is not clear whether the increase in episterol reflects a direct effect on the enzyme involved in the introduction of the 5(6)-double bond or, less likely, the reduction of the 7(8)-double bond. Nevertheless, results suggest that in *E. graminis* f.sp. *tritici*, fenpropimorph has a target site that affects the conversion of episterol in ergosta-5,24(28)-dien-3β-ol. Similar results have been reported for tridemorph and fenpropidin (Senior, 1991). Treatment of *E. graminis* f.sp. *hordei* and f.sp. *tritici* with sterol 14α-demethylation inhibitors (DMIs) led to an increase of eburicol, obtusifoliol and also episterol (Kwok and Loeffler, 1993; Senior *et al.*, 1995). These results suggest that DMIs inhibit sterol 14α-demethylation and, in addition, affect the conversion of episterol into ergosta-5,24(28)-dien-3β-ol. This may suggest that morpholines and DMIs have a common target site in the final part of the sterol biosynthesis pathway. It is possible that this target site also plays a role in the toxic activity of SBIs against powdery mildews. This target site is probably not involved in the mechanism of resistance to fenpropimorph, since no substantial difference in sterol composition of conidia from fenpropimorph-treated FS- and FR-isolates was observed.

Fenpropimorph treatment did not completely inhibit sterol synthesis since ergosta-5,24(28)-dien-3β-ol remained the major sterol in all experiments. This may be explained by the quality of the conidia analyzed. Probably, fungal biomass produced after the fungicide treatment partly originated from conidia that had escaped toxic effects of fenpropimorph, tested at its EC_{50} value. This assumption also explains why the sterol composition of conidia is stronger affected after curative than after preventive treatment. Application of fenpropimorph at lethal concentrations might have caused a more pronounced effect on sterol composition but would have hampered recovery of fungal biomass.

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Chapter 7

Genetic analysis of resistance to fenpropimorph in *Aspergillus niger*

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Abstract. Resistance to the morpholine-fungicide fenpropimorph was studied in *Aspergillus niger* and *Aspergillus nidulans*. Mass selection of conidia of *A. nidulans* on agar amended with the fungicide at different concentrations did not yield resistant mutants, even after UV-treatment of the conidia. In contrast, similar experiments with *A. niger* generated many fenpropimorph-resistant mutants. The mutants displayed cross-resistance to fenpropidin and showed, generally, wild-type sensitivity to the unrelated toxicants fenarimol and cycloheximide. Genetic analysis of fenpropimorph-resistance in *A. niger* was carried out by means of the parasexual cycle. In the mutants tested, two genes were involved in fenpropimorph-resistance which were located on linkage group II. Dominance tests showed that resistance to fenpropimorph in *A. niger* is recessive.

Introduction

Morpholine fungicides are sterol biosynthesis inhibitors (SBIs) with a dual mode of action. Their antifungal activity is based on inhibition of both sterol Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization in the synthesis of demethyl sterols such as ergosterol, a major component of fungal membranes (Mercer, 1991). A commercially important fungicide of this group is fenpropimorph. This compound is widely used for control of powdery mildews and rusts in cereals.

Development of resistance to fungicides with a specific mode of action is a major threat for effective chemical control. This danger also accounts for the fungicide fenpropimorph used to control *Erysiphe graminis* f.sp. *tritici* in wheat. Fenpropimorph was predicted to have a relatively low risk for resistance development because of its dual mode of action. The present sensitivity of cereal mildews confirms this prediction since, to date, no case of field resistance to fenpropimorph has been reported. In other fungi, laboratory mutants resistant to fenpropimorph were easily selected (Barug and Kerkenaar, 1984; Nuninger-Ney et al., 1989; Lasseron et al., 1991). This indicates that resistance development to fenpropimorph in cereal mildews remains a serious threat. Indeed, recent field trials indicated a small but significant decrease in the sensitivity to fenpropimorph within populations of *E. graminis* f.sp. *tritici* (Engels et al., 1996).

Little is known about the genetic background of fungal resistance to fenpropimorph and results from literature are contradictory. Polygenic resistance to fenpropimorph has been observed in *Nectria haematococca* var. *cucurbitae* (Demakopoulou et al., 1989; Lasseron et al., 1991), *Pyrenophora teres* (Peever and Milgroom, 1993) and *Pseudocercosporella herpotrichoides* (Hocart and McNaughton, 1994). However, in

Erysiphe graminis f.sp. *hordei* only one or a very limited number of genes seems to control the observed low degree of reduced sensitivity to fenpropimorph (Brown et al., 1996). To our knowledge, a similar genetic analysis of the decrease in sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph is not possible (Brown, pers. comm.). In view of this, we decided to analyze in vitro development of resistance to fenpropimorph in more detail in the model fungi *Aspergillus niger* and *A. nidulans*. These fungi are genetically well-characterized, which enables rapid genetic analysis of fungicide resistance (Van Tuyl, 1977). Mutations can relatively easy be assigned to linkage groups. In following studies such mutants will be used as tools to study the biochemical mechanism of resistance to morpholine fungicides.

The objectives of the present research were (a) to isolate fenpropimorph-resistant mutants of *A. niger* and *A. nidulans*, (b) to assess whether these mutants reveal cross-resistance to other compounds, (c) to determine whether fenpropimorph-resistance is mono- or polygenic and (d) to determine the linkage group on which the resistance gene(s) are located. It is expected that these type of studies will contribute to a better evaluation of the risk of resistance development in target plant pathogens.

Materials and Methods

Fungal strains and culturing conditions. Strains used in this research are listed in Table 7.1.

Table 7.1. Genotype of *Aspergillus niger* and *Aspergillus nidulans* strains used in experiments to study the genetics of resistance to fenpropimorph.

Strain	Linkage group							
	I	II	III	IV	V	VI	VII	VIII
A. niger								
N423					*nicA1*			
N522	*fwnA1*				*metB11*			
N890	*fwnA1*	*argH12*	*pyrA5*	*leuA1*	*pheA1*	*lysD25*	*oliC2*	*crnB12*
N891		*argH12*	*pyrA5*	*leuA1*	*pheA1*	*lysD25*	*oliC2*	*crnB12*
A. nidulans								
WG096	*yA2* *pabaA1*							
WG132	*pabaA1*	*wA3* *acrA1*		*pyroA4*				

Gene symbols: colour of conidia: *fwn*, fawn; *y*, yellow; *w*, white; deficiencies: *nic*, nicotinamide; *met*, methionine; *arg*, arginine; *pyr*, pyrimidine; *leu*, leucine; *phe*, phenylalanine; *lys*, lysine; *paba*, *p*-aminobenzoic acid; *pyro*, pyridoxine; resistances: *oli*, oligomycin; *crn*, chlorate; *acr*, acriflavin.

The strains were grown on minimal medium (MM), supplemented minimal medium (SM), or complete medium (CM) (pH 5.8) at 30°C (*A. niger*) and 37°C (*A. nidulans*) in the dark (Pontecorvo et al., 1953). All media contained traces of $CuSO_4$, $FeSO_4$, $MnCl_2$ and $ZnSO_4$. The carbon source (0.05 M glucose) was autoclaved separately and added after autoclaving. CM was also complemented with a vitamin solution after sterilization. Conidial suspensions were made in saline (distilled water with 0.8% NaCl)-Tween (0.05% Tween 80, w/v), vigorously shaken to break conidial chains and filtered through sterile glass wool to remove mycelial fragments.

Fungicides. Fenpropimorph was kindly provided by Ciba-Geigy, Roosendaal, the Netherlands. Stock solutions were made in dimethyl sulfoxide (DMSO) and added to molten growth medium at concentrations indicated. The amount of DMSO never exceeded 1‰ (v:v) in treated and control samples. Benomyl (Du Pont de Nemours & Co., Wilmington, DE, USA), cycloheximide (Merck, Amsterdam, The Netherlands), fenpropidin (Ciba-Geigy, Basel, Switzerland) and fenarimol (Dow Elanco, Benelux), supplied by their manufacturers, were also dissolved in DMSO, except for benomyl which was dissolved in acetone.

Sensitivity tests. Sensitivity of strains was determined in radial growth tests. Droplets (5 μl) of conidial suspensions (10^4 conidia ml^{-1}) were transferred to CM-agar plates supplemented with the test compound at different concentrations. Radial growth was assessed 3 days after incubation at 30°C in the dark. Colony diameters after treatments were calculated as percentages of control treatments (100%) and transformed to probits. Using the software-program 'Lotus 123', percentages were plotted against compound concentration on a logarithmic scale and regression analysis of inhibitor response data was performed. The inhibitory activity of compounds was expressed as EC_{50} values (concentration of the fungicide which inhibits radial mycelial growth by 50%). The degree of resistance of mutant strains is given by the Q_{50} value, which is the ratio between the EC_{50} value of resistant and corresponding wild-type strain.

In addition, sensitivity of strains was assessed in colony formation tests in which approximately 100 conidia were plated per Petri dish on CM-agar supplemented with the test compound at different concentrations. Plates were evaluated 3 to 4 days after incubation for colony formation. The average number of developing colonies was calculated for each concentration, expressed as percentages of control treatment and transferred to probits. Linear regression was carried out to calculate EC_{50} values (concentration of the fungicide which reduces colony formation by 50%). Both tests were carried out in three repetitions.

Selection of fenpropimorph-resistant mutants. Spontaneous fenpropimorph-resistant mutants were selected by inoculating conidia in sterile saline onto CM-agar (25 ml) supplemented

with fenpropimorph (0, 25, 50, 100, 200 μg ml^{-1}) in Petri dishes. Conidial densities ranged between 10 and 10^6 per dish. Induced mutants were obtained by ultraviolet irradiation (120 J m^{-2}) of conidial suspensions (10^7 conidia ml^{-1}). This resulted in about 70-80% survival of conidia. Irradiated conidia were plated on fenpropimorph-containing CM-agar, as described above, and incubated at 30°C (*A. niger*) and 37°C (*A. nidulans*) for 3 to 6 days. Developing colonies on CM-agar supplemented with fenpropimorph were transferred to CM-agar with fenpropimorph at 100 and 200 μg ml^{-1}. The sensitivity of the strains to fenpropimorph was established in radial growth tests (in triplicate).

Allocation of fenpropimorph-resistance genes to linkage groups in A. niger. Master strains (N890 and N891) of *A. niger* with markers on each linkage group, were used to assign putative fenpropimorph-resistance gene(s) (*fpm*) to linkage groups. This genetic analysis was carried out by means of the parasexual cycle which involves heterokaryon formation, followed by karyogamy to form a heterozygous diploid. Subsequently, haploid segregants obtained from such diploids were analysed for their genetic markers (Bos et al., 1988).

Heterokaryons were obtained by incubation of mixtures of conidia of the master strain N890 and mutant strains selected from N423 and, in a similar way, from master strain N891 and N522 or mutant strains selected from N522, in liquid CM (2 ml) at 30°C for one day. The resulting mycelium mat was washed in saline and fragments of the mat were transferred to MM-agar and incubated for 4 to 5 days. Heterokaryons were observed as vigorously growing sectors.

Heterozygous diploids were isolated from heterokaryons by mixing conidia from heterokaryons suspended in saline-Tween (10^4 to 10^7 conidia per dish) with MM (8 ml) in a Petri dish. After solidification, the agar was overlaid with MM ($\pm$ 30 ml) and incubated at 30°C for 5 to 7 days. Based on their prototrophic growth and their colour (grey/ black), conidia from putative diploid colonies were transferred to MM-agar to obtain monospore colonies. The diploidy of strains was checked by determining their conidial size with a Coulter counter (model ZF with channelyzer). The diploid strains were maintained at 4°C in the dark and served as starting material for genetic analysis and sensitivity tests.

Heterozygous diploids were haploidized by inoculating on CM master plates supplemented with benomyl (0.3 μg ml^{-1}). The plates were incubated for 7 days and haploid segregants were isolated on CM. Segregants were purified by transfer of conidia from one conidiophore to CM and tested for genetic markers and fenpropimorph-resistance. About 250 haploids per combination were tested to allocate fenpropimorph-resistance gene(s) to linkage groups.

Dominance tests. In order to establish whether fenpropimorph-resistance in *A. niger* is dominant, recessive or intermediate, expression of mutations was determined in heterozygous diploids by calculating the percentage dominance. This percentage dominance

was calculated as the ratio between the resistance factor of the heterozygous diploid and that of the resistant haploid multiplied by 100% (Van Tuyl, 1977). The resistance factor was defined as the ratio between the EC_{50} value of the resistant haploid (or heterozygous diploid) minus that of the sensitive haploid (or diploid) and the EC_{50} value of the sensitive haploid (or diploid) (Van Tuyl, 1977). Tests were carried out for heterozygous diploids N423K//N522, N423P//N522, N423Y//N522, N423//N522A, N423//N522T and N423//N522AI.

Allelism tests. Diploids were constructed of pairs of fenpropimorph mutants. If two independent fenpropimorph mutations are allelic (and recessive), resistance of the diploid will be at the same level as the individual haploids. If two mutations are non-allelic (and recessive), resistance of the diploid will be at the level of the non-mutant (control).

Data analysis. Mean EC_{50} and Q_{50} values were calculated for each strain and diploid and significance was determined by the Tukey test following Analysis of Variance (ANOVA).

Results

Selection of fenpropimorph-resistant mutants. Non-treated and UV-irradiated conidia of *A. niger* readily developed colonies on CM-agar supplemented with fenpropimorph at different concentrations. The frequency of developing colonies (putative mutants) was dependent on the strain, the number of plated conidia and the fenpropimorph-concentration (Table 7.2). All putative mutants, obtained independently, displayed a decreased level of sensitivity to fenpropimorph. Their Q_{50} values ranged from 2 to 8 with an average of 6.0 ± 1.6 indicating that variation in sensitivity to fenpropimorph was limited. The decreased sensitivity of the putative mutants was stable and, therefore, they were regarded as true fenpropimorph-resistant mutants. Six mutants were selected for further studies (Table 7.3).

In similar experiments with *A. nidulans* colonies did not develop on fenpropimorph-supplemented CM-agar. However, at sublethal concentrations, colonies showed sectors with a relatively fast growth. Isolates of these sectors had a wild-type sensitivity to fenpropimorph. Hence, no resistant mutants of *A. nidulans* were selected.

Table 7.2. Mutation frequency for resistance to fenpropimorph (fpm) in *Aspergillus niger* strains N423 and N522.

Strain	Fpm (μg ml^{-1})	Number of conidia plated	Mutation frequency[1] SP[2]	UV[2]
N423	100	$3.5 \cdot 10^5$	2.3	2.6
	100	$3.5 \cdot 10^6$	0.4	0.7
	200	$3.5 \cdot 10^5$	0.9	1.1
	200	$3.5 \cdot 10^6$	0.3	0.4
N522	100	$2.9 \cdot 10^5$	0	1.0
	100	$2.9 \cdot 10^6$	0.03	1.3
	200	$2.9 \cdot 10^5$	0	0.7
	200	$2.9 \cdot 10^6$	0.03	0.9

[1] Per 10^5 surviving conidia.

[2] SP and UV: spontaneous and UV-irradiated, respectively.

Table 7.3. Fenpropimorph-resistant mutants of *Aspergillus niger* used in genetic studies.

Mutant	SP/UV[1]	Q_{50}[2]	Test[3]
N423K	SP	4	dominance, allelism
N423P	SP	6	cross-resistance, allocation, dominance, allelism
N423Y	UV	5	dominance, allelism
N522A	SP	5	dominance, allelism
N522T	UV	4	cross-resistance, allocation, dominance, allelism
N522AI	UV	8	cross-resistance, allocation, dominance, allelism

[1] SP/UV: Mutants obtained spontaneously or after UV-treatment, respectively.

[2] Q_{50} value: Degree of reduced sensitivity to fenpropimorph; ratio between EC_{50} value of resistant mutant and corresponding wild-type isolate.

[3] Test to which the mutants were subjected.

Sensitivity tests. Sensitivity data show that mutants N423P, N522T and N522AI were less sensitive to fenpropimorph than the corresponding wild-type strains (Table 7.4).

Table 7.4. Sensitivity of *Aspergillus niger* strains to fenpropimorph (fpm), fenpropidin (fpd), fenarimol (fen) and cycloheximide (cyclo) in radial growth tests.

Strain[1]	EC_{50} value ($\mu g\ ml^{-1}$)			
	fpm	fpd	fen	cyclo
N423	7.4 a[2]	3.4 a	1.2 a	24.2 a
N522	8.3 a	4.2 a	1.1 a	44.6 b
N423P	43.2 b (5.8)[3]	14.4 bc (4.2)	2.3 ab (1.9)	35.1 ab (1.5)
N522T	34.9 b (4.2)	16.3 c (3.9)	2.2 ab (2.0)	45.9 b (1.0)
N522AI	64.5 c (7.8)	13.5 b (3.2)	3.2 b (2.9)	49.5 b (1.1)
$Tukey_{0.05}$	10.3	2.6	1.2	17.6

[1] N423 and N522: wild-types; N423P, N522T and N522AI: fenpropimorph-resistant mutants.

[2] Means followed by the same letter in the same column do not differ significantly, Tukey, $P < 0.05$.

[3] Q_{50} value: Degree of reduced sensitivity to fenpropimorph (ratio between EC_{50} value of resistant mutant and corresponding wild-type strain).

Data demonstrate that EC_{50} values of fenpropidin were higher for fenpropimorph-resistant mutants than for the wild-type strains. The strains tested hardly differed in sensitivity to fenarimol and cycloheximide. One strain (N522AI), however, with a relatively high EC_{50} value for fenpropimorph, also showed a significantly reduced sensitivity to fenarimol. N423-strains seemed to be more sensitive to cycloheximide than N522-strains. Similar data were obtained in experiments to determine EC_{50} values for colony formation (data not shown).

Dominance tests. The dominance of the mutants tested varied between 14 and 28% (Table 7.5) demonstrating that resistance to fenpropimorph in *A. niger* is recessive, although not completely.

Allelism tests. Heterozygous diploids selected from combinations between two resistant mutants were tested to study whether more than one gene is involved in fenpropimorph-resistance. One group of heterozygous diploids of two resistant mutants (N423K//N522AI, N423P// N522AI, N423Y//N522A and N423Y//N522T) had relatively low Q_{50} values (1.8 - 2.7) which did not differ but were significantly lower than those of the component strains (Table 7.6).

Table 7.5. Dominance relationship of mutations for *fpm*-resistance in *Aspergillus niger*.

Strain	Resistance factor[1]		Percentage dominance[2]
	Resistant haploid	Heterozygous diploid	
N423K	2.9	0.4	14
N423P	4.8	0.7	15
N423Y	3.8	0.6	16
N522A	4.3	1.2	28
N522T	3.2	0.9	28
N522AI	6.8	1.1	16

[1] The resistance factor was defined as the ratio between the EC_{50} value of the resistant haploid (or heterozygous diploid) minus that of the sensitive haploid (or diploid) and the EC_{50} value of the sensitive haploid (or diploid).
[2] The percentage dominance was calculated as the ratio between the resistance factor of the heterozygous diploid and that of the resistant haploid, multiplied by 100% (Van Tuyl, 1977).

Table 7.6. Q_{50} values[1] of fenpropimorph of diploids of *Aspergillus niger* in radial growth tests.

Diploid	Q_{50} value of component strains[2]	Q_{50} value of diploids[3]
N423//N522	1//1	1.0 a
N423K//N522A	4//5	4.6 c
N423K//N522T	4//4	5.5 c
N423K//N522AI	4//8	1.8 ab
N423P//N522A	6//5	5.1 c
N423P//N522T	6//4	5.3 c
N423P//N522AI	6//8	2.0 ab
N423Y//N522A	5//5	2.7 b
N423Y//N522T	5//4	1.9 ab
N423Y//N522AI	5//8	5.1 c
$Tukey_{0.05}$		1.1

[1] Q_{50} value: Degree of reduced sensitivity to fenpropimorph (ratio between EC_{50} value of resistant mutant and corresponding wild-type strain).
[2] Data from Table 7.3.
[3] Q_{50} value based on EC_{50} value of N423//N522 (7.6 μg fenpropimorph ml^{-1}). Means followed by the same letter do not differ significantly, Tukey, $P < 0.05$.

These Q_{50} values did also not differ from the Q_{50} value of the wild-type diploid except for N423Y//N522A. These results indicate that two, more or less recessive genes are involved in resistance to fenpropimorph in *A. niger*. Alternatively, but less likely, intragenic complementation might be present within one gene. Another group of the diploids (N423K//N522A, N423K//N522T, N423P//N522A, N423P//N522T and N423Y//N522AI) showed relatively high Q_{50} values (4.7 - 5.5) which significantly differed from the wild-type but were at the same level as those of the component strains. These results indicate that *fpm*-genes in each of these diploids were allelic. Hence, it can be deduced that *fpm*-genes can be assigned to two groups, one comprising the mutants N423K, N423P, N522A and N522T and the other the mutants N423Y and N522AI.

Allocation of fenpropimorph-resistance genes to linkage groups. Heterozygous diploids of different mutants with master strains were isolated at a frequency of about one in a million conidia of the heterokaryons. Diploids from reference strains (N423//N890 and N522// N891) were tested to optimize the experimental conditions. Then, allocation experiments were performed with diploids N423P//N890, N522T//N891 and N522AI//N891. N423P and N522T are representatives of one allelic group and N522AI is a representative of the other allelic group. In all three cases, the fenpropimorph resistance loci (*fpmA1* and *fpmA2* in N423P and N522T, respectively; *fpmB3* in N522AI) were clearly linked to the *argH* locus on linkage group II (Table 7.7), while showing free recombination with markers of the other linkage groups (results not shown). These results indicate that the *fpm* loci can be allocated to linkage group II in all mutants tested.

Table 7.7. Assignment of *fpm* genes of *Aspergillus niger* to linkage groups[1].

Diploid strain	Linked markers		Phenotype of segregants[2]				Recombinants (%)	Linkage group
	a	b	ab	ab^+	a^+b	a^+b^+		
N423P//N890	*fpmA1*	*argH12*	10	72	127	10	9.1	II
N522T//N891	*fpmA2*	*argH12*	1	38	195	24	9.7	II
N522AI//N891	*fpmB3*	*argH12*	7	31	137	10	9.2	II

[1] Only results of linked markers are shown. Markers of other linkage groups gave free recombinations (35-65% recombinants). The master strains N890 and N891 are described in Table 7.1.

[2] The numbers of segregants of each phenotype is given.

Discussion

Mutants of *A. niger* resistant to fenpropimorph could be readily selected upon mass selection of untreated and UV-irradiated conidia with the fungicide. Their degree of resistance (Q_{50} value: 2-8) was relatively low compared to fenpropimorph-resistance in laboratory mutants of *Ustilago maydis* (> 10) (Barug and Kerkenaar, 1984), *Monilinia fructicola* (5-18) (Nuninger-Ney et al., 1989) and *N. haematococca* (10-50) (Lasseron et al., 1991), but comparable with field isolates of the target pathogen *E. graminis* f.sp. *tritici* (≤ 10) (Felsenstein, 1994). The mutation frequencies for spontaneous and UV-induced mutants of strain N423 and UV-induced mutants of N522 were in the same order of magnitude as observed in *N. haematococca* (Lasseron et al., 1991) and about 15 times higher than in *M. fructicola* (Nuninger-Ney et al., 1989). In contrast, the mutation frequency of spontaneous mutants in strain N522 was much lower. Both strains originate from the same wild-type strain. Therefore, the strain specific response is hard to explain, but may relate to phenotypic (colour) or genetic (deficiencies) differences.

Fenpropimorph-resistant mutants of *A. nidulans* could not be detected. An explanation for this discrepancy is not obvious. It may be that the mutation frequency in *A. nidulans* for fenpropimorph-resistance is so low that mutants were not present in the population screened. This would imply that the mutation frequency for resistance in *A. nidulans*, even after induction with ultraviolet irradiation, is lower than 10^{-8}.

Resistance to fenpropimorph also conferred resistance towards fenpropidin, a fungicide with a similar mode of action. Comparable results were obtained for *E. graminis* f.sp. *hordei* (Brown and Evans, 1992) and f.sp. *tritici* (De Waard et al., 1992). These data indicate that rotational use of both fungicides is not likely to reduce the risk of resistance development in plant pathogens. Cross-resistance to the fungicide fenarimol (a sterol demethylase inhibitor) and the antibiotic cycloheximide (a protein synthesis inhibitor) was absent, suggesting that multidrug resistance does not operate as a mechanism of resistance.

Fenpropimorph-resistance in *A. niger* was recessive and *fpm*-genes are determined by loci located on linkage group II. This is the first time that such data are provided for fungal resistance to a morpholine fungicide. It appears that two genes are involved in fenpropimorph-resistance in the mutants of *A. niger* tested, since allelic and non-allelic interactions were apparent when heterozygous diploids of different pairs of resistant mutants were tested. Whether these genes are closely linked or at some distance on linkage group II is not easy to test in the imperfect fungus *A. niger*, because intrachromosomal recombination is rare. In *E. graminis* f.sp. *hordei*, only one or a few genes seem to control reduced sensitivity to fenpropimorph (Brown, 1996). By contrast, resistance to *N. haematococca* var. *cucurbitae* (Demakopoulou et al., 1989; Lasseron et al., 1991), *P. teres* (Peever and Milgroom, 1993) and *P. herpotrichoides* (Hocart and McNaughton, 1994) was polygenic. These data indicate that not one single genetic model for fenpropimorph-

resistance is appropriate for all fungi. This implies that fungicides cannot simply be classified in two major categories with respect to resistance development in plant pathogens, viz. monogenic or polygenic resistance, as is usually done (Georgopoulos and Skylakakis, 1986). Both mechanisms may operate in the same class of fungicides, viz. morpholines. A similar phenomenon has been described for resistance to fungicides which belong to the sterol 14α-demethylation inhibitors (DMIs). Genetic analysis of both field isolates and laboratory-induced mutants revealed that resistance to DMIs can be monogenic (Stanis and Jones, 1985; Kalamarakis et al., 1989) or polygenic (Van Tuyl, 1977; Hollomon et al., 1984).

We propose that model organisms may be useful to predict resistance development in target pathogens, since, in retrospect, the results described for *A. niger* corroborate the data described for resistance development in *E. graminis* f.sp. *hordei* to fenpropimorph (Brown, 1996). In addition, the fenpropimorph-resistant mutants described may serve to analyse the biochemical and genetic background of fenpropimorph-resistance. However, generalizations should not be made since results with the model organism *A. nidulans*, where resistance could not be induced, would have led to different conclusions. Therefore, studies aimed to assess the risk of resistance development to fungicides in plant pathogens should, by preference, be carried out with the target organism. If impossible, as in the case for morpholines in powdery mildews, various model organisms should be tested. A quantitative assessment of the resistance risk, as proposed by Gisi and Staehle (1988), remains difficult since the mutation frequency may differ between model organisms and target pathogens and even between different strains of the same fungus. Despite these restrictions, we suppose that genetic analysis of morpholine resistance in *A. niger* bears implications for proper resistance management of the fungicide in practice. Our results agree with a management strategy that involves rotational use of fungicides with a different mode of action and the use of mixtures (Russell, 1995).

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Chapter 8

Biochemical analysis of resistance to fenpropimorph in *Aspergillus niger*

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Abstract. The mechanism of resistance to the morpholine fungicides fenpropimorph and tridemorph and the morpholine-related fungicide fenpropidin was studied in cell-free sterol biosynthesis assays from fenpropimorph-sensitive and -resistant isolates of *Aspergillus niger*. Fecosterol accumulated in sensitive and resistant isolates to a similar extent, indicating no significant change in sensitivity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase, a target enzyme in sterol biosynthesis. A sterol, tentatively identified as ergosta-8,14,24(28)-trien-3β-ol, accumulated to a relatively high level in fenpropimorph-treated mycelium of the wild-type isolates only. This trienol is a derivative of 4,4-dimethyl-ergosta-8,14,24(28)-trien-3β-ol, the substrate of sterol Δ^{14}-reductase, another target enzyme of morpholines in sterol synthesis. These results suggest that reduced sensitivity of sterol Δ^{14}-reductase can play a role in morpholines resistance. Results with fenpropidin were comparable with those of fenpropimorph, although inhibitory effects on fecosterol accumulation were relatively weak, thus confirming that fenpropidin is a weaker inhibitor of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase than fenpropimorph. Sterol patterns in tridemorph-treated assays from sensitive and resistant isolates were similar. For both types of isolates, tridemorph treatment caused a marked accumulation of fecosterol only, indicating an exclusive inhibition of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase. Initial accumulation of fenpropimorph in mycelium of sensitive and resistant isolates was similar, suggesting that increased efflux as a mechanism of resistance is not relevant.

Introduction

The morpholine fungicides fenpropimorph and tridemorph and the morpholine-related piperidine fungicide fenpropidin are important compounds for control of powdery mildews and rusts on cereals in Europe. Their fungitoxicity is caused by inhibition of sterol biosynthesis which reduces the formation of ergosterol and related sterols, and enhances the accumulation of abnormal sterol intermediates. These effects ultimately result in malfunctioning of mycelial membranes and inhibition of growth. Most investigations on the mode of action of morpholines have been carried out with intact mycelium, cells or conidia. Results reveal that these fungicides have a dual mode of action. They inhibit, to different degrees, both sterol Δ^{14}-reductase and sterol $\Delta^8 \rightarrow \Delta^7$-isomerase depending on the fungicide and the fungal species (Mercer, 1991). To investigate the differential sensitivity of the enzymes to both groups of fungicides, studies on their inhibitory potency in cell-free assays are necessary. Such studies with yeast reveal that tridemorph specifically inhibits activity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase while fenpropimorph and fenpropidin strongly inhibit both sterol Δ^{14}-reductase and $\Delta^8 \rightarrow \Delta^7$-isomerase activity (Baloch and Mercer, 1987).

Development of resistance in target organisms to fungicides with a specific mode of action is a major threat for effective chemical control. This holds also for morpholine and morpholine-related fungicides. The most important morpholine fenpropimorph has a relatively low risk for resistance development due to its dual mode of action (Baloch and Mercer, 1987). The present sensitivity of cereal mildews confirms this prediction since, to date, 18 years after its introduction, no case of field resistance to fenpropimorph has been reported. However, fenpropimorph-resistant mutants of various fungi can be readily selected under laboratory conditions (Barug and Kerkenaar, 1984; Nuninger-Ney *et al.*, 1989; Lasseron-De Falandre *et al.*, 1991; Engels *et al.*, 1998), indicating that the potency to overcome the toxic activity of morpholines is present.

The mechanism of reduced sensitivity to morpholines is unknown. In a previous study, laboratory-generated mutants of *Aspergillus niger* with reduced sensitivity to fenpropimorph were characterized genetically (Engels *et al.*, 1998). These mutants were used to study two putative mechanisms of resistance which may operate in these isolates, viz. reduction in sensitivity of the target enzyme and increased efflux of the fungicide from mycelium. The first mechanism of resistance is common in many plant pathogens to different classes of fungicides (Henry, 1992). The latter mechanism mentioned results in a reduction of the intracellular concentration of a fungicide by which their target enzymes are less readily inhibited. This mechanism operates in resistance of laboratory-generated mutants of *Aspergillus nidulans* (De Waard and Van Nistelrooy, 1979), *Penicillium italicum* (De Waard and Van Nistelrooy, 1984) *Candida albicans* (Ryley *et al.*, 1984), *Monilia fructicola* (Ney, 1988), *Nectria haematococca* var. *cucurbitae* (Kalamarakis *et al.*, 1991), and *Botrytis cinerea* (Stehmann and De Waard, 1995) to sterol 14α-demethylation inhibitors (DMIs).

The present paper reports on investigations on the effects of inhibition of fenpropimorph, tridemorph and fenpropidin on sterol biosynthesis in cell-free assays from the filamentous fungus *Aspergillus niger*. To test whether modification of the target enzymes plays a role in resistance to fenpropimorph, the effect of the fungicides on sterol biosynthesis in cell-free extracts from genetically-characterized resistant isolates were investigated as well. As a potential mechanism of resistance, reduced accumulation was tested by comparing accumulation of fenpropimorph in sensitive and resistant isolates.

Materials and Methods

Isolates and culturing conditions. Isolates N423, N423P, N522 and N522AI of *A. niger* were used. Isolates N423 and N423P produce black conidia and are nicotinamide deficient, while N522 and N522AI produce fawn coloured spores and are methionine deficient. Isolates N423 and N522 have a wild-type sensitivity to fenpropimorph and N423P and

N522AI have a reduced sensitivity to this compound with resistance levels of 6 and 8, respectively (Engels *et al.*, 1998). Isolates were maintained on malt agar in bottles (25 ml) at 4°C in the dark. Conidia of *A. niger* were obtained from malt agar cultures (30 ml) in Petri dishes (diameter 9 cm) incubated at 30°C for 3-5 days in the dark. Flasks (2 l) with 1 l malt extract (17 g l^{-1} water) medium were inoculated with washed conidia collected from these plates (initial density $2x10^6$ conidia ml^{-1}). Liquid cultures for production of standard germlings to be used in cell-free assays were incubated in a rotary shaker (180 rpm) at 30°C for 11 (N423 and N522) or 12 h (N423P and N522AI) in the dark. Incubation time for production of germlings to be used in accumulation experiments was 1 h less.

Chemicals. [^{14}C]fenpropimorph, fecosterol and ignosterol were generously provided by BASF AG (Limburgerhof, Germany). Fenpropimorph, propiconazole and captan, (Ciba-Geigy, Roosendaal, the Netherlands), fenpropidin (Ciba-Geigy, Basel, Switzerland), tridemorph (BASF AG, Limburgerhof, Germany) and fluazinam (ISK Biosciences Co., Mentor, Ohio, USA) were supplied by their manufacturers and used as pure active ingredients. All compounds were dissolved in 1000x concentrated solutions in dimethyl sulfoxide (DMSO). [2-^{14}C]mevalonate, dibenzethylenediamine salt in ethanol (sp. act. 1.9 GBq $mmol^{-1}$) was purchased from Amersham International, UK. Nicotinamide-adenine dinucleotide (NAD^+), nicotinamide-adenine dinucleotide phosphate ($NADP^+$, NADPH), adenosine 5'-triphosphate (ATP), glucose-6-phosphate, L-methionine, oxytetracycline, ergosterol, lanosterol, eburicol and squalene were purchased from Sigma (St. Louis, Mo., USA). Carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) and cetylpyridinium bromide (CPB) were purchased from Sigma (St. Louis, Mo., USA).

Preparation of cell-free extracts. Cell-free extracts were prepared according to a modified method described by Guan *et al.* (1992). Standard germling suspensions of *A. niger* were made by filtering liquid cultures (harvest about 4 g wet weight l^{-1} medium) over a 0.2 mm pore sieve to remove clusters of mycelium. Filtrates containing the germlings were collected on a 0.05 mm pore stainless steel sieve. Germlings were washed extensively with running cold tap water followed by 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 (22 mg dry weight) ml^{-1} buffer. Subsequent steps were carried out at 0 - 4°C. A 32-ml Bead-Beater vessel (Biospec Products, Bartlesville, Ok., USA) containing glass beads (15 g; diameter 0.5 mm) was completely filled with germling suspension and 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 90 V. Resulting homogenates were 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 debris. Resulting supernatants were used immediately in sterol biosynthesis assays. The absence of any intact cells was verified by microscopic observation and mycelial growth tests on malt agar.

Sterol biosynthesis assays. Standard sterol biosynthesis assays were carried out according to a modified method of Ballard *et al.* (1990). 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 solutions were combined (normally 20 ml), adjusted to pH 7.3 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) or DMSO-solutions of the fungicide tested (1 μl) were added to the incubation mixtures. Sterol biosynthesis was started by addition of [2-^{14}C]mevalonate (5 μl, 18.5 kBq, 8.5 nmol). Mixtures were incubated in a reciprocal water bath shaker (80 strokes min^{-1}) at 36°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 and water (90 + 10 by volume).

Saponification, sterol extraction and analysis. Saponification of incubation mixtures, extraction and analysis of the non-saponifiable lipids (NSLs) by thin layer chromatography (TLC; silica gel plate F_{254}, Merck, Darmstadt, Germany) and radio-high performance liquid chromatography (radio-HPLC) were carried out as described by Guan *et al.* (1992). Incorporation of [2-^{14}C]mevalonate into sterols was determined by counting the extracted samples for radioactivity in a liquid scintillation spectrophotometer (Beckman LS 5800). Samples were eluted with 95% methanol (HPLC grade, J.T. Baker B.V., Deventer, the Netherlands) by reverse phase HPLC 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 (Henry, 1992). Eburicol, ergosterol, fecosterol, ignosterol and lanosterol were identified by radio-HPLC with authentic standards. All experiments were carried out in duplicate. The ratio of sterols formed (%) in assays was calculated after integration of the area of the peaks 3-8 observed in radio-HPLC analysis.

Accumulation of fenpropimorph. Experiments were carried out according to the method described by De Waard and Van Nistelrooy (1979). Standard germlings, collected on a 0.05 mm pore stainless sieve as described before, were washed three times by resuspending 1.0 g wet mycelium in 23.4 mM potassium phosphate buffer (pH 7.0, 50 ml) containing 0.1 mM $CaCl_2$ and 10 g D-glucose l^{-1}. These standard mycelium suspensions (70 ml in 300 ml

Erlenmeyer flasks) were shaken on a reciprocal shaker at 30°C for 20 min. Experiments were initiated by adding [^{14}C]fenpropimorph (30 or 100 μM initial external concentration; initial external activity 0.1 kBq ml^{-1}) from a 100x concentrated stock solution in methanol. Accumulation of fenpropimorph was determined in germlings collected from 5 ml samples taken at intervals. Samples were washed 5 times with 5 ml buffer on a Millipore sampling manifold apparatus within 30 s. 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 (Beckman 2800 LS).

Results

Identification of sterols formed in sterol biosynthesis assays. Incorporation of [2-^{14}C]mevalonate into NSLs of sensitive and fenpropimorph-resistant isolates accounted for 30.3 ± 5.2% and 30.1 ± 4.9% of the total radiolabel added, respectively. Separation of the NSLs by TLC resulted in a typical pattern of bands as illustrated by the autoradiogram in Figure 8.1.

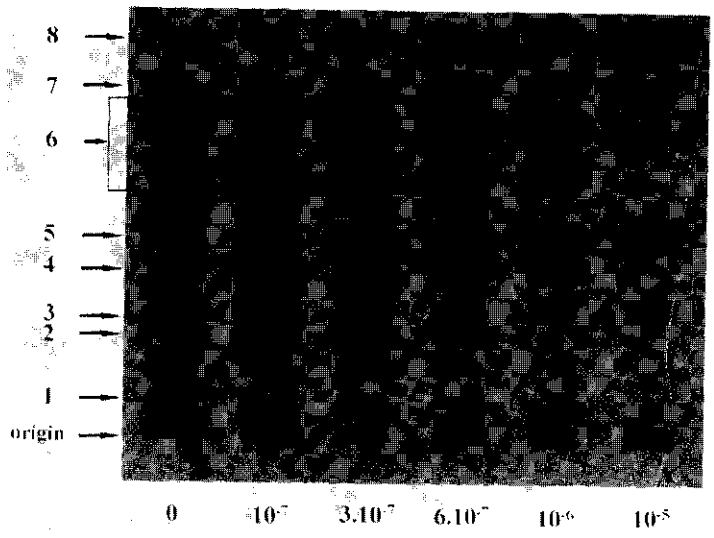


Figure 8.1. Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C] mevalonate in cell-free assays from *Aspergillus niger* (N423P) in the absence and presence of fenpropimorph. Bands 2 and 3 contain C4-demethyl sterols, and bands 4, 5, 7 and 8 C4-monomethyl sterols, C4,4-dimethyl sterols, 2,3-oxidosqualene (tentative) and squalene, respectively. The identity of compounds in bands 1 and 6 is not known.

According to literature data (Ryder *et al.*, 1984; Stehmann and De Waard, 1995), compounds in bands 7 and 8 are probably 2,3-oxidosqualene and squalene, respectively. By comparison with literature data (Ballard *et al.*, 1990; Stehmann and De Waard, 1995) and radio-HPLC analysis of compounds extracted from bands on TLC plates, sterols in bands 2 and 3 were identified as C4-demethyl sterols and in bands 4 and 5 as C4-monomethyl and C4,4-dimethyl sterols, respectively. The identity of compounds in bands 1 and 6 is not known.

NSLs analysed by radio-HPLC revealed eight peaks with retention times of 8.6 (peak 1), 9.9 (peak 2), 14.1 (peak 3), 16.3 (peak 4), 17.0 (peak 5), 19.1 (peak 6), 22.3 (peak 7) and 25.0 min (peak 8) (Figure 8.2). Peaks 4, 5, 7 and 8 had retention times identical to those of authentic ergosterol, fecosterol, lanosterol and eburicol, respectively. This identity was confirmed by UV detection at 210, 250 and 280 nm. Authentic ignosterol had a retention time of 17.2 min and hence, did not account for the identity of peaks 1, 2, 3 or 6. Identification of peaks 1 and 2 was not pursued. The putative identification of peaks 3 (compound A) and 6 (compound B) is described in the discussion.

Effects of fungicides in assays from the wild-type isolate. The main sterol in the wild-type isolate N423 was ergosterol. With increasing concentrations of the fungicides tested, the ergosterol content decreased (Table 8.1). At relatively low concentrations of fenpropimorph (10^{-7} to 10^{-6} M), fecosterol and to a lesser extent a new sterol, compound A, accumulated. With increasing concentrations (10^{-6} to 10^{-5} M) fecosterol accumulation decreased and compound A increased. The highest concentrations tested also affected the biosynthesis of lanosterol and eburicol.

Treatment with relatively low concentrations of fenpropidin (10^{-7} to 10^{-6} M) resulted in an increase of both fecosterol and compound A. With increasing concentrations, biosynthesis of fecosterol only slightly decreased while compound A strongly increased.

Treatment with tridemorph also revealed an enhanced accumulation of fecosterol, but the effect on compound A was less pronounced. At the highest concentration tested (10^{-4} M), a new sterol, compound B, with a retention time of 19.1 min was detected. Analysis of TLC bands indicated that this sterol might be a C4-demethyl sterol.

Propiconazole, an inhibitor of sterol 14α-demethylase activity, inhibited biosynthesis of ergosterol and caused accumulation of eburicol (Table 8.1).

Figure 8.2. Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from cell-free synthesis assays from fenpropimorph-sensitive isolate N423 (A en B) and fenpropimorph-resistant isolate N423P (C en D) of *Aspergillus niger*. Control-treatments (A and C); fenpropimorph-treatment B (10^{-6} M) and D (10^{-5} M). Identity of peaks 4, 5, 7 and 8: ergosterol, fecosterol, lanosterol and eburicol, respectively. Identity of peaks 1, 2, 3 and 6: not known.

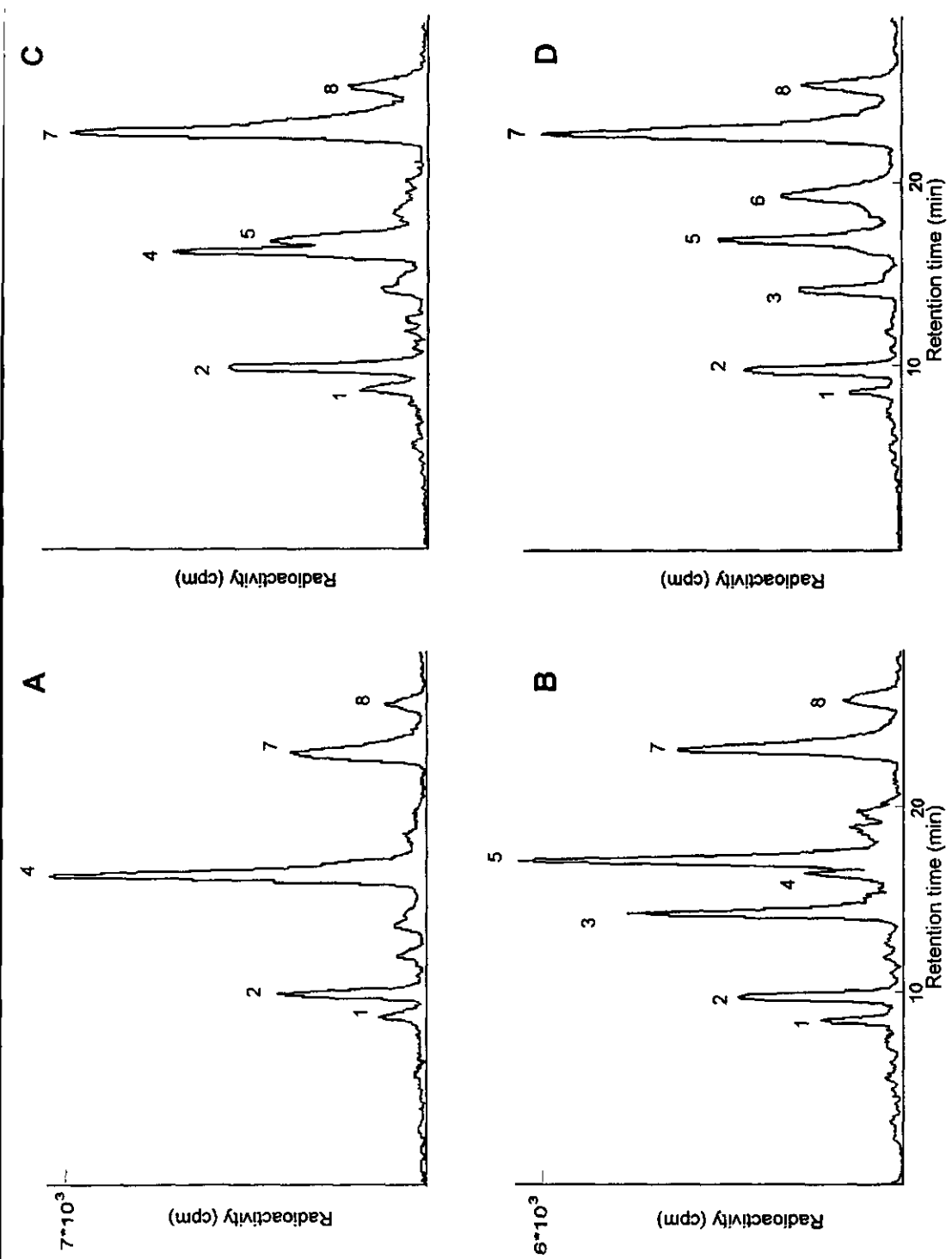

A
B
C
D
Radioactivity (cpm)
7*10^3
6*10^3
Retention time (min)
10
20
1
2
3
4
5
6
7
8

Table 8.1. Effect of fungicides on sterol biosynthesis of the wild-type isolate N423 and the fenpropimorph-resistant isolate N423P in cell-free extracts from *Aspergillus niger*.

Concentration	Sterol ratio (%)[1]											
(M)	Ergosterol		Fecosterol		Compound A[2]		Compound B[3]		Lanosterol		Eburicol	
	N423	N423P	N423	N423P	N423	N423P	N423	N423P	N423	N423P	N423	N423P
Fenpropimorph												
0	64.8	24.0	-[4]	4.9	-	9.3	-	-	27.8	49.4	7.4	12.4
$1 \cdot 10^{-7}$	39.0	14.3	22.7	20.1	7.3	6.5	-	-	24.3	48.8	6.7	10.3
$3 \cdot 10^{-7}$	14.5	5.1	38.4	29.2	12.7	6.4	-	-	26.6	50.1	7.8	9.2
$1 \cdot 10^{-6}$	6.4	-	32.4	24.4	27.3	11.3	-	8.8	26.8	46.2	7.1	9.3
$3 \cdot 10^{-6}$	-	-	18.8	17.1	47.4	13.6	-	16.0	26.5	44.0	7.3	9.3
$1 \cdot 10^{-5}$	-	-	10.9	8.2	53.3	15.0	-	16.3	26.8	43.0	8.9	17.5
$1 \cdot 10^{-4}$	-	-	-	-	-	-	-	-	36.4	57.6	63.6	42.4
Fenpropidin												
0	66.1	32.5	-	-	1.9	9.3	-	-	25.4	41.8	6.6	16.4
$1 \cdot 10^{-7}$	48.9	24.0	10.0	7.5	11.2	6.4	-	-	23.9	40.2	6.0	21.9
$3 \cdot 10^{-7}$	31.6	19.8	16.5	13.2	21.6	7.5	-	6.3	24.0	37.5	6.3	15.7
$1 \cdot 10^{-6}$	14.1	15.4	12.1	14.5	41.9	10.3	-	7.8	25.3	38.4	6.6	13.6
$3 \cdot 10^{-6}$	-	4.8	10.2	9.4	58.9	18.1	-	16.9	24.4	38.3	6.5	12.5
$1 \cdot 10^{-5}$	-	4.8	8.4	6.8	58.6	17.3	-	21.6	26.5	36.7	6.5	12.8
$1 \cdot 10^{-4}$	-	-	-	-	39.8	-	-	9.0	51.8	68.9	8.4	22.1
Tridemorph												
0	58.2	24.8	-	10.4	-	2.7	-	-	31.7	41.4	10.1	20.7
$1 \cdot 10^{-7}$	48.2	27.1	8.0	18.1	-	2.3	-	-	33.0	35.3	10.8	17.2
$3 \cdot 10^{-7}$	21.8	21.0	31.1	31.4	-	3.5	-	-	32.9	31.4	14.2	12.7
$1 \cdot 10^{-6}$	16.7	23.6	33.8	30.9	-	4.5	-	-	34.0	27.5	15.5	13.5
$3 \cdot 10^{-6}$	11.1	12.0	37.5	41.0	11.6	5.5	-	-	29.6	28.5	10.2	13.0
$1 \cdot 10^{-5}$	6.5	8.6	54.0	45.7	9.5	6.2	-	-	22.5	26.5	7.5	13.0
$1 \cdot 10^{-4}$	10.5	-	-	4.9	19.5	12.2	18.8	8.1	35.9	43.9	15.3	30.9
Propiconazole												
0	81.0		-		-		-		14.8		4.2	
$1 \cdot 10^{-8}$	80.3		-		-		-		14.9		4.8	
$3 \cdot 10^{-8}$	79.2		-		-		-		16.1		4.7	
$1 \cdot 10^{-7}$	77.2		-		-		-		14.5		8.3	
$3 \cdot 10^{-7}$	38.4		-		-		-		12.5		49.1	
$1 \cdot 10^{-6}$	17.6		-		-		-		9.9		72.5	

[1] Average of 2 experiments
[2] Non-identified sterol with a retention time of 14.1 min
[3] Non-identified sterol with a retention time of 19.1 min
[4] Not detectable

Effect of fungicides on resistant isolates. Ergosterol biosynthesis in cell-free assays from the resistant isolate N423P was relatively low and varied between 24.0 and 32.5%. In contrast, the biosynthesis of lanosterol was relatively high. Increasing concentrations of fenpropimorph and fenpropidin had an obvious transient effect on fecosterol accumulation, while the biosynthesis of compound A was less as compared to tests with isolate N423. Treatment with the fungicides also resulted in biosynthesis of compound B (Table 8.1).

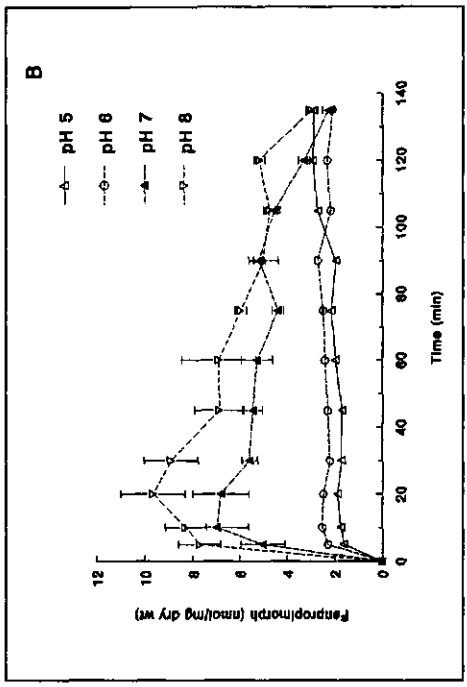
Tridemorph affected sterol biosynthesis in cell-free assays from the resistant isolate in a similar way as described for the wild-type isolate.

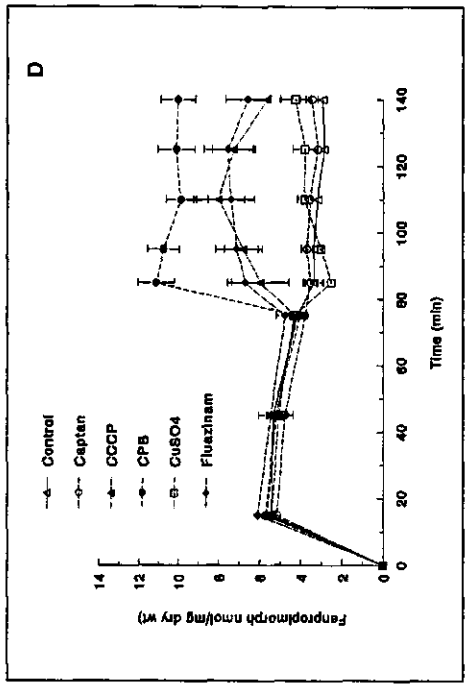
Effects of fenpropimorph on sterol biosynthesis were also studied in cell-free assays from isolates N522 (wild-type) and N522AI (resistant). Results were similar as described for N423 and N423P, respectively (data not shown).

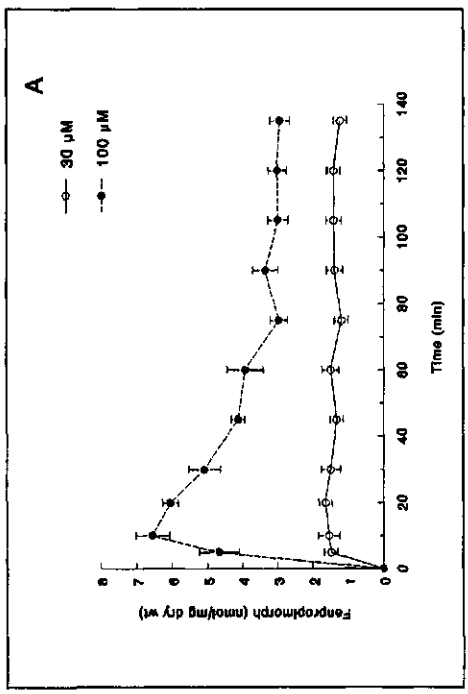
Accumulation. Accumulation of fenpropimorph at an initial external concentration of 30 μM by the wild-type isolate N423 was constant in time ($\pm$ 1.5 nmol mg^{-1} dry weight mycelium) (Figure 8.3). At 100 μM, accumulation became transient with a maximum ($\pm$ 7.0 nmol mg^{-1} dry weight) after about 10 min of incubation. The external pH affected the accumulation level of fenpropimorph. At pH 5 and 6, accumulation of fenpropimorph was relatively low and remained constant in time ($\pm$ 2 nmol mg^{-1} dry weight), whereas at pH 7 and 8, accumulation was relatively high and transient (Figure 8.3).

Accumulation of fenpropimorph by the laboratory-generated resistant isolates N423P and N522AI was compared with that of the corresponding wild-type isolates N423 and N522. At standard conditions used (external concentration 100 μM; pH 7), the resistant isolate N423P showed a transient accumulation pattern which did not differ significantly from that of the wild-type isolate N423. Isolate N522AI also showed a transient accumulation pattern but accumulated significantly lower levels of fenpropimorph after 30-120 min of incubation than the wild-types N423 and N522 (Figure 8.3).

Isolates N423 and N423P were selected to study effects of test compounds on accumulation of fenpropimorph. At 100 μM, the accumulation in both isolates stabilized during the second hour of incubation at about 3 nmol fenpropimorph mg^{-1} dry weight. Under these equilibrium conditions test compounds were added. CPB and, to a lesser extent, CCCP and fluazinam enhanced accumulation of fenpropimorph while captan and $CuSO_4$ had no effect (Figure 8.3). No differences were observed between the effect of the inhibitors on accumulation of fenpropimorph by both isolates tested (results not shown).


B
pH 5
pH 6
pH 7
pH 8
Fenpropimorph (nmol/mg dry wt)
Time (min)


D
Control
Captan
CCCP
CPB
CuSO4
Fluazinam
Fenpropimorph nmol/mg dry wt)
Time (min)


A
30 µM
100 µM
Fenpropimorph (nmol/mg dry wt)
Time (min)

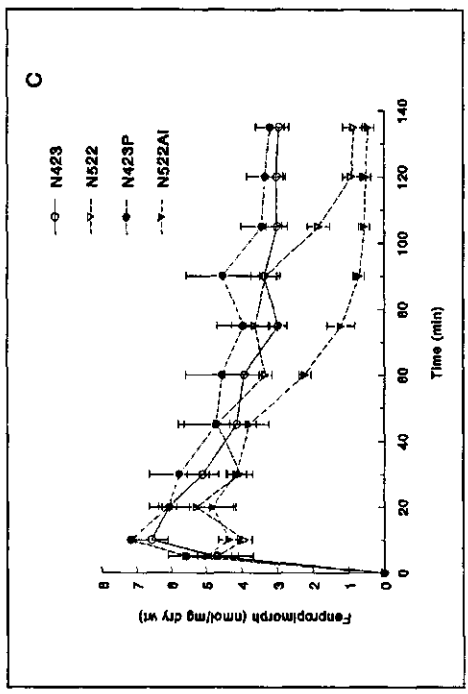

C
N423
N522
N423P
N522AI
Fenpropimorph (nmol/mg dry wt)
Time (min)

Figure 8.3. Accumulation of [^{14}C]fenpropimorph by germlings of *Aspergillus niger*. A: Accumulation by fenpropimorph-sensitive isolate N423 at different fenpropimorph-concentrations (pH 7). B: Accumulation by fenpropimorph-sensitive isolate N423 at different pH values (fenpropimorph 100 μM). C: Accumulation by fenpropimorph-sensitive N423 and N522 and -resistant isolates N423P and N522AI (fenpropimorph 100μM; pH 7). D: Effect of test compounds on accumulation by isolate N423 (fenpropimorph 100μM; pH 7).

Discussion

The transient accumulation of fenpropimorph by germlings of *A. niger* and the effect of different inhibitors on accumulation suggest that the accumulation level of the fungicide is determined by passive influx and energy-dependent efflux of the fungicide as described for accumulation of DMIs in several fungi (De Waard and Van Nistelrooy, 1980; Ryley *et al.*, 1984; Ney, 1988; Kalamarakis *et al.*, 1991; Stehmann and De Waard, 1995). However, the energy-dependent efflux of fenpropimorph seems to be lower as compared to that of DMIs since the transient pattern was less obvious and inhibitors tested were less potent in inducing accumulation. Another characteristic of fenpropimorph accumulation was the pH effect of the medium. A low pH of the medium will lead to protonation of fenpropimorph (pK=7), which probably impaired passive influx and caused the relatively low accumulation level. The relatively high accumulation level at pH 7 and 8 may explain the relatively high fungitoxicity of the fungicide under basic conditions (results not shown). The initial accumulation level of fenpropimorph was similar in wild-type and resistant isolates. This indicates that increased efflux in resistant isolates is probably not responsible for resistance as observed for DMIs in DMI-resistant laboratory-generated mutants of several fungi (De Waard and Van Nistelrooy, 1980, 1984, 1988; Stehmann and De Waard, 1995).

The major sterol synthesized in cell-free assays from *A. niger* isolate N423 is ergosterol, indicating that the metabolic pathway involved proceeds effectively, as described before for cell-free assays from *P. italicum* (Guan *et al.*, 1992) and *B. cinerea* (Stehmann *et al.*, 1994). The ergosterol biosynthesis in cell-free assays from resistant isolates was significantly lower than in assays from sensitive isolates. This cannot be ascribed to a reduction of the incorporation of [2-^{14}C]mevalonate into NSLs. The enhanced accumulation of lanosterol indicates that a reduced capacity of side-chain alkylation may be responsible for this observation.

Incubation with the fungicides tested led to the accumulation of newly synthesized sterols with retention times of 14.1 (compound A), 17.0 (fecosterol) and 19.1 min (compound B) as observed in radio-HPLC analysis (Figure 8.2). Compound A accumulated to $\pm$50-60% after treatment with fenpropimorph (10^{-5} M) and fenpropidin (3-10x10^{-6} M) and to $\pm$20% after treatment with tridemorph (10^{-4} M). Fenpropimorph and fenpropidin are regarded as strong inhibitors of activity of sterol Δ^{14}-reductase and tridemorph as a strong

inhibitor of $\Delta^8 \rightarrow \Delta^7$-isomerase (Baloch *et al.*, 1984; Berg *et al.*, 1984). This information suggests that compound A can be the substrate of sterol Δ^{14}-reductase, *i.e.* 4,4-dimethyl-ergosta-8,14,24(28)-trien-3β-ol or one of its metabolic conversion products such as ignosterol or ergosta-8,14,24(28)-trien-3β-ol (Figure 8.4). However, the identity of compound A is probably not 4,4-dimethyl-ergosta-8,14,24(28)-trien-3β-ol since, under the reverse phase HPLC conditions used, the retention time of compound A is 14.1 minutes suggesting a relatively polar character as compared to ergosterol (Rodriguez and Parks, 1985). This is not the case for 4,4-dimethyl-ergosta-8,14,24(28)-trien-3β-ol because of the presence of the C4,4-dimethyl groups. Ignosterol was found to accumulate to high quantities in *Ustilago maydis* after treatment with fenpropimorph (Baloch *et al.*, 1984). However, compound A is not identical to ignosterol since under the test conditions used its retention time is 17.2 min. Probably, the identity of compound A is ergosta-8,14,24(28)-trien-3β-ol, a sterol identified in fenpropimorph-treated mycelium of *Rhynchosporum secalis* (Girling, 1991), *Neurospora crassa* (Ellis *et al.*, 1991) and *Fusarium* (Debieu *et al.*, 1992).

The sterol with retention time 19.1 (compound B) might be ergosta-8-en-3β-ol, a metabolic conversion product of fecosterol, formed upon reduction of the C24 double bond (Figure 8.4). This newly formed sterol was also found in morpholine-treated cells of *U. maydis* (Baloch *et al.*, 1984). Experiments to identity compounds A and B conclusively are in progress.

Results of the cell-free assay experiments indicate that the fungicides tested inhibit sterol biosynthesis in *A. niger* in different ways. The accumulation of fecosterol at a relatively low concentration of fenpropimorph indicates that sterol $\Delta^8 \rightarrow \Delta^7$-isomerase is a highly-sensitive target enzyme of this fungicide. Accumulation of compound A would indicate that activity of sterol Δ^{14}-reductase becomes inhibited as well. Since sterol Δ^{14}-reductase proceeds the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase in the sterol biosynthesis pathway, full inhibition of sterol Δ^{14}-reductase activity would explain the transient accumulation of fecosterol. The effect of fenpropidin on fecosterol biosynthesis is relatively low as compared to that of fenpropimorph. In contrast, accumulation of compound A is relatively high. These results suggest that fenpropidin has an effect on both enzymes but that the inhibitory activity of this fungicide on sterol Δ^{14}-reductase is relatively strong as compared with that of fenpropimorph. Tridemorph predominantly inhibits activity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and not sterol Δ^{14}-reductase since fecosterol accumulation is not transient, even not at relatively high fungicide concentrations. The results described confirm those reported earlier for assays from *U. maydis* and yeast (Baloch *et al.*, 1984; Baloch and Mercer, 1987).

Incubation of cell-free assays with the DMI propiconazole resulted in the accumulation of eburicol. This result confirms that propiconazole has an inhibitory effect on activity of sterol 14α-demethylase. Similar results have been described for triazoles in cell-free assays with *P. italicum* (Guan *et al.*, 1992) and *B. cinerea* (Stehmann *et al.*, 1994).

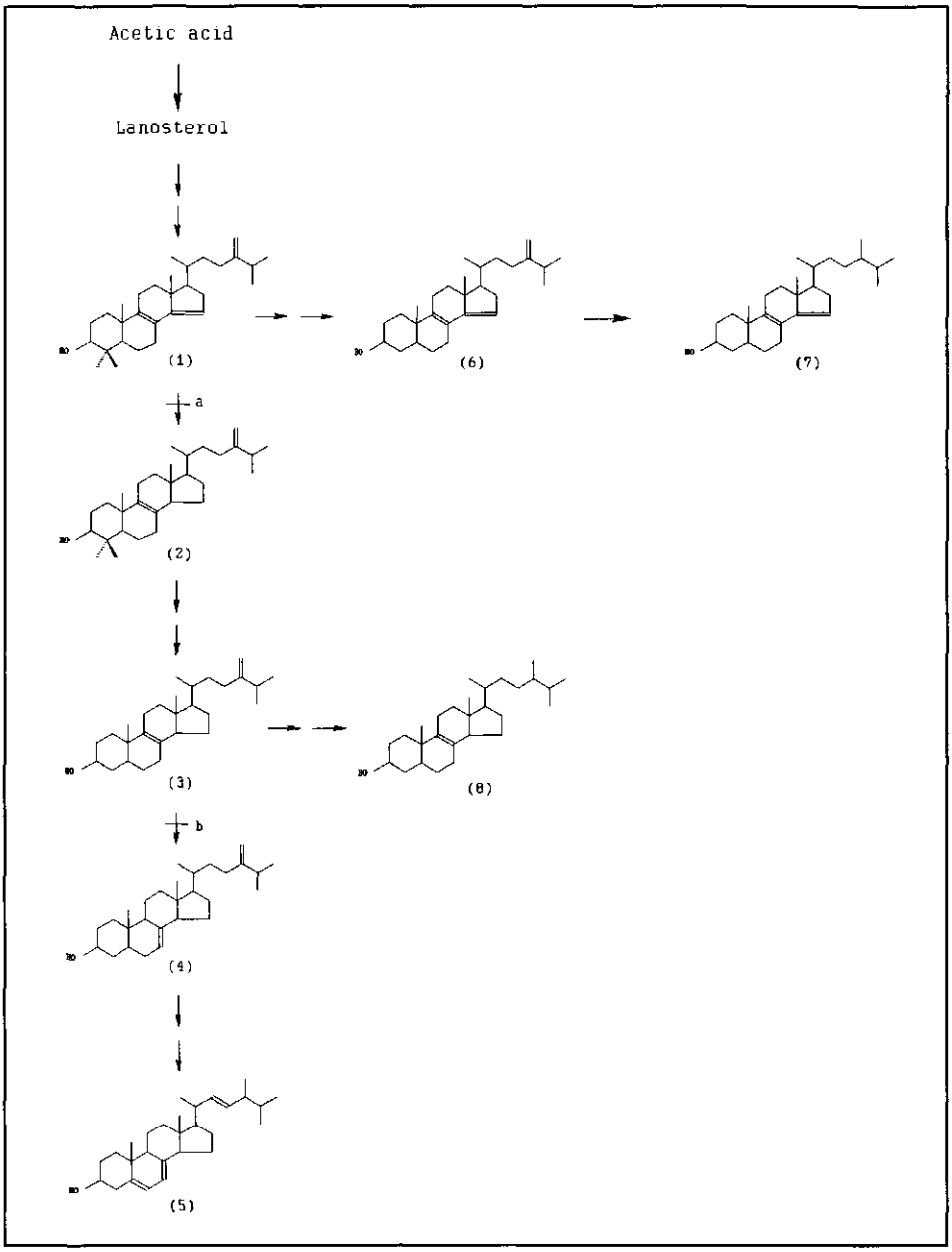


Figure 8.4. Sterol biosynthesis pathway of *Aspergillus niger*. 1: 4,4-dimethylergosta-8,14,24(28)-trien-3β-ol; 2: 4,4-dimethylergosta-8,24(28)-dien-3β-ol; 3: ergosta-8,24(28)-dien-3β-ol (fecosterol); 4: ergosta-7,24(28)-dien-3β-ol (episterol); 5: ergosta-5,7,22-trien-3β-ol (ergosterol); 6: ergosta-8,14,24(28)-trien-3β-ol; 7: ergosta-8,14-dien-3β-ol (ignosterol); 8: ergosta-8-en-3β-ol; a: sterol Δ^{14}-reductase; b: sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase.

Incubation of cell-free assays from resistant isolates with fenpropimorph and fenpropidin resulted in a pronounced accumulation of fecosterol while accumulation of compound A remained low, even at relatively high concentrations. This observation suggests that these fungicides predominantly inhibited activity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase while activity of sterol Δ^{14}-reductase remained largely unaffected. This would imply that the sterol Δ^{14}-reductase from the resistant isolates has a relatively low binding affinity to fenpropimorph. A reduction in sensitivity of sterol Δ^{14}-reductase has also been suggested as a mechanism of resistance to fenpropimorph in *Fusarium* spp and *Pseudocercosporella herpotrichoides* (Debieu *et al.*, 1995).

Cell-free extracts from sensitive and resistant isolates treated with relatively high concentrations of tridemorph contained compound B, which might be 5α-ergosta-8-en-3β-ol, a metabolic conversion product of fecosterol (Baloch *et al.*, 1984). The observation that compound B is also formed in cell-free assays from resistant isolates incubated with fenpropimorph and fenpropidin would confirm that the primary target site of these fungicides in these isolates is sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and that the affinity of this enzyme in the resistant isolate to the fungicides remains unchanged.

The metabolic effects described above for the fungicides tested can explain the cross-resistance between fenpropimorph and fenpropidin in *A. niger* as described before (Engels *et al.*, 1998). Similar results have been observed for *E. graminis* f.sp. *tritici* (De Waard *et al.*, 1992) and *Erysiphe graminis* f.sp. *hordei* (Brown *et al.*, 1996) indicating that *A. niger* is a good model organism to study the mode of action of morpholine fungicides. The dual mode of action observed for the fungicides would explain why resistance development is low and, if present, proceeds stepwise. The data support a proper design of strategies to counteract resistance development.

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Chapter 9

General Discussion

General Discussion

The aim of the present research was to evaluate strategies to avoid or decrease the risk for resistance development to fenpropimorph in *Erysiphe graminis* f.sp. *tritici*, the causal agent of wheat powdery mildew, and to study putative mechanisms of resistance.

Fenpropimorph is a modern, systemic fungicide, widely used in integrated crop protection in cereals since 1980. A potential problem of such a modern fungicide is resistance development in target organisms. Evolution of resistance can jeopardise the considerable investments made by agrochemical companies to market new fungicides. Therefore, the risk of resistance development in target organisms to new fungicides should be evaluated carefully before their introduction in practice (Gisi and Staehle, 1988). An early assessment of the resistance risk will help to ensure the longest possible life-time of a new active ingredient (Georgopoulos, 1994). To prevent or delay fungicide resistance, agrochemical companies cooperate in the Fungicide Resistance Action Committee (FRAC) (Russell, 1995a). This committee provides useful information on anti-resistance strategies in giving advice to farmers for a proper use of crop protection products. A basic principle of anti-resistance strategies is a reduction in selection pressure, thereby decreasing the risk of selection of resistant mutants. A useful additional positive effect of anti-resistance strategies is a reduction in use of pesticides. The importance of avoiding or decreasing the risk for resistance development has been recognized in the harmonized registration process of the European Union, which includes requirements for the provision of information on resistance (Anonymous, 1994).

In the context of integrated control of plant diseases, farmers in various parts of Europe started using fungicides in cereals at dosages lower than recommended to reduce fungicide input in agriculture. Results from field trials indicate that use of split applications and low dosages increases the rate at which *E. graminis* f.sp. *tritici* can become less sensitive to fenpropimorph (Chapter 3 and 4). A second disadvantage of the application of low dosages is a reduction in control of other foliar pathogens. Therefore, the use of fungicides at the recommended dosage is implemented by FRAC as an element of proper resistance management (Russell, 1995a). This contradicts the advice of extension services of various European countries to apply fungicides at reduced dosages because of environmental considerations.

The success of anti-resistance strategies depends on its effective and consistent implementation by the end user of the product. Do farmers and extension services really put anti-resistance strategies into practice? For the farmer, anti-resistance strategies can be costly and often of little direct value. The strategies may help to extend the useful lifetime of a fungicide but this is generally of limited direct concern to the farmer. Usually, this is only the case when fungicide resistance has a direct financial impact on farm business (Clark, 1995). Cereal growers are also well aware of the fact that their anti-resistance

strategies will have little or no effect on the sensitivity of the pathogen population, unless all surrounding farmers adopt these strategies (Clark, 1995). This situation creates a dilemma between general interest and the overall economics of production. Farmers may well be more interested in reducing inputs by restricting spray applications and using reduced dosages than supporting anti-resistance strategies (Russell, 1995b). A change in this attitude may require a concerted action of extension officers and industrial representatives.

Fungicides with a different mechanism of resistance are valuable tools in counteracting evolution of resistance. Currently, for powdery mildew control cereal growers rely almost entirely on fungicides from only two fungicide groups, viz. the azoles and morpholines. New classes of fungicides with different modes of action have been developed for disease control in cereals. Among these are the anilinopyrimidine (Heye *et al.*, 1994) and strobilurin fungicides (Ammermann *et al.*, 1992; Godwin *et al.*, 1994). In addition, plant activators may become important (Kessmann *et al.*, 1996). One plant activator, a benzothiadiazole derivative, has already been introduced for powdery mildew control in winter wheat in Germany in 1996. The availability of these chemicals will make the design of anti-resistance strategies based on mixtures or rotational use easier.

A parameter influencing resistance development in fungi to fungicides is the fitness of resistant subpopulations. Genetic changes conferring resistance can be linked to lower fitness in the absence of the fungicide. This condition may significantly slow down resistance development (Chin, 1987; Milgroom *et al.*, 1989; Shaw, 1989). Results from field and climate room experiments indicate that isolates of *E. graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph indeed have a lower fitness than wild-type isolates (Chapter 5).

The sterol composition of isolates of *E. graminis* f.sp. *tritici* was determined to study whether reduced sensitivity would relate to an altered sterol composition (Chapter 6). It appeared that in the absence of fenpropimorph, isolates with wild-type and reduced sensitivity to fenpropimorph possessed a similar sterol composition. This indicates that an altered sterol composition cannot explain the reduced sensitivity to fenpropimorph in isolates with reduced sensitivity. After treatment with fenpropimorph, accumulation of episterol was noticed, suggesting that fenpropimorph has a target site in *E. graminis* f.sp. *tritici* affecting the conversion of episterol in ergosta-5,24(28)-dien-3β-ol. Since this phenomenon has been found also after treatment of *E. graminis* f.sp. *hordei* and f.sp. *tritici* with several sterol biosynthesis inhibitors (Senior, 1991; Kwok and Loeffler, 1993; Senior *et al.*, 1995), it is possible that this target site plays a role in the mode of action of these fungicides in powdery mildews. As a consequence, it may be that besides sterol Δ^{14}-reduction and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerization (Baloch *et al.*, 1984; Mercer, 1991), a third target site may be relevant for fenpropimorph. An additional target site for fenpropimorph, viz. squalene epoxidase, has also been described in *Nectria haematococca* var. *cucurbitae* (Ziogas *et al.*, 1991). It might be that the presence of multiple target sites of fenpropimorph relates to the slow

evolution of resistance to this fungicide in plant pathogens.

Biochemical studies on the mechanism of resistance to fenpropimorph in *E. graminis* f.sp. *tritici* are virtually impossible because of the obligate character of this fungus. Genetic studies are not feasible since methods to cross isolates of the pathogen under controlled laboratory conditions are not known. Therefore, the filamentous fungus *Aspergillus niger* was used to study the genetic and biochemical mechanism of resistance to fenpropimorph. Genetic analysis of fenpropimorph-resistance in *A. niger* revealed two genes involved, located on linkage group II (Chapter 7). In *E. graminis* f.sp. *hordei*, only one or a few genes seem to control reduced sensitivity to fenpropimorph (Brown *et al.*, 1996). In contrast, resistance to *N. haematococca* var. *cucurbitae* (Demakopoulou *et al.*, 1989; Lasseron-De Falandre *et al.*, 1991), *Pyrenophora teres* (Peever and Milgroom, 1993) and *Pseudocercosporella herpotrichoides* (Hocart and McNaughton, 1994) is polygenic. These data indicate that not one single genetic model for fenpropimorph-resistance is applicable for different fungi. This implies that fungicides cannot simply be classified in two major categories with respect to resistance development in plant pathogens, viz. monogenic or polygenic resistance, as is usually done (Georgopoulos and Skylakakis, 1986). Both mechanisms can be operative for fungicides from the same class, viz. morpholines. Therefore, genetic studies aimed to assess the risk of resistance development to fungicides in plant pathogens should, by preference, be carried out with the target organism. If impossible, as is the case for *E. graminis* f.sp. *tritici*, model fungi may be used. In retrospect, it seems that *A. niger* is useful in this respect.

Results of cell-free assays suggest that sterol Δ^{14}-reductase from isolates with reduced sensitivity to fenpropimorph has a reduced affinity to this compound (Chapter 8). A reduction in sensitivity of sterol Δ^{14}-reductase has also been suggested as a mechanism of resistance to fenpropimorph in *Fusarium* spp and *P. herpotrichoides* (Debieu *et al.*, 1995). Obviously, reduced sensitivity of this target enzyme to morpholines is a common mechanism of decreased sensitivity. This is also true for many other classes of fungicides (Lyr, 1995). A major difference is that the level of resistance to morpholines is relatively low.

Results generated in this thesis have provided a better understanding of a possible evolution of resistance to morpholine fungicides in *E. graminis* f.sp. *tritici* and other plant pathogens. Till now, reduction in sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph has not resulted in failure of disease control in practice. This study indicates that a combination of factors may explain this favourable situation. In summary, major factors involved can be the oligosite mode of action of the fungicide, the oligo- or polygenic nature of fungal resistance to the fungicide and the lower competitive ability of isolates with reduced sensitivity in the absence of the fungicide. These conditions may assure that proper anti-resistance strategies may be relatively effective as compared to other classes of fungicides.

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Summary

In the last three decades, plant disease control has become heavily dependent on fungicides. This practice increased yield significantly but had also negative side-effects on the environment. In many countries, integrated control programs have been initiated in order to reduce pesticide use and their side-effects (Chapter 2). In addition, modern systemic fungicides have been discovered with a high selective and potent activity. These compounds can be applied in relatively low dosages and are less harmful to the environment than conventional fungicides. A problem of these modern fungicides is resistance development in their target organisms since they have a site-specific mode of action. This risk also applies for the morpholine fungicide fenpropimorph, the active ingredient of Corbel, in the control of *Erysiphe graminis* f.sp. *tritici*. This pathogen is the causal agent of wheat powdery mildew, which is one of the most common and severe foliar diseases of wheat in Europe.

In the context of integrated control, farmers started using fenpropimorph in cereals at dosages lower than recommended. Adopted approaches are the use of split applications which is defined as a treatment in which the recommended dosage is divided over a number of applications, and the use of reduced dosages. To study the effect of these strategies on resistance development, a four-year-field trial was conducted in the south of the Netherlands. In the first three years (1992-1994), the effect of split applications of Corbel (active ingredient fenpropimorph) and of Tilt Top (active ingredients fenpropimorph and propiconazole) on sensitivity of *E. graminis* f.sp. *tritici* was investigated (Chapter 3). In 1995, the effect of reduced dosages of Corbel was studied (Chapter 4). The sensitivity of isolates collected was assessed in foliar spray tests. A change in sensitivity to fenpropimorph of the mildew population in plots treated with applications at the recommended dosage (2x1.0 l ha^{-1}) was not observed, except for 1995 when a minor change was noticed. In contrast, powdery mildew isolates from plots treated with split applications (5x0.4 l ha^{-1}) displayed a significant decrease in sensitivity. Reduced dosages (2x0.25 l ha^{-1}) may also increase the rate of resistance development. Since maintenance of sensitivity of the pathogen population is valued highly, the split application strategy and the use of reduced dosages is not recommended for wheat powdery mildew control. Another disadvantage of the application of low dosages is reduced disease control of other foliar pathogens.

A parameter influencing resistance development in fungi to fungicides is the fitness of resistant subpopulations (Chapter 5). Fitness is a rather complex characteristic with many parameters involved. If resistance is correlated with reduced fitness, it may significantly slow down resistance development. Therefore, the competitive ability and survival in winter of isolates of *E. graminis* f.sp. *tritici* with wild-type and reduced sensitivity to fenpropimorph was investigated. In addition, a broad range of fitness components, such as

germination, latent period, colony forming ability and production of conidia were studied. Under crowded infection conditions, isolates with reduced sensitivity showed a lower competitive ability than wild-type isolates. The lower competitive ability could not be ascribed to changes in germination, latent period, colony forming ability or production of conidia. Hence, reduction in competitive ability seems to be a complex change in phenotype which cannot easily be assessed by characterization of individual fitness components.

Use of mildew isolates containing fungicide-sensitivity and virulence markers made it possible to study the survival of *E. graminis* f.sp. *tritici* in winter in field trials (Chapter 5). Results revealed that isolates with a reduced sensitivity to fenpropimorph survived the winter. The competitive ability of these isolates seemed to be slightly lower than that of a mildew population which migrated naturally into the experimental field. The data indicate that in practice, in periods without selection pressure, the frequency of isolates with reduced sensitivity to fenpropimorph may decrease.

Changes in sterol composition may play a role in the reduced sensitivity of *E. graminis* f.sp. *tritici* to fenpropimorph (Chapter 6). Therefore, sterols were extracted from mildew conidia and analyzed by gas chromatography and mass spectrometry. Isolates of *E. graminis* f.sp. *tritici* with a wild-type and reduced sensitivity to fenpropimorph had the same sterol composition, viz. ergosta-5,24(28)-dien-3β-ol ($\pm$90%) and episterol ($\pm$10%). Following treatment with fenpropimorph, the content of episterol increased in conidia of all isolates tested while that of ergosta-5,24(28)-dien-3β-ol decreased. These results suggest that fenpropimorph, under the test conditions used, did not inhibit activity of sterol Δ^{14}-reductase or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase but rather interfered with the final part of the demethyl sterol synthesis. However, modifications in this part of the pathway are probably not responsible for the decreased sensitivity of the pathogen to the fungicide.

Genetic studies on resistance of *E. graminis* f.sp. *tritici* are not feasible since crossings cannot be made under controlled laboratory conditions. As an alternative, *Aspergillus niger* and *Aspergillus nidulans* were used as model fungi (Chapter 7). Treatment of conidia with UV-light did not result in the detection of fenpropimorph-resistant mutants of *A. nidulans*. In contrast, resistance to fenpropimorph was readily induced in *A. niger*. The degree of resistance varied between 2 and 8. Dominance tests showed that resistance to fenpropimorph is recessive. Genetic analysis of fenpropimorph-resistance in this fungus was carried out by means of the parasexual cycle. Resistance to fenpropimorph involved two genes, located on linkage group II.

Biochemical studies on the mechanism of resistance to fenpropimorph in *E. graminis* f.sp. *tritici* are virtually impossible because of the obligate character of the pathogen. Therefore, the genetically characterized mutants of *A. niger* described in Chapter 7 were used to study effects of morpholine fungicides on cell-free sterol synthesis and on fenpropimorph-accumulation in mycelium (Chapter 8). Cell-free extracts of *A. niger* actively synthesized C4-demethyl sterols from [2-^{14}C]mevalonate. Sterol analyses by means of thin-

layer chromatography and high-performance liquid chromatography displayed that the main sterol formed in cell-free assays from wild-type isolates was ergosterol. Inhibition of sterol biosynthesis in cell-free bioassays by the morpholine fungicides fenpropimorph and tridemorph, the morpholine-related fungicide fenpropidin and the triazole propiconazole was investigated. Results suggest that, in sensitive isolates, fenpropimorph and fenpropidin inhibited activity of both sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and sterol Δ^{14}-reductase. Tridemorph proved to be a relatively strong inhibitor of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase activity only. In cell-free bioassays from resistant isolates fenpropimorph and fenpropidin predominantly inhibited activity of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase while activity of sterol Δ^{14}-reductase remained less affected. Tridemorph inhibited sterol biosynthesis from sensitive and resistant isolates in a similar way. Therefore, the biochemical mechanism of resistance to fenpropimorph in the isolates tested is probably related to reduced affinity of sterol Δ^{14}-reductase to the fungicide. Accumulation of fenpropimorph by wild-type and resistant isolates did not differ significantly, indicating that differential efflux is not responsible for resistance to fenpropimorph.

It is concluded that the results presented provide a better understanding of a possible evolution of resistance to morpholine fungicides in *E. graminis* f.sp. *tritici* and other plant pathogens. The results also support the relevance of strategies aimed to counteract development of resistance to morpholine fungicides.

Samenvatting

In de afgelopen drie decennia is de bestrijding van plantenziekten sterk afhankelijk geworden van fungiciden. Deze ontwikkeling verhoogde de opbrengsten van gewassen significant maar had ook negatieve bijwerkingen op het milieu. In diverse landen zijn daarom geïntegreerde programma's gestart om het gebruik van gewasbeschermingsmiddelen en hun bijwerkingen te verminderen (Hoofdstuk 2). Bovendien zijn moderne en actieve systemische fungiciden ontdekt met een zeer selectieve werking. Systemische fungiciden kunnen in relatief lage doseringen toegediend worden en zijn minder schadelijk voor het milieu dan conventionele fungiciden. Een probleem van deze moderne middelen is resistentieontwikkeling in hun doelorganismen. Dit is een gevolg van hun specifieke werkingsmechanisme. Het risico van resistentieontwikkeling geldt ook voor fungiciden die zijn afgeleid van morfolinen. Het belangrijkste middel van deze groep is fenpropimorf, de werkzame stof van Corbel. Corbel wordt toegepast bij de bestrijding van *Erysiphe graminis* f.sp. *tritici*. Dit pathogeen is de veroorzaker van tarwemeeldauw, één van de meest voorkomende en schadelijke ziekten van tarwe in Europa.

In het kader van geïntegreerde bestrijding gebruiken akkerbouwers steeds vaker lage doseringen van Corbel voor de bestrijding van meeldauw in granen. In sommige Europese landen worden vaak split applicaties toegediend. Hierbij wordt de aanbevolen dosering verdeeld over een aantal toepassingen. Ook vindt reductie van de dosering plaats terwijl het aantal applicaties niet verandert. Om het effect van deze strategieën op resistentie-ontwikkeling te bestuderen is gedurende vier jaar een veldproef uitgevoerd in het zuiden van Nederland. In de eerste drie jaar werd het effect van split applicaties van Corbel en Tilt Top (werkzame stoffen fenpropimorf en propiconazool) op de gevoeligheid van *E. graminis* f.sp. *tritici* bestudeerd (Hoofdstuk 3). In 1995 werd het effect van gereduceerde doseringen van Corbel getoetst (Hoofdstuk 4). De gevoeligheid van de verzamelde isolaten werd bepaald in spuitproeven. In de meeldauwpopulatie van plots behandeld met de aanbevolen dosering (2x1.0 l ha^{-1}) werd geen verandering in gevoeligheid voor fenpropimorf waargenomen, behalve in 1995 toen een kleine verandering optrad. Daarentegen vertoonden meeldauwisolaten van plots behandeld met split applicaties (5x0.4 l ha^{-1}) een significante afname in gevoeligheid. Ook het gebruik van verlaagde doseringen (2x0.25 l ha^{-1}) versnelden de snelheid van resistentieontwikkeling. Op grond van deze resultaten wordt split applicatie en het gebruik van gereduceerde doseringen afgeraden voor de bestrijding van tarwemeeldauw. Het in stand houden van de gevoeligheid van de meeldauwpopulatie wordt namelijk van grote waarde geacht. Een tweede nadeel van het toepassen van lage doseringen is een verminderde effectiviteit in de bestrijding van overige graanziekten.

Een parameter die resistentieontwikkeling in schimmels tegen fungiciden beïnvloedt is de fitness van resistente subpopulaties (Hoofdstuk 5). Resistentieontwikkeling kan vertraagd worden als resistentie gecorreleerd is met verminderde fitness. Daarom is de

fitness van resistente isolaten van *E. graminis* f.sp. *tritici* onderzocht in competitieproeven met wild-type isolaten en door bestudering van de overleving gedurende de winter. Bij hoge infectiedruk vertoonden isolaten met een verminderde gevoeligheid voor fenpropimorf een lager competitief vermogen dan de wild-type isolaten. Deze eigenschap kon niet worden toegeschreven aan veranderingen in kiemsnelheid, latente periode, vermogen tot kolonievorming en conidiënproductie. De vermindering van het competitief vermogen lijkt daarom een complexe verandering in fenotype die niet gemakkelijk bepaald kan worden door karakterisering van individuele fitnesscomponenten.

Gebruik van meeldauwisolaten met merkers voor gevoeligheid voor fungiciden en virulentie maakte het mogelijk om de overwintering te bestuderen in veldproeven (Hoofdstuk 5). De resultaten toonden aan dat isolaten met een verminderde gevoeligheid voor fenpropimorf de winter overleefden. Het competitief vermogen van deze isolaten leek iets lager dan dat van een meeldauwpopulatie die van buiten in het proefveld migreerde. De gegevens tonen aan dat het voorkomen van isolaten met een verminderde gevoeligheid voor fenpropimorf kan afnemen in periodes zonder selectiedruk.

De verminderde gevoeligheid van *E. graminis* f.sp. *tritici* voor fenpropimorf kan gebaseerd zijn op veranderingen in sterolcompositie (Hoofdstuk 6). Daarom werden sterolen geëxtraheerd uit meeldauwconidiën en geanalyseerd met behulp van gaschromatografie en massaspectrometrie. Isolaten van *E. graminis* f.sp. *tritici* met een wild-type en een verminderde gevoeligheid voor fenpropimorf hadden dezelfde sterolcompositie, te weten ergosta-5,24(28)-dien-3β-ol ($\pm$90%) en episterol ($\pm$10%). Na behandeling met fenpropimorf nam het gehalte aan episterol toe en dat van ergosta-5,24(28)-dien-3β-ol af. Dit effect werd in alle geteste isolaten waargenomen. Deze resultaten suggereren dat fenpropimorf, onder de gebruikte proefomstandigheden, de activiteit van sterol Δ^{14}-reductase of $\Delta^{8}\rightarrow\Delta^{7}$-isomerase niet remt maar interfereert met het laatste deel van de synthese van demethylsterolen. Deze mogelijke aangrijpingsplaats is waarschijnlijk niet betrokken bij het mechanisme van verminderde gevoeligheid van het pathogeen voor het fungicide.

Genetische studies met betrekking tot resistentie van *E. graminis* f.sp. *tritici* zijn niet mogelijk omdat onder laboratoriumomstandigheden geen kruisingen kunnen worden gemaakt. Als alternatief zijn *Aspergillus niger* en *Aspergillus nidulans* gebruikt als modelschimmels (Hoofdstuk 7). Behandeling van de conidiën met UV-licht resulteerde niet in de detectie van fenpropimorf-resistente mutanten van *A. nidulans*. Daarentegen werd resistentie tegen fenpropimorf gemakkelijk geselecteerd in *A. niger*. De resistentiegraad varieerde tussen 2 en 8. Dominantieproeven lieten zien dat resistentie tegen fenpropimorf recessief is. De genetische analyse van fenpropimorf-resistentie werd uitgevoerd in *A. niger* door middel van de parasexuele cyclus. Resistentie tegen fenpropimorf bleek te berusten op twee genen, beide gelokaliseerd op linkage groep II.

Biochemische studies betreffende het resistentiemechanisme tegen fenpropimorf in *E. graminis* f.sp. *tritici* zijn vrijwel onmogelijk wegens het obligate karakter van het

pathogeen. Daarom werden de genetisch gekarakteriseerde mutanten van *A. niger*, beschreven in Hoofdstuk 7, gebruikt om het effect van morfolinen op de sterolsynthese in celvrije proeven te bestuderen (Hoofdstuk 8). Celvrije extracten van *A. niger* vertoonden synthese van C4-demethylsterolen bij gebruik van [2-^{14}C]mevalonaat als substraat. Sterolanalyses met behulp van dunnelaag chromatografie en hoge druk vloeistof chromatografie toonden aan dat ergosterol het belangrijkste sterol is dat in celvrije proeven van wild-type isolaten wordt gevormd. Fenpropimorf en fenpropidin remden zowel de activiteit van sterol $\Delta^8 \rightarrow \Delta^7$-isomerase als van sterol Δ^{14}-reductase in gevoelige isolaten, terwijl tridemorf vrijwel alleen de activiteit van sterol $\Delta^8 \rightarrow \Delta^7$-isomerase remde. In celvrije bioassays van resistente isolaten remden fenpropimorf en fenpropidin vooral sterol $\Delta^8 \rightarrow \Delta^7$-isomerase activiteit terwijl de activiteit van sterol Δ^{14}-reductase veel minder werd beïnvloed. Tridemorf remde de sterolbiosynthese in gevoelige en resistente isolaten op dezelfde wijze. De resultaten wijzen erop dat het resistentiemechanisme tegen fenpropimorf in de geteste isolaten gerelateerd is aan verminderde gevoeligheid van sterol Δ^{14}-reductase voor het fungicide. Accumulatie van fenpropimorf in wild-type en resistente isolaten verschilde niet significant. Dit resultaat duidt er op dat differentiële efflux niet verantwoordelijk is voor resistentie tegen fenpropimorf.

De beschreven resultaten verschaffen een beter inzicht in een mogelijke evolutie van resistentie tegen morfoline fungiciden in *E. graminis* f.sp. *tritici* en andere plantenpathogenen. De resultaten ondersteunen ook de relevantie van strategieën die resistentieontwikkeling tegen morfoline fungiciden tegengaan.

Curriculum vitae

Antonius Johannes Gerardus (Tonnie) Engels werd geboren op 29 november 1967 te Venray en groeide op in Merselo (Limburg). In 1986 behaalde hij het Gymnasium-ß diploma aan het Boschveldcollege te Venray. Van september 1986 tot en met juni 1992 volgde hij de studie Landbouwplantenteelt aan de Landbouwuniversiteit Wageningen. De doctoraalstudie bestond uit de afstudeervakken Theoretische Produktie Ecologie, Landbouwplantenteelt en Statistiek. De stageperiode voor Landbouwplantenteelt werd doorgebracht op Cornell University te Ithaca NY, USA. Aansluitend aan zijn studie werd hij aangesteld als assistent in opleiding op de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen en verrichtte het onderzoek dat is beschreven in dit proefschrift. Van 1993 tot en met 1996 vertegenwoordigde hij Nederland in het managementcomité van COST-actie 817 getiteld 'Population studies of airborne pathogens on cereals as a means of improving strategies for disease control' van de Europese Unie. Gedurende ruim twee jaar (1993-1995) was hij coördinator van de werkgroep 'Epidemiological Parameters' van deze actie. Sinds 1 oktober 1996 is hij in dienst bij de firma Huntjens B.V. te Gronsveld onderdeel van de Belgische AVEVE-groep.